Understanding the molecular response of rice to biotic (brown planthopper) and abiotic (nitrogen deficiency) stress

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Abstract

Rice, one of the world's most important food crops and staple food for more than half of the world's population, is constantly exposed to severe biotic and abiotic stresses, resulting in significant yield losses of up to 70%. The present study investigated the role of β-1,3-glucanase (*Gns5*) in host plant susceptibility to brown planthopper (BPH) using RNAi-based approaches (Chapter 2). Knock-down of *Gns5* in these RNAi lines (using two different promoter systems) exhibited enhanced resistance in terms of survival, fecundity and developmental rate compared to controls lines (BPH-susceptible TN1; empty vector transformants). Microscopic studies demonstrated that the RNAi lines exhibited higher levels of callose accumulation post BPH-infestation, compared to TN1 or empty vector lines. Electronic Penetration Graphs (EPG) suggested that the probing frequency by BPH was significantly greater ($p<0.05$), but the duration of phloem ingestion was significantly shorter, on the RNAi lines compared to control lines. This study showed that knockdown of *Gns5* prevents callose decomposition and therefore increases the resistance of the commercially grown TN1 cultivar towards BPH. Physiological and molecular responses of rice to nitrogen (N) deficiency and the combination of N deficiency and BPH infestation were also investigated. N stress was shown to cause significant ($p<0.05$) reductions in shoot height, number of tillers and leaves, leaf area, root length, relative water content and chlorophyll content in a dose-dependent manner in both TN1 (susceptible to BPH) and IR70 (resistant to BPH) (Chapter 3). Further studies were carried out to identify transcriptions factors (TFs) involved in the response to N deficiency. Twelve TFs, previously reported to potentially be involved in the response of rice to BPH were up-regulated in IR70 compared to ten TFs up-regulated in TN1; furthermore, the magnitude of the response was significantly greater in IR70 (Chapter 4). Expression profiles of the combined stress (N + BPH) showed that more TFs were down-regulated in IR70 at different time points post BPH infestation compared to the susceptible TN1 (Chapter 5). Two of these N-responsive TF genes, *Os02g0214500* and *Os03g0437200* showed an interesting pattern of expression whereby those genes were down-regulated at most time points post BPH infestation in IR70 in response to reduced levels of N, in contrast to TN1 where both these TFs genes were up-regulated. These findings provide a platform for developing stress-tolerant rice cultivars.

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List of Abbreviations

µL	microliter
$(NH_4)_6$ MO_7O_{24}	ammonium molybdate tetrahydrate
µM	micromolar
ABA	Abscisic Acid
ABRE	ABA-responsive cis-acting elements
ANOVA	analysis of variance
AP2	Activating Protein 2
B.C	Before Christ
BIERF	BENZOTHIADIAZOLE-INDUCED ERF
bp	base pair
BPH	brown planthopper
bZIP	basic leucine zipper
C2H2	Cys2-His2
$CaCl_2$	calcium chloride
CalS	callose synthase
cDNA	complementary DNA
CRT	C-repeat
CT	cycle threshold
$CuSO_4$	copper sulphate
cv.	cultivar
DNA	Deoxyribonucleic acid
DRE	Dehydration-responsive element
DREB	Dehydration-responsive element binding protein
dsRNA	Double-stranded RNA

EPG	Electrical Penetration Graph
ERF	ethylene response factors
EST	Expressed Sequence Tag
ET	ethylene
FAO	Food and Agriculture Organization of the United Nations
FC	Fold change
$FeCl_3$	Iron (III) chloride
Fe-EDTA	ferric ethylenediaminetetraacetic acid
g	gram
GA	Gibberellins
GO	Gene Ontology
h	hours
H_3BO_3	Boric acid
ha	hectare
IR70	Indica Rice70
IRGSP	International Rice Genome Sequencing Project
JA	jasmonic acid
K	Potassium
K_2SO_4	potassium sulphate
kb	kilobase
KCl	potassium chloride
kg	kilogram
kgN ha^{-1}	kilogram nitrogen per hectare
log_2	logarithm base 2
M	molar
mA	milliampere
MAS	Marker-aided selection
Mb	Millions of base pairs

MeJA	Methyl jasmonic acid
mg	milligram
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
mins	minutes
mL	millilitre
mm	millimetre
mM	millimolar
mmol	millimoles
$MnCl_2$	manganese chloride
mRNA	messenger RNA
Mt	Million tonnes
MYB	Myeloblastosis
N	Nitrogen
n	number of replicates
N/ha	Nitrogen per hectare
N/L	Nitrogen per litre
N_2O	Nitrous oxide
NACRS	NAC recognition sequence
NaH_2PO_4	Sodium phosphate monobasic
$NaSiO_3$	Sodium silicate
NH_4NO_3	Ammonium nitrate
NO3-	Nitrate ion
NPR1	Pathogenesis-related genes 1
NUE	Nitrogen Utilization Efficiency
O.D.	optical denisty
^oC	degrees Celsius

OsBIERF	BIERF2 gene or protein in rice
OsbZIP	OsbZIP gene or protein in rice
OsMYB	MYB gene or protein in rice
OsNAC	NAC gene or protein in rice
OsWRKY	WRKY gene or protein in rice
P	Phosphorus
PBS	phophate-buffered saline
PCR	polymerase chain reaction
PD	Plasmodesmata
pmol	picomol
PR	Pathogenesis-related
qPCR	quantitative real-time polymerase chain reaction
QTL	Quantitative Trait Loci
R^2	Coefficient of determination
RAP	Rice Annotation Project
RAV	Related to AB13/VP1
RFLP	Restriction Fragment Length Polymorphism
RGSV	Rice Grassy Stunt Virus
RHT	Rathu Heenati
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
rRNA	ribosomal RNA
RRSV	Rice Ragged Stunt Virus
RWC	Relative Water Content
s	second
S.D	standard deviation

s^{-1}	second power $^{-1}$ (Hertz)
SA	salicylic acid
SAR	Systemic Acquired Resistance
SEL	Size exclusion limit
SEL	Size exclusion limit
siRNA	Small interfering RNA
SNAC	Stress-responsive NAC
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeat
SuSy	Sucrose synthase
t/ha	Tonne per hectare
TAE	Tris Acetate-EDTA
TCA	Tricarboxylic acid
TF	Transcription factor
TGA	TGACG motif binding
TN1	Taichung Native1
UGT1	UDP- glucose transferase
US	United Sates
USDA	United States Department of Agriculture
UV	Ultraviolet
V	Volts
WRKY	Amino acids in the conserved WRKYGQK signature motif contact
ZF	Zinc finger
ZFP	Zinc Finger Proteins
Zn	Zinc
$ZnSO_4$	zinc sulphate

β	Beta
ΔC_T	C_T (target gene) - C_T (endogenous control)

Chapter 1. General Introduction

1.1 Global food security

Food security is one of the most important and challenging issues which has drawn the attention of policy-makers, researchers and farmers across the world. This is due to the dramatic increase in human population in both the developing and the developed countries. The global population is estimated to reach 9.7 billion by 2050 and the demand for food will inevitably increase with this (Melorose *et al*., 2015). In 1996, the World Food Summit defined food security as “the availability of people to have economic and physical access to consume safe and nutritious food at all time to meet their dietary needs to lead a healthy life” (Pinstrup-Andersen, 2009).

The demand for crops is forecast to increase by approximately 100% to 110% from 2005 to 2050. However, recent studies have revealed that there will not be an increase in crop yield in many regions of the world growing important food crops. These regions failed to achieve the estimated range of 24%-39% of crop yield increase due to many socio-economic factors (Ray *et al.*, 2013). Many factors affect global food production including climate change, competition for arable agriculture land, water scarcity and energy. Farmers with less technical knowledge and skills also contribute towards the low yield of crop productivity (Godfray *et al.*, 2012).

A recent study suggested that by 2050, the world food consumption has to be increased by 70% to 100% from the current level to feed the growing world population (Godfray *et al.*, 2012). The consumption of cereals for both food and animal feed is projected to reach three billion tonnes by 2050 (FAO, 2009). Therefore strategies to increase food production are important to ensure sustainable food security globally.

As a solution to increase food production, more land was cleared for growing agriculture crops. However, over the past five decades, crop production has increased but the arable land for agriculture has only expanded by 9% globally. This is due to usage of new land for activities such as urbanization and protection of ecosystems. Other losses of land were due to climate change, soil erosion and issues of unsustainable land management. The demand from the biofuel industry to

produce good quality fuel has also been the cause for reduction of agriculture land (Godfray *et al.*, 2012).

According to the EU biofuel directive (2008), 10% of all transport fuel should come from biofuel by 2050. A certain percentage of the global cereal crop, sugarcane and palm oil production will be used in the biofuel industry. This requirement has increased the competition for land usage and hence global food prices. Many recent studies have also suggested that the land used for farming and conservation management should be separated. This approach will increase the yield of crops and ensure a sustainable environment (Tscharntke *et al.*, 2012).

In order to overcome all these challenges, as well as to sustain the global food production, new farming technologies and introduction to biotechnology traits and development of new breeding techniques will have to be adopted to increase crop production. Development of these technologies globally will help to fulfil the demand for feed, fuel, and food without using large land areas (Edgerton, 2009).

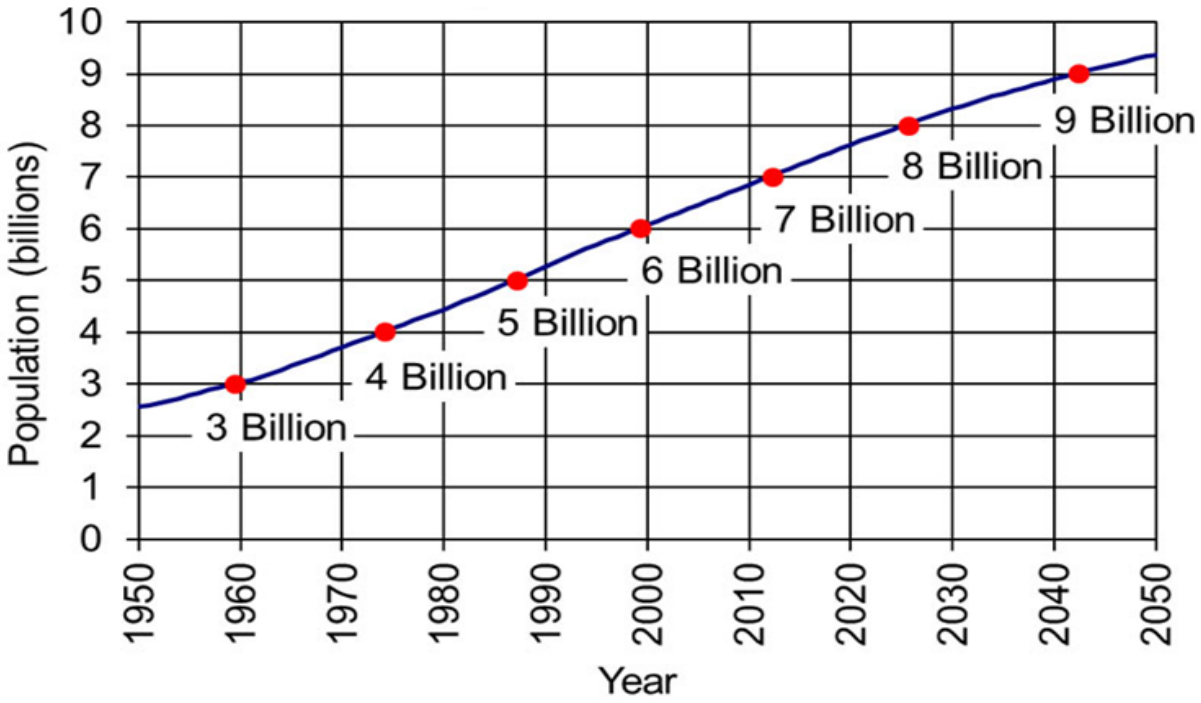


Figure 1.1 World Population from 1950 to 2050 (U.S. Census Bureau, International Data Base, 2016).

1.2 Rice

1.2.1 *Origin and Distribution of Rice*

Rice is one of the most important cereal crops in the world after wheat. There is evidence that this crop has been grown for more than 6500 years in many countries. Rice was first cultured in China around 5000 B.C followed by Thailand around 4500 B.C and then Cambodia, Vietnam and Southern India (Gnanamanickam, 2009).

Rice is now grown in every continent except Antarctica. The domesticated rice consists of two species which are *Oryza sativa* and *Oryza glaberrima*. *O. sativa* is widely grown in Asia, North and South America, the Middle East and Africa while *O. glaberrima* is grown in Africa (Muthayya *et al.*, 2014). *O. sativa* is used in many research studies because it is the most cultivated rice in many rice growing regions (Izawa and Shimamoto, 1996).

Recent studies showed that *O. sativa* is grown in more than 100 countries. It is made up of two main subspecies, the Japonica and Indica varieties. Japonica is an irrigated rice which is grown in temperate areas while Indica is grown in warm tropical regions (Gnanamanickam, 2009). These rice cultivars are usually classified according to their grain shape and texture. The long-grained Indica rice variety is widely grown in tropical and subtropical Asian countries and the short/medium-grained rice is usually cultivated in regions such as Japan and northern China. The medium-grained Japonica rice is grown in the Philippines and the mountainous areas of Madagascar and Indonesia (Muthayya *et al.*, 2014).

The origin of rice has been debated for a long time however, the domestication of rice is one the most important developments in the history of rice which remains the largest grown cereal crop in the world (Gnanamanickam, 2009).

1.2.2 *Rice as a global staple food*

Rice is one of the leading food crops in the world and provides more than 50% of all calories consumed by the entire human population. Every year around 154 million ha of rice is harvested globally (www.knowledgebank.irri.org). Rice is also the staple food and the main source of energy for more than half of the world's population. It is also an important food commodity in India, China, and Asia where 92% of the world's rice is grown (Gnanamanickam, 2009).

The demand for rice consumption is estimated to increase by 2025 as 4.6 billion people are predicted to consume rice in their daily diet (Gnanamanickam, 2009). One study also projected that the world will need approximately 880 million tonnes of rice by 2025 which is 92% higher than the amount of rice consumed in 1992 (FAO, 1999). In sub-Saharan Africa, the consumption of rice has doubled since 1970. The countries in the Caribbean and Latin America regions also reported an increase in rice consumption among their population (Muthayya *et al.*, 2014). Hence, rice production needs to be increased rapidly to fulfil the demands of the growing global population.

1.2.3 *World rice production*

An increase in global rice production has been observed over the last three decades of the twentieth century. Annual global production of paddy rice is approximately 715 million tonnes and there are 15 countries around the world which are major contributors to world rice harvest. China and India account for 50% of the global rice-growing areas. Overall China, India, Indonesia, Bangladesh, Vietnam, Myanmar, Thailand, Philippines, Japan, Pakistan, Cambodia, the Republic of Korea, Nepal and Sri Lanka and other Asian countries contribute 90% of the world total rice production. Other countries such as Brazil, the United States, Egypt, Madagascar, and Nigeria also contributes 5% of the world rice production (Muthayya *et al.*, 2014).

According to the FAO, the world rice production in 2015/16 was 491.7 million tonnes which showed a reduction of 2.7 million tonnes compared to the world rice production (494.4 million tonnes) in 2014/15. This reduction was due to the El Nino phenomenon which affected rice production in Latin America, the Caribbean and in Oceania. The largest rice production in 2016 was in India and Thailand. Countries such as Indonesia, Malaysia, and Vietnam also showed a decline in the rice production due to drought and abnormally high temperatures during the planting and development stage of the crop. The FAO estimated that world rice production will reach 499.3 million tonnes in 2016/17. This record shows an increase of 7.6 million tonnes in the global rice production (FAO, 2016).

Asia is anticipated to produce 454.4 million tonnes in 2017 which is 0.6% higher compared to 2016. Indonesia is also expected to show an increase in rice production by 46.6 million tonnes (2%) in 2017 due to the expansion of land area and enhanced

irrigation infrastructure. China also showed an increase of 0.4% (142.3 million tonnes) in 2017 compared to 2016. This is due to the lower procurement prices by the government of China. Other countries such as Malaysia, Myanmar, Nepal, Pakistan, the Philippines, Thailand and Turkey also showed positive progress in rice production in 2017. However, Vietnam and Bangladesh did not show much increase in rice production due to flash floods in the rice growing areas in both these countries (FAO, 2017).

The USDA estimated that rice production in the Philippines for 2016/17 will remain unchanged at 11.5 million metric tons, which only showed an increase of 1% since 2015. This is due to typhoons in October and December 2016 which affected the most important agriculture regimes of the country. Sri Lanka was forecast for 2.35 million tonnes of rice production in 2016/17 which was down by 29% from the previous year. The decline in the production was due to drought and major floods at the end of May (USDA, 2017). Global rice production is forecast at 502.6 million tonnes for 2017/18 which is 0.7% (3.3 million tonnes) more than 2016/17 (FAO, 2017).

Ray *et al.* (2013) estimated that by 2050, global rice production should be increased by 42% to meet the demands of the growing population. Global rice production between 2015/16 and 2016/17 rose by only 0.9% and dropped to 0.7% between 2016/17 and 2017/18 (FAO, 2016 and FAO, 2017). The increase in global rice production achieved between these years is less than the estimated annual yield increase. Therefore rice production across the world needs to be rapidly increased within the limited land available. Many factors such as the expansion of agricultural land for growing rice, climate change, irrigation systems and government policies will contribute towards future rice production globally (FAO, 2017).

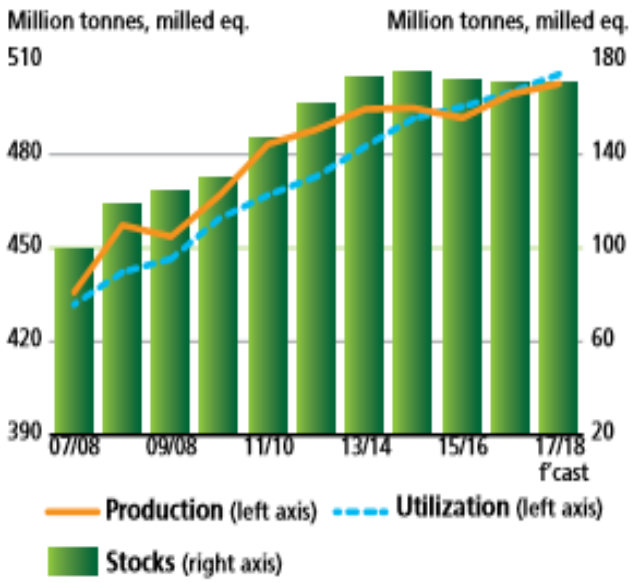


Figure 1.2 Rice production, utilization and stock from 2007/2008 until 2017/2018 (forecast) (FAO, 2017).

1.2.4 *Rice as a model plant for genomics studies*

Several studies have predicted that consumption and demand for rice will increase with the rise of global human population. Therefore, application of molecular techniques to improve rice production is in great demand. Besides being an important cereal food crop, rice has emerged as a model cereal for molecular biology studies (Goff, 1999). Rice is a monocotyledon plant with a small genome (430 Mb) compared to other cereal crops such as maize (2500 Mb) and barley (4900 Mb) (Bennetzen, 2002). Its genome size is three times larger than Arabidopsis which is a model dicotyledon plant (Ashikari and Matsuoka, 2002). Over the years, researchers have developed important tools for genetic analyses and genetic transformation techniques in rice (Bennetzen, 2002).

Sequencing of the *O. sativa ssp. Japonica* cultivar Nipponbare was initiated by the International Rice Genome Sequencing Project (IRGSP) in 1998 and the complete genome of this cultivar was publicly available in 2004. In the same year, the Rice Annotation Project (RAP) was launched and this RAP provides standardized and highly accurate annotation of the rice genome (Ohyanagi and Tanaka, 2006). Completion of the rice genome enabled great advances in rice improvement. The number of molecular markers identified was increased and their physical order was clearly understood (Jackson, 2016). Knowledge of sequence-based analysis of

variation in cultivated and wild rice gave a better understanding to the breeders to explore the genetic variation (McCouch *et al.*, 2012). The understanding of molecular genetic traits such as N- and P- use also drive rice research into the ‘Green Super Rice' project which will help to fulfil the demand of the growing world population (Zhang, 2007).

Genetic linkage maps of the rice genome have been used to develop molecular markers such as Restriction Fragment Length Polymorphism RFLP (Kishimoto *et al.*, 1993). High-resolution rice genetic-linkage maps have been constructed using Expressed Sequence Tag (EST) clones as Restriction Fragment Length Polymorphism (RFLP) probes. There were approximately 50,000 ESTs available in the EST database of rice genes (Shimamoto and Kyozuka, 2002). The Rice Genome Research Program (RGP) was started in 1991 to increase the application of genetic tools to four main groups which consist of cDNA analysis, genetic mapping, physical mapping and informatics (Sasaki, 1998). The genetic informatics of rice will help to increase yields and develop improved rice varieties that are tolerant to biotic and abiotic stress.

1.3 Biotic stress in rice

Many studies have shown that crop production for human consumption has been affected by insect pests. Crop losses caused by pests have an impact on the global agricultural economy. Crops such as soybean, wheat, and cotton were estimated to have losses between 26 -29%, 31% in maize, 37% in rice and 40% in potatoes due to insect pests (Oerke, 2006).

According to the report by the International Rice Research Institute (IRRI), it was estimated that 37% of loss in rice yield was due to pests and disease infection. Sparks *et al*. (2012) put this loss at between 29% and 41% based on the rate of production. Rice plants are infested by more than 100 species of insects. However, only 20 of these are categorized as serious pests and have shown significant damage to rice crops. Some of the major insect pests of rice are white-backed planthopper, *Sogatella furcifera*, rice brown planthopper, *Nilaparvata lugens*, the rice leaf folder, *Cnapalocrocis medinalis* and the green leafhopper, *Nephotettix virescens* (Ooi, 2015). The stem borrers cause approximately 70% yield loss of rice. Yield losses due to leaffolders were estimated to be between 63% and 80% (Gianessi,

2014). BPH was one of the major rice pests which contributed to 60% of crop losses globally (Prasannakumar *et al*., 2014).

Insect-resistant crops are an effective strategy in controlling pest in crops and have led to the reduction in insecticide use which helps to protect the environment and human health (Ansari *et al.*, 2015). In order to reduce crop losses from plant diseases and meeting the demand of the growing population, protection of crops against plant diseases is very important (Savary *et al.*, 2012). This can be achieved through developing rice varieties with broad-spectrum resistance to insect pests and diseases.

1.3.1 *Brown Planthopper, Nilaparvata lugens*

Brown planthopper, *Nilaparvata lugens* (BPH) is one of the most serious rice pests across the world especially in the temperate and tropical regions of East and Southeast Asia. BPH is a migratory, monophagous rice herbivore. The adult BPH are differentiated according to the length of their wings. The short-winged BPH biotypes cannot migrate but produce the larger number of eggs whereas the BPH with long wings is able to fly. The wing type is genetically controlled by insulin receptor genes. Both the short and long-winged BPH have caused severe crop loss across all rice growing areas in the world (Hu *et al*., 2016).

BPH sucks the rice phloem sap and causes direct damage to the plant by abstraction of nutrients, resulting in "hopperburn". "Hopperburn" is a phenomenon whereby the whole rice plant turns brown and completely dies. The early symptoms of "hopperburn" are yellowing of the older leaf blades of the rice plants than to all the other parts of the plant (Sogawa, 2015). Previous studies have shown that the physiology of the rice plants such as plant height and the number of tillers were affected by BPH infestation (Du *et al.*, 2009). These authors also reported that BPH infestation in rice caused reductions in leaf area, photosynthesis rate, leaf and stem nitrogen concentration, chlorophyll contents and dry weight of susceptible rice plants.

However, more importantly, BPH causes indirect damage as it transmits rice viruses such as Rice Ragged Stunt Virus (RRSV) and Rice Grassy Stunt Virus (RGSV) (Jena and Kim, 2010). Previous reports have revealed that BPH had affected the rice production in China and other countries in Asia as early as 1968 (Hu *et al.*, 2014). BPH outbreak in China caused a combined yield loss of 2.7 million tonnes of rice in

2015 and 2008. In Vietnam, 0.4 million tons of rice yield loss was due to RGSV and RRSV transmitted by BPH (Jena and Kim, 2010). Nagadhara *et al.* (2003) reported that the damage caused by BPH has been estimated at 250 million US dollars annually in South-east Asia.

One of the major factors responsible for the increase of BPH in the rice crop is the extreme usage of chemical pesticides among farmers. Farmers have extensively used chemical pesticides to control BPH infestation in the rice field. In 1988, 15% of the total world insecticides were used in rice production. The overuse of chemical pesticides has increased the total production cost and destroyed the natural predators of BPH, *Anagrus nilaparvatae* and also caused damage to the environment (Ghaffar *et al*., 2011).

Continuous use of insecticides also has resulted in BPH resistance to insecticides in many countries such as Taiwan, Japan and Philippines. Throughout Asia, insecticides are an important component of BPH control especially in countries where commercial resistant varieties are not available. Application of certain insecticides is reported to attract more macropterous hoppers immigrating into the rice fields, hence showed an increase in feeding, reproduction and longevity of BPH. Studies have shown that BPH outbreaks in farmers' fields were induced by insecticides (Chelliah and Heinrichs, 1981). Therefore, identification and development of rice varieties resistant to BPH are important to reduce rice crop losses and also maintain the agro-ecosystem in the rice field.

Figure 1.3 Brown planthopper, *Nilaparvata lugens* affecting rice crop (www.knowledgebank.irri.org).

Figure 1.4 “Hopperburn” due to damage caused by BPH (www.knowledgebank.irri.org).

1.3.2 *Mechanism of BPH feeding*

BPH is one of the most serious pests of rice and causes significant economic damage. Many studies have been carried out to understand the mechanism of BPH feeding which is important for rice planthopper management. Knowledge of the feeding behaviour is important to develop resistant varieties. BPH is a phloem-feeding insect pest in rice. One of the most important elements of the BPH is the stylet. The stylet consists of the piercing and sucking organ. It is approximately 650-700 µm long and includes an outer pair of mandibular and an inner pair of maxillary, stylets (Sogawa, 2015*)*. The BPH penetrates into the phloem sap of the plant using its stylet and probes for the phloem sieve-tube. The stylet of the BPH contains a salivary sheath which is made of solid saliva that is released during probing (Ghaffar *et al*., 2011). The salivary gland of BPH consists of the principle glands, accessory glands and salivary ducts, which play important roles in saliva secretion (Huang *et al.*, 2015).

Previous studies suggested that the mechanism of BPH feeding is divided into two phases. The first phase involves the movement of the stylet tips across the plant tissues and the second phase is the feeding process. In the feeding process, the stylet enters the vascular bundle and ingests the phloem sap (Ghaffar *et al*., 2011). The sheath material is watery or diffusible saliva, which contains digestive enzymes which are secreted by the salivary glands. The salivary glands also release α-glucosidase which hydrolyzes sucrose and trehalose and β-glucosidase which acts on phenolic glucosides such as arbutin and salicin (Sogawa, 2015).

Several studies have investigated the feeding behaviour of BPH using the Electrical Penetration Graph (EPG) technique. This technique is important to explore the feeding patterns of BPH across different type of rice varieties. Comprehensive studies on BPH feeding on rice varieties exhibiting different types/levels of resistance will help to elucidate the feeding mechanism of this insect pest which will be useful to identify new targets for its control (Ghaffar *et al*., 2011).

1.3.3 *Brown planthopper biotypes*

Different biotechnology methods have been developed to control rice yield losses. One of the most effective methods to control BPH damage and increase productivity in crops is by implementing the host-plant resistance strategy (Jena and Kim, 2010).

The first BPH resistance in rice was identified in 1967. There were 573 cultivated rice accessions identified to have resistance to at least one BPH biotype. A total of 484 accessions (92.5%) were identified to have resistance to biotype 1 and 80 accessions (15.3%) were resistant to biotypes 1, 2 and 3 (Hu *et al*., 2016).

In 2010, 21 major genes in rice for BPH resistance had been identified by IRRI. These genes were then used to distinguish resistance or susceptibility of rice genotypes to BPH biotypes. BPH biotypes are defined as a population or an individual which is different from other populations or individuals by nonmorphological traits such as adaptation and development to a particular host, host preference for feeding or oviposition or both. The biotypes of the BPH can be distinguished by their different virulence pattern in the rice genotypes. This study showed that there are four biotypes of BPH in rice. Biotypes 1 and 2 are found in Southeast and East Asia. Biotype 3 was identified after rearing BPH on the resistant ASD7 which carries the *bph 2* gene for resistance. Biotype 4, also known as the South Asian biotype, is one of the most destructive biotypes and was identified in the Indian subcontinent (Jena and Kim, 2010).

Rice cultivars with *Bph1* gene have been found to have resistance to biotype 1 and 3 but are susceptible to biotype 2. The *bph2* gene has resistance to biotype 1 and 2 but not to biotype 3 whereas the *Bph3* and *bph4*, *bph8* and *Bph9* genes confer resistance to all four biotypes (Jena and Kim, 2010). Some genes such as *bph5*, *Bph6* and *bph7* confer resistance to biotype 4 only. Elucidation of the genetics of BPH resistance will help in developing rice cultivars with a broad spectrum of BPH resistance, which is important for rice breeding programmes for resistance to this devastating pest (Hu *et al*., 2016).

1.4 Abiotic stress in rice

Over the years, the rice crop has been constantly exposed to various environmental stresses which resulted in significant reduction of rice production globally. Abiotic stresses are unfavourable environmental conditions that limit plant growth and productivity (Sarwat *et al*., 2017). Previous studies have shown that environmental constraints have affected plant growth and crop production. It was estimated that 50% of crop yield loss is due to abiotic stress (Wang *et al.*, 2016). In 2007, FAO reported that only 3.5% of the global land area is not affected by environmental

stresses (Cramer *et al.*, 2011). Hence, abiotic stress is a serious problem which needs to be overcome to enhance crop production.

Abiotic stresses such as salinity, water deficiency, chilling and heavy metals have affected the growth and physiological processes of plants (Basu and Roychoudhury, 2014). Two major abiotic stresses that affected rice yield are drought and salinity (Sperotto, 2014). Studies have shown that rice yield has declined by up to 68% due to salinity problems in the soil. Rise in sea levels due to global warming has led to surplus irrigation; this scenario increases salt stress in many rice growing areas. It has been predicted that if this scenario continues, 50% of the agricultural land will be lost by 2050 (Wani and Sah, 2014). In South and Southeast Asia, approximately 90 million ha of rice growing land was uncultivated due to the unsuitable conditions of the soil such as high levels of salinity, alkalinity, and strong acidity or excessive of organic matter (Ali *et al.*, 2006).

Zhao and Fitzgerald (2013) reported that high temperatures have caused a reduction in rice yield. This is due to the higher loss of carbon through increased respiration in the plant (Sarwat *et al*., 2017). In most of the rice growing areas, rice is cultivated at an optimal temperature (28/22°C). Therefore, any future increase in temperature will affect the growth and yield production of this crop (Korres *et al.*, 2017). In some countries, low temperature is also a limiting factor in rice production. Losses due to these factors can range between 0.5 to 2.5 t/ha and grain yield can be reduced by up to 26% (Sperotto, 2014). Another common abiotic stress in rice is submergence stress. Submergence stress has caused many symptoms such as elongation of leaf and stems, leaf degeneration and dry mass loss (Gao *et al*., 2007).

Nutrient deficiency has been shown to deleteriously affect rice production (Takehisa *et al.*, 2013). Zinc (Zn) deficiency is a constraint for rice crop after nitrogen (N) and phosphorus (P). Zn deficiency in rice affects the yield of the rice due to low solubilisation of Zn in the soil. P deficiency affects the uptake of P by the plant roots and also affects plant growth and hence yield (Lafitte *et al*., 2004). N is also one of the most limiting nutrients in rice which causes significant losses to the yield of rice (Zhou *et al*., 2010).

Irrespective of reduction in the yield, plants are able to adapt to the different extreme environmental conditions by triggering different mechanisms to cope with these

conditions. Studies have shown that genetic and physiological traits involved in tolerance to abiotic stress are difficult to be identified at the organ or tissue level (Sarwat *et al*., 2017).

Conventional breeding programmes such as hybridization, hybrid breeding, wide hybridization and ideotype breeding have developed rice varieties which are tolerant against salinity and drought. However, the success rate of conventional breeding is not sufficient to fulfil the demands of the world population (Wani and Sah, 2014). Therefore a greater understanding of the physiology and molecular biology of stress tolerance to abiotic stress is necessary to build improved stress-tolerant rice varieties (Gao et al., 2007).

1.4.1 *Nitrogen: Abiotic stress in rice*

Approximately 45% of the global rice growing area is affected by various abiotic stresses which have limited rice production (Lafitte *et al*., 2004). Nutrients are the second major abiotic constraint factor after water stress in many rice fields and have contributed to low productivity of rice crop (Haefele *et al.*, 2008). One of the nutrients which have an impact on the rice production is nitrogen (N). It is also one of the most important macronutrients required for growth and development of plants which is required for the production of high-yield rice crop (Vinod and Heuer, 2012). However, it has been reported that N is the most yield-limiting nutrient in irrigated rice production area around the world (Tayefe *et al*., 2014).

It has been estimated that rice removes around 16-17 kg N from the soil for the production of each tonne of rough rice (Kennedy, 2006). Therefore adequate levels of N in the soil are important to achieve optimal rice yields, especially in the lowland areas. Most of the flooded rice fields have low N content and only a small amount of N enters the soil through natural precipitation and biological N fixation. Although there is a significant amount of N (2-20 t ha^{-1}) available in the soil only a limited amount is absorbed by the plants (Vinod and Heuer, 2012). Crop plants are able to utilize only 30% - 40% of the applied N, which is present in soil in the form of nitrate and ammonium ions. Thus, more than 60% of the N in the soil is lost through leaching, denitrification, volatilization and microbial consumption (Kant *et al*., 2010). Reduction in the soil N supply causes N deficiency and contributes towards the decline of rice yields globally (Eagle *et al.*, 2000).

Deficiency of this nutrient results in symptoms such as the stunted and slow growth of the plants. Chlorosis in the leaves with a small leaf area has also been observed. In order to overcome this limitation, N fertilizer has been extensively used in the rice growing areas across the world (Kennedy, 2006). Cassman *et al.* (1998) estimated that the total amount of N fertilizer required in irrigated rice will have to be increased threefold in the next 25 to 30 years to achieve 60% of the increase in rice production required to sustain the expanding world population.

1.4.2 *Consumption of Nitrogen Fertilizers in Rice Crop*

Many countries in the world are facing challenges to produce sufficient food to sustain the demand of the growing population in their countries. Due to the high rate of industrial development, there is a rapid reduction of agriculture land for food crop production. Therefore, increasing the yield of the food crop will be the best solution to increase the food production. The common solution to increase the food production is by using fertilizers, especially N fertilizers (Zhu and Chen, 2002). Over the past 60 years, a large amount of N fertilizer has been used to increase crop productivity (Zhang *et al.*, 2015). Previous studies reported that 50% of fertilizers were used in human food production and the need for the fertilizer has increased to about 7.3 million tons worldwide every year (Khan *et al.*, 2017).

Research conducted in the 1960s and 1970s demonstrated that adequate N fertilizer is required to increase grain protein in improved rice varieties at the panicle initiation stage (Blumenthal *et al.*, 2008). One of the studies showed that there was an increase in plant height, the number of panicles, panicle length, grain and straw yield when the rate of nitrogen fertilizer was increased up to 80kg N/ha. In general, rice requires 1kg of nitrogen to produce 15-20 kg of grain. Therefore, additional N fertilizers need to be applied to rice grown in the lowland in order to produce higher rice yields (Tabar, 2013).

In China, the application of chemical fertilizers has increased rapidly since the 1950s. The total amount of chemical fertilizers exceeded 55.3 million tonnes (Mt) in 2005 which is 30% of the global agricultural N consumption (Qiao *et al.*, 2012). Dekhane *et al.* (2014) also reported that the Asian rice production has increased by 24% from 1965 to 1980 which was due to the usage of a higher rate of fertilizers mainly N-fertilizers. In the temperate zone in the United States, N fertilization has improved

annual rice production between 40-50% as it is one of the most critical nutrients in crops (Mikkelsen, 1987).

Previous studies have also reported that appropriate use of fertilizers can rapidly increase the yield and improve the quality of rice. However, application of the increased rate of N fertilizers may increase the yield but reduce the quality of the grain. Therefore, it is important to know the best dosage required in each variety of rice as this also influences other agronomic parameters such as the cycle, plant height, loading and moisture content of the grain (Tayefe *et al*., 2014).

1.4.3 *Environmental impact of N fertilizer*

Overdosage of N fertilizers to increase global rice production has resulted in high environmental pollution. In China, most of the farmers have resorted to using higher (500-600 kgN ha^{-1} year) than the recommended doses of N fertilizers to maintain previously attained yield levels which have resulted in reduced N recovery rate and environmental pollution (Talpur *et al.*, 2013).

The application of large amounts of nitrogenous fertilizer has caused severe pollution impact to aquatic ecosystems (Cai *et al.*, 2009). The high content of N which flows to the aquatic ecosystems caused an enormous growth of blue-green algae species which dominates the lakes, rivers, and streams (David, 1999). The increased use of N chemical fertilizers reduced the number of phytoplankton species which serve as a food source for fish and increased the growth of algae in the aquatic ecosystem. This excess algal growth creates anaerobic conditions which may result in the killing of fish populations (Ghosh and Bhat, 1998). Excessive usage of N fertilizers also leads to loss of biodiversity in the ecosystems, and can also affect the composition of species due to the susceptibility of certain organisms to the N compounds. It can also have serious health effects on humans and other animals (Zahoor *et al.*, 2014).

Around 65% of N is lost from the plant-soil framework through emission of gasses, erosion, and leaching. This loss has caused severe environmental impacts such as the greenhouse effect, diminishing ozone, acid rain and also leads to changes in the global N cycle and nitrate pollution (Tabar, 2013). The underground water which was contaminated with NO_3-N caused pollution due to the leaching down of N fertilizers through the soil. Drinking water containing more than 45 mg NO_3- N/L of water is dangerous to human and animal health (Ghosh and Bhat, 1998). Respiratory

problems such as ‘bluebaby syndrome’ and cancer are linked to nitrate contamination in drinking water (Hashim *et al.*, 2015). A high level of nitrate intake by cattle and sheep through water and plant sources has resulted in methemoglobinemia (Ghosh and Bhat, 1998).

Therefore loading of nitrogenous compounds to the ecosystems has resulted in unfavourable environmental conditions in rice growing areas and particularly in the developing nations (Ghosh and Bhat, 1998). Several approaches are being taken to reduce the use and environmental impact of fertilizer-N while maintaining, or even increasing plant productivity. Plant breeding approaches to improve nitrogen use efficiency in crops have been extensively developed. Understanding and increasing plant breeding techniques will also help to increase sustainability and reduce the impact of N-fertilizer on the environment. Therefore, breeding programmes with efficient use of nitrogen in crops are important to produce a crop with high yield and lessen the impact of environmental pollution (Cai *et al.*, 2009).

1.5 Plant-Insect Interactions

In the ecosystem, plants and insects interact with each other in a complex way. There are many defence mechanisms in plants which are used to reduce insect attack. These mechanisms involve chemical and physical barriers such as induction of defensive proteins, volatiles attracted by insect herbivores and production of secondary metabolites (Mello and Silva-Filho, 2002). Studies using microarray technologies to investigate the herbivore-induced transcriptome have revealed the novel discoveries of plant-insect interactions (Ferry *et al.*, 2004).

Overall, plant defence against insect herbivores is divided into constitutive defences and induced defences. The insect herbivore triggers the induced defence system of plants, which involves signalling pathways such as systemin, jasmonate, oligogalacturonic acid and hydrogen peroxide. Plants also produce volatiles in response to insect attack to induce defence responses (Gatehouse, 2002).

Many studies have identified the role of phytohormones in the regulatory mechanisms of the plant defence response. There are three important plant hormones involved in herbivore-induced defence responses: jasmonic acid (JA), salicylic acid (SA) and ethylene (ET). Cytokines, abscisic acid, gibberellins, and auxin

are also important hormones that play a role in herbivore-induced defence signalling (Stam *et al.*, 2014).

SA triggers the responses of plants against phloem-feeding insects and biotrophic plant pathogens. Accumulation of SA positively regulates the pathogenesis-related gene 1 (NPR1). The regulatory expression of SA responsive genes occurs downstream of NPR1 which interacts with the TGA type and WRKY transcription factor genes. The expression of these genes leads to activation of gene expression and the production of pathogenesis-related (PR) protein. JA is one of the most important regulators of defence responses against chewing insects, necrotrophic pathogens, and cell content feeders. Upon herbivory, JA is produced via the octadecanoid pathway. SA accumulates in plants upon interaction with aphids and whiteflies (Stam *et al.*, 2014).

Plants have developed defence mechanisms to protect themselves against herbivore insect pests. Studies have shown that application of signalling molecules, salicylic acid (SA), methyl jasmonate (MJ) and abscisic acid (ABA), to insect-free plants, demonstrated the association of these genes with specific defence-response pathways. Up-regulation of a SA-induced gene related to lipoxygenases that are involved in jasmonic acid (JA)-biosynthesis is suggestive of positive cross-talk between SA- and JA-mediated signalling pathways (Sardesai *et al.*, 2005). For an example, a study on aphid-wheat interaction showed that activities of key enzymes which belong to both JA and SA-signalling pathways and the relative transcript levels of key defence genes in the signalling pathways increased significantly with aphid-feeding. These authors proposed that aphid-feeding could activate both JA and SA -signalling transduction pathways (Zhao *et al.*, 2009). A comprehensive understanding of the connection between plants and insects is an essential key to create successful biological natural control of these insect pests (Mello and Silva-Filho, 2002 and Lu *et al.*, 2018).

1.6 Plant Responses to Combined Biotic and Abiotic Stresses

Plants have been constantly exposed to a broad range of environmental stresses. Plants under natural conditions are affected by the combination of two or more stresses. In many cases, plants under abiotic stress contribute towards infection by pathogen and herbivore pests. For example, increasing temperature in agriculture land is known to enhance crop susceptibility to pathogen infection and hence

increase pathogen spread. Simultaneous occurrence of different biotic and abiotic stresses resulted in complexity in plant responses, as the responses to these combination stresses are largely controlled by different signalling pathways that may interact and inhibit one another. Metabolic and signalling pathways are involved in these responses and include TFs and hormone signalling. To date, the majority of mechanisms underlying the tolerance of plants to a combination of different stresses remain elusive and further studies are required to better understand these complex responses (Suzuki *et al.*, 2014).

Breeding for resistance to combinatorial stress is challenging. Currently, there are limited studies on combined abiotic and biotic stress tolerance. However, recent studies on crop plants have shown that differentially regulated genes provide interesting candidates for understanding combined stress tolerance (Kissoudis *et al.*, 2014). The combination of two stresses (abiotic-biotic) does not always lead to negative impact on plants. For example, some stress combinations negate the effect of each other and caused a positive impact on the plant. One stress may also provide endurance to plants against another stress and hence the yield of the plants is not always affected (Pandey *et al.*, 2017). Pandey *et al*. (2015) reported that abiotic stresses such as drought, heat, salinity and nutrient stress have drastically altered the response of plants to biotic stresses. Similarly, exposure of plants to pathogen attack has shown to affect its response to abiotic stresses. These interactions can either provide resistance or susceptibility towards any of the two stresses depending on the plant species, pathogen and stress intensity. In *O. sativa*, low temperature decreased the resistance of plants to blast pathogen *Magnaporthe oryzae*.

The biotic and abiotic signal transduction results in a complex arrangement of interacting factors. There are certain genes that are involved in both biotic and abiotic stress signalling and therefore control the specificity of the response to multiple stresses. Many studies using transcriptomic tools have helped to increase our knowledge of such processes (Atkinson and Urwin, 2012). Therefore, understanding the plant responses to simultaneous stresses is crucial in providing opportunities for developing breeding programmes with broad-spectrum stress-tolerant crops. Chapter 5 of this study investigates the molecular responses of two rice cultivars, one resistant and one susceptible, to the combination of N deficiency stress and BPH infestation.

1.7 Molecular Breeding Strategy towards Improvement of Biotic and Abiotic Stress in Rice

Several studies have revealed that biotic and abiotic stresses are the major limiting factors in food crop production, which is a great challenge to achieve food security by 2050. Therefore, adoption of different breeding strategies to improve stress tolerance in plants has become very important to increase crop productivity (Ashkani *et al.*, 2015). This approach is defined as an application of molecular biotechnological strategies which are genotypic assays to improve or alter plant traits. Molecular breeding has resulted in the development of crop plants resilient to the different types of biotic and abiotic stresses (Gazal *et al.*, 2016).

Molecular breeding strategies have also proven to be more efficient compared to traditional breeding in improving tolerance to multiple stresses in plants (Gazal *et al.*, 2016). Many molecular breeding strategies such as the use of molecular markers, expressed sequence tags (ETSs), microarrays and genetic transformations have been applied to crop plants to produce plants with enhanced levels of stress tolerance. The development of DNA markers such as Quantitative Trait Loci (QTLs) mapping, marker-aided selection (MAS) and genetic transformation have enabled the production of high-quality rice crops (Ashkani *et al.*, 2015).

The accessibility of DNA sequence databases and complete physical maps in plants are vital tools to study integrated genomics. Extensive studies on gene expression were used to identify the molecular process and the interaction between gene functions and stress adaption. Technologies such as microarrays, RNA sequencing, microRNA sequencing and downstream analyses have been used to study transcriptomes in the development of stress tolerant rice plants. Differential regulated expression resulted in the development of improved rice plants which has led to generating transgenic rice plants with altered expression of the genes. Over the years, the genome transcriptome sequences which are available in the database were used to generate SSR (simple sequence repeat) and SNP (single nucleotide polymorphism) markers at a genome-wide scale in Indica and aromatic rice (Agarwal *et al.*, 2016).

Functional genomics techniques have been explored to identify the functions of genes and the interactions between genes to generate improved varieties of plants. This information has been used to inform the development of stress tolerant crops via

the inactivation or overexpression of candidate genes for the desired traits (Akpinar *et al*., 2013). RNA interference or silencing (RNAi) is another technology which has been used for crop improvement against many different biotic and abiotic stresses. The present study has used transcription factors (TFs) and RNAi technology to enhance tolerances to both abiotic and biotic stresses, which will be discussed in detail in the next chapters.

1.8 Transcription factors in rice and their role in biotic and abiotic stress

Plants have developed various efficient strategies to respond, adapt and survive under stress situations. Responses to biotic and abiotic stresses have lead the rice plants to develop defence mechanisms from the molecular level to the physiological level to adapt to this environment (Gao *et al*., 2008). In plants, the gene regulatory network and plant physiology or morphology can be altered if any changes are triggered in the TF regulation, their sequences or their target DNA sequences (Khong *et al.*, 2008). The response mechanisms of plants are regulated by many genes that encode regulatory proteins. One example of a regulatory protein is a transcription factor (TF) (Alves *et al.*, 2014). Many studies have reported that stress-tolerant rice cultivars were developed based on their phenotypes and physiological responses under various stresses. These studies have revealed that TFs from different families have been reported to be involved in stress-response pathways (Shankar *et al*., 2016).

TFs have drawn particular interest from plant breeders because they are good candidates for genetic engineering to develop stress-tolerant crops. TFs are able to regulate many stress-responsive genes. Hence, these TFs have been used in model and crop plants to improve tolerance to multiple stresses under field conditions (Alves *et al.*, 2014).

TFs are DNA-binding proteins that control gene expression by binding to specific DNA sequences called cis-elements which are located in the promoter region of the gene. A previous finding reported the role of TFs in growth, development, and defence of the plant (Seo *et al*., 2015). Xiong *et al.* (2005) also reported that regulation of gene expression is essential in response to environmental conditions, regulation of metabolic pathways and defence against pathogens.

Several TFs were shown to play an important role in stress signalling either by acting as positive or negative regulators of stress-responsive genes. Hence, understanding the transcriptional response of plants to stress requires extensive studies for better understanding of plant growth and development patterns (Roy, 2016).

Many TFs in rice have been shown to play roles in abiotic and biotic stress responses. There are approximately 7% of coding sequences which consist of TFs in the plant genomes. The TFs are divided into different families based on their distinct signatures in structure (Thao *et al.*, 2014). TFs such as AP2/ERF, bZIP, Zn-finger, NAC, MYB and WRKY have been identified to play a major role in abiotic and biotic stress tolerance in rice (Santos *et al.*, 2011).

Although extensive research has been carried out on the functions of these genes, most of the research carried out to date has been focused on a single gene with a single stress response. Due to the complexity of the regulation of stress responses, many studies have not been done on the combination of abiotic and biotic stresses that enhance plant stress tolerance (Wang *et al.*, 2016). Therefore, research focused on identification of TFs which play a role in both abiotic and biotic stresses is an important strategy to generate improved stress-resilient rice cultivars. Chapter 4 and chapter 5 of this study have investigated the response of TFs from different families to N stress, and the response of these same TFs to a combination of both N and BPH stress in susceptible and resistant rice cultivars.

1.8.1 AP2/ERF TFs

The AP2/ERF is one of the largest of the TF families. The presence of these TFs has been reported in bacteria, bacteriophage, ciliate and plants. There are many AP2/ERF TFs which have been isolated from rice. The AP2/ERF TFs have one or two AP2 domains consisting of approximately 60 conserved amino acids (Seo *et al*., 2015). This TFs family is divided into four subfamilies based on the similarity of the DNA binding domain which consists of AP2 (Apetala), RAV (Related to AB13/VP1), ERF (Ethylene responsive factor) and DREB (dehydration-responsive element-binding protein) (Wang *et al.*, 2016).

The DREB and ERF subfamilies contain a single AP2 DNA-binding domain which acts as regulators of biotic and/ or abiotic stress responses. The DNA binding specificity of the DREB and ERF are different. The DREB proteins interact with the

DRE/CRT cis-element which is present in the promoter of genes and are involved in abiotic stresses such as cold, drought and high salinity. ERF proteins are reported to bind to the GCC box element which is present in most pathogenesis-related (PR) genes (Santos *et al.*, 2011).

Previous studies reported that the ERF and DREB subfamilies are involved in plant responses to biotic and abiotic stresses. AP2/ERF TFs also play important roles in plant development processes, abiotic and biotic stress responses and plant hormone responses. There were 163 AP2/ERF TFs identified in rice (Wang *et al.*, 2016). For example, the overexpression of *OsBIERF2* (BENZOTHIADIAZOLE- INDUCED ERF) in rice enhanced tolerance to drought, high salinity, and low temperature. *OsBIERF1*, *OsBIERF3*, and *OsBIERF4* were induced by pathogen infection and also by abiotic stresses such as cold, drought and salt. In rice, *OsDREB1B* and *OsDRED1A* were differentially expressed in response to cold, drought and high salt conditions. Overexpression of *OsDREB1B* in tobacco was shown to confer high resistance to virus infection and induced the expression of several PR genes (Santos *et al.*, 2011).

1.8.2 MYB Family

The MYB TFs in plants are characterized by the presence of a highly conserved MYB domain which consists of MYB repeats (R). These MYB repeats (R) are involved in DNA binding and protein-protein interactions. MYB proteins are divided into three subfamilies R-MYB, R2R3-MYB and R1R2R3-MYB (MYB3R) depending on the presence of one to three tandem MYB repeats. Studies have shown that the MYB protein is involved in responses to biotic and abiotic stresses (Santos *et al.*, 2011).

Over the years, 183 members of MYB TFs have been identified in rice. Many MYB TFs have been identified to be involved in the physiological and biochemical processes of the plant such as cell development and the cell cycle, primary and secondary metabolism, hormone synthesis signal transduction and responses to the different types of biotic and abiotic stresses (Wang *et al.*, 2016). The rice MYBS3 protein consists of a single MYB repeat (MYB1R), whose encoding gene is activated by ABA and induced by cold and salt. Overexpression of MYBS3 in rice has increased the tolerance of the plant to cold (Santos *et al.*, 2011). *OsMYB2* in rice was induced by salt, cold and dehydration stress and transgenic rice over-expressing *OsMYB2* showed significant tolerance to various stresses (Wang *et al.*, 2016).

Another study showed that *OsJMyb* encoding MYB TF plays a role in JA-mediated abiotic and biotic stress responses in rice. The *OsJMyb* overexpressing transgenic rice lines enhanced resistance to the rice blast pathogen, *Magnaporthe grisea* and could be useful for engineering rice crops for blast resistance (Cao *et al.*, 2015). However, only a few reports have identified these TFs to be involved in both abiotic and biotic stress responses in rice. Only a few OsMYB have been identified for their role in abiotic stress responses especially to cold stress response mechanisms (Santos *et al.*, 2011).

1.8.3 NAC family

The NAC TF family is one of the largest TF families. The NAC proteins contain a highly conserved N-terminal DNA binding domain (NAC domain) and a variable C-terminal domain that plays a major role in the regulation of transcription as an activator or repressor (Santos *et al.*, 2011). The NAC TFs interact with the NAC recognition sequence (NACRS) which consists of CACG core-DNA binding motif located in the promoter of these genes (Wang *et al.*, 2016). NAC TFs are involved in flower development, the formation of secondary walls and cell division, shoot apical meristem formation, leaf senescence and biotic and abiotic stress responses (Wang *et al.*, 2016).

The NAC TFs can be divided into seven subfamilies based on their gene functions. Recent studies revealed that proteins produced by pathogens interfere with the function of NAC TFs (Alves *et al.*, 2014). In rice the NAC protein was classified into two major groups, A and B. The A and B groups are then subdivided into 7 and 9 subgroups, respectively. The TFs responsible for development and stresses belong to group B (Santos *et al.*, 2011). Seo *et al*. (2015) reported that the responses to biotic stresses are closely related to the response to abiotic stress and or to hormone signalling.

One hundred and fifty-one NAC TFs have been identified in rice species. However, out of this large number, only 20 OsNAC genes have been identified in response to abiotic and biotic stresses. Extensive research has been done on the rice NAC TF genes but only 7 genes of this family which are the *OsNAC5*, *OsNAC6/SNAC2*, *OsNAC10*, *OsNAC19/SNAC1*, *OsNAC045*, *OsNAC52*, and *OsNAC063* have been reported to be involved in abiotic stresses such as drought and salt (Santos *et al.*,

2011). In rice, 26 NAC TFs were upregulated following infection by ‘rice stripe virus' or ‘rice tungro spherical virus'. These findings demonstrated that NAC TFs play an important role in the plant defence response (Seo *et al*., 2015).

1.8.4 bZIP family

The bZIP family comprises of approximately 60 to 80 amino acids with a conserved bZIP domain. This domain consists of a DNA-binding basic region and a leucine zipper (Seo *et al*., 2015). bZIP TFs also plays an important role in development processes of the plants as well as in response to abiotic stress such as drought, high salinity, and cold. There are 89 bZIP TFs identified in the rice to date (Wang *et al.*, 2016).

OsbZIPs in rice plays an important role in improving tolerance to stresses such as high salinity and drought. Many studies have shown that the stress response mediated via the bZIP TFs is activated by ABA which regulates the expression of stress. This occurs through interaction with specific ABA-responsive cis-acting elements (ABRE) in their promoter region. A previous study showed that *OsbZIP71* has a high tolerance to drought and salinity (Wang *et al.*, 2016).

1.8.5 WRKY family

The WRKY family of TFs is one of the largest families among all the transcriptional regulators in plants. These TFs have one or two WRKY domains consisting of 60 amino acids. The WRKY domain contains a conserved WRKYGQK motif at the N-terminus and a C2H2 or C2HC zinc-finger motif at the C-terminus, which binds specifically to W-box cis-elements with a sequence of TTGACC/T located at the promoter regions of many target genes (Wang *et al.*, 2016).

The WRKY TF family is divided into three groups based on the number of domains. Group I consists of two domains and groups II and III have one WRKY domain each (Santos *et al.*, 2011). WRKY TFs have been shown to play an important role in various processes in plants including plant growth seed development, leaf senescence and responses to biotic and abiotic stresses. There were 102 WRKY TFs identified in rice (Wang *et al.*, 2016). For example, *OsWRKY11* gene showed tolerance to heat and drought (Wang *et al.*, 2016) and *OsWRKY62.1* showed significant defence against the pathogen *Xanthomonas oryzae pv. oryza* (Seo *et al*.,

2015). Many studies have shown that *OsWRKY13* and *OsWRKY45* are involved in biotic stress resistance in rice (Santos *et al.*, 2011).

1.8.6 Zinc fingers

The zinc-finger protein plays an important role in many cellular pathways which are important in root development, flower development, carbohydrate metabolism and hormonal signalling. The C2H2-type Zn finger TFs are one of the most abundant Zn finger TFs which are involved in different forms abiotic stress. In rice, the C2H2-type TFs were differentially regulated by several abiotic stresses (Santos *et al.*, 2011).

These TFs are also known as TFIIIA-type zinc finger TFs which are characterized by two cysteine and two histidine-residues that bind to a zinc ion to form a structure that binds to the major groove of DNA. Although many genes encoding C2H2-type Zn finger TFs have been identified in rice only a few have been functionally characterized e.g. the ZINC FINGER PROTEINS 182 (ZFP182), ZFP245, ZFP252 and drought salt tolerance (DST). The overexpression of ZFP182, ZFP245, and ZFP252 increased tolerance to abiotic stress conditions. In rice, overexpressing ZFP252 was reported to have altered levels of *OsDREB1A*. Therefore C2H2-type TFs are signalling components that can be located either up-or-downstream of the DREB1/CBF genes (Santos *et al.*, 2011).

1.9 RNA interference (RNAi)

Over recent years, RNA interference (RNAi) has been a focal point in the field of plant molecular biology. This technology has proven to play an important role in crop improvement in most important food crops. RNAi has turned out to be a powerful and reliable tool to inhibit the expression of targeted gene sequences (Younis *et al.*, 2014).

Extensive studies using RNAi technology has resulted in breeding lines with desired traits such as resistance to pests and pathogens, tolerance to biotic and abiotic stresses and improved nutritional quality (Tyagi and Mohanty, 2000). The discovery of novel genes and their expression pattern in response to abiotic stress has provided a platform to engineer effective strategies for greater stress tolerance. Alterations in plant architecture such as plant height, shoot branching, stem elongation and leaf morphology have shown a great impact on agronomic traits in

terms of yield, physiological and biochemical processes and resistance to environmental stresses (Jagtap *et al*., 2011).

In rice, suppression of *OsPINI* expression resulted in changes in tiller numbers and the shoot/root ratio. This suggested that *OSPINI* played an important role in auxin-dependent adventitious root emergence and tillering. RNAi was also used to suppress the expression of *OsGA20ox2* gene in rice which encodes the regulatory enzyme GA 20-oxidase. This transgenic rice exhibited reduced levels of endogenous biologically active GA1 which decreased plant height and resulted in semi-dwarf phenotype (Jagtap *et al*., 2011). Expression of *OsPL14* exhibited a decrease in tiller number and enhanced yield of rice grain (Kamthan *et al.*, 2015).

Crop losses due to insect pests have caused several impacts on crop production. Ferry and Gatehouse (2010) reported that more than 500 species of insects and mites are resistant to one or more insecticides. This indicates that novel strategies to control insect pests are highly important. Many studies have demonstrated that the development of systemic RNAi in controlling insect pests has been a successful strategy to control insect pests in major crops such as rice, wheat, maize, potatoes and cotton and soybean (Koch and Kogel, 2014). For example, the silencing of *NIHI1* gene in the midgut of *Nilaparvata lugens* has reduced virulence of this devastating pest in the rice crop (Koch and Kogel, 2014). Recent findings revealed that RNAi-mediated suppression of rice gene *OsSS12* has increased resistance towards *M.oryzae* and *X.oryzae* (Onaga and Wydra, 2016). RNAi strategies also have a high potential for developing plants with improved resistance to abiotic stresses. A novel stress-related miRNA from rice seedling was reported to show high tolerance to cold dehydration and salt (Jagtap *et al*., 2011).

Although RNAi has been successfully proven to be a powerful tool in understanding the function of genes in plants, this technology has not been fully exploited in crop protection. This technology will lead to the novel discovery of plants with high tolerance to various stresses and resistance to insect pests in this crop, which will be an important factor in increasing rice productivity. Chapter 2 of this study investigates in detail the effects of silencing the β-1,3-glucanase gene which we hypothesise to be involved in the defence system of the susceptible rice cultivar, TN1.

1.9.1 Mechanism of RNAi in plants

RNAi is a biological mechanism which occurs during post-transcriptional gene silencing (PTGS) and is triggered by double-stranded RNA (dsRNA) molecules to prevent the expression of specific genes (Younis *et al.*, 2014). Introduction of dsRNA molecule into the cytosol resulted in degradation of this dsRNA into small interfering RNAs (siRNAs) by a RNase III Dicer, which is about 20 -25 basepair long. A RNA-induced silencing complex (RISC) is then used to distinguish between the two siRNA strands as either sense or antisense. The sense strands, which have the exact same sequence as the target gene, are degraded. The antisense strands are assembled to the RISC and are used as a guide to target messenger RNAs (mRNA) in a sequence-specific manner. The mRNA is then cleaved by RISC. The activated RISC can repeatedly participate in mRNA degradation, which inhibits protein synthesis. This results in knock-down, or knock-out, of protein expression (Sheikh *et al*., 2014).

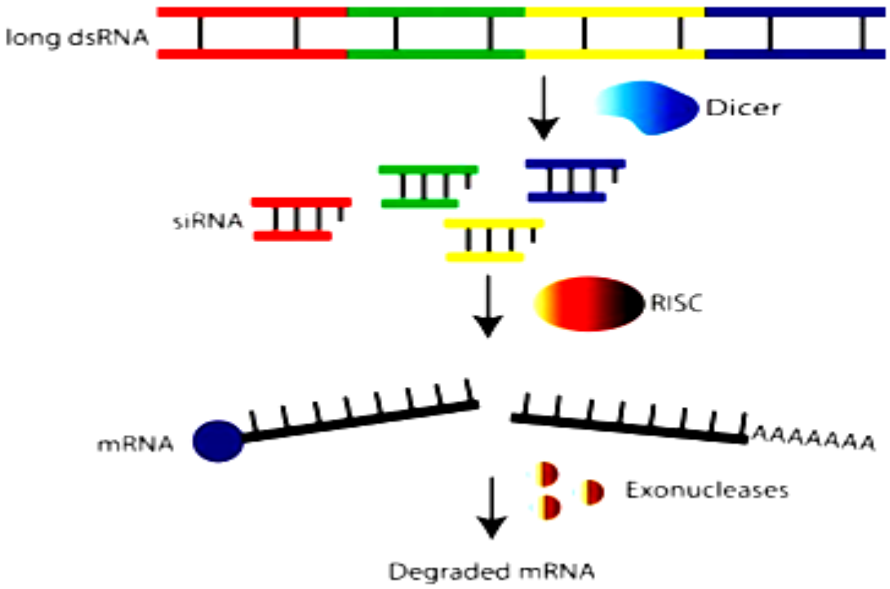


Figure 1.5 Mechanism of RNA interference (RNAi) (Sheikh *et al*., 2014).

1.10 β-1,3-glucanase gene in rice

β-1,3-glucanases are ubiquitous in various plant species including Arabidopsis, rice, tobacco and soybean. Their encoding genes have formed a complex and diverse families in plants and are involved in several physiological and development processes in addition to defence against pathogen infection (Xu *et al.*, 2016). Expression of β-1,3-glucanase genes in combination with other antifungal genes has

been reported to be a potential strategy to develop resistance against fungal pathogens in crop plants (Balasubramanian *et al.*, 2012).

Previous studies reported that there were 14 β-1,3-glucanase genes found in rice. These genes together with other monocot β-glucanase genes are classified into four subfamilies: endo-1,3- β-glucanases (EC 3.2.1.39 subfamily A), endo-1,3:1,4- β-glucanases (EC 3.2.1.73 subfamily B) and subfamilies C and D. β-glucanases in subfamily A are predicted to play important roles in plant defence and development. Two tandem gene clusters have been identified in subfamily A which includes Gns2-Gns3-Gns4 and Gns5-Gns6. *Gns5* is presumed to encode a PR-2 protein which is involved in defence against pathogen attack and is induced by BPH in the susceptible rice plants (Wan *et al.*, 2011).

Hao *et al.* (2008) reported that out of six β-1,3-glucanases genes analysed (*Osg1*, *Gns2*, *Gns3*, *Gns4*, *Gns5* and *Gns6*), only four genes (*Osg1*, *Gns4*, *Gns5* and *Gns6*) showed differential expression profiles in response to BPH infestation in the susceptible TN1 and resistant B5 rice cultivars. The author reported that *Osg1* and *Gns5* were up-regulated to a higher magnitude in the susceptible TN1 compared to the resistant B5 rice cultivar upon BPH infestation and these genes were presumed to play an important role in defending the plant against BPH attack.

Another study also reported that *Gns5* showed an increase in gene expression in the susceptible TN1 rice lines in response to BPH, but remained unchanged in the resistant Bph15 rice lines (Wei *et al.*, 2009). The results of this finding demonstrated that the *Gns5* gene is induced during BPH infestation in the susceptible TN1 rice plants suggesting that *Gns5* potentially plays an important role in callose deposition.

1.11 Role of callose deposition in plants

Plants under severe stress conditions have developed various chemical and physical defence mechanisms to protect themselves against pathogen attack. The cell wall is one of the most important components in the plant cell which protect plants from pathogen invasion. In plant cells, callose containing cell-walls are called papillae and are reported to play an important role as a barrier to protect the plant at the early stages of pathogen invasion (Luna *et al.*, 2011).

Callose is a polysaccharide made up of β-1,3-glucan with some β-1,6-branches and can be found in many species of higher plants. The callose synthesis process is

catalyzed by the multisubunit enzyme complex which is related to the plasma membrane. An important component of this enzyme complex is known as callose synthase (CalS). This enzyme complex also consists of UDP-glucose transferase (UGT1) and sucrose synthase (SuSy) which is important for the synthesis of callose (Piršelová and Matušíková, 2013).

Callose is involved in various stages of plant development and also in the response to multiple biotic and abiotic stresses. Previous studies indicated that callose is synthesized by a class of enzymes called callose synthases. In plants, callose deposition occurs at the plasmodesmata (PD) which regulates cell movement of molecules by controlling the size exclusion limit (SEL) of PD. Callose is also deposited when plants are wounded following infection by pathogens and other physiological stresses (Chen and Kim, 2009).

Callose deposition occurs at certain regions of the cell wall surrounding the plasmodesmata and its degradation is important in regulating the transport of substances through the symplast. Callose is also involved in formation and closing of the pores of the sieve plates and therefore is involved in intercellular communication in the plants. Previous studies suggest the importance of plant hormones and secondary metabolites in the synthesis of pathogen-induced callose. One such finding showed that increased concentrations of abscisic acid suppress the transcription of genes encoding β-glucanases and increases callose deposition in the plant tissues. Hence, an increase in callose deposition resulted in increased plant resistance (Piršelová and Matušíková, 2013). According to Fujita *et al*. (2013) callose deposition occurs through the activities of callose synthase and the callose-hydrolyzing enzyme β-1,3-glucanase and has been detected following planthopper attack.

Callose deposition may be different according to the degree of synthesis and subsequent degradation by β-glucanases. Deposition of callose in plant tissue is one of the ways plants defend themselves against pathogen attack. Callose deposition in sieve plate pores after wounding by herbivores reduces phloem conductivity. Wound callose is first deposited in the sieve plates and then spreads to surrounding wall regions. The induced callose will produce cells with new physical barriers that seal the injured plant tissues (Piršelová and Matušíková, 2013).

Du *et al.* (2009) reported that callose deposition on sieve plants is an important defence mechanism that prevents BPH from ingesting phloem sap. Genes encoding the callose-hydrolyzing enzyme β-1,3-glucanase, *Gns5* and *Gns9* were slightly down-regulated in transgenic plants expressing *Bph14* (a BPH resistance gene); this down-regulation prevented callose decomposition and resulted in the sieve tubes being occluded. In another study, a β-1,3-glucanase gene, *Osg1* was found to be induced by brown planthopper attacking the leaf sheaths of susceptible rice plants. Further studies have shown that silencing of the *Osg1* gene is important for callose degradation in pollen development and results in male sterility (Wan *et al.*, 2011).

Previous studies reported that *Osg1* and *Gns5* were induced by BPH infestation and play an important role in callose decomposition and thus facilitate ingestion of phloem sap by BPH from susceptible rice plants (Hao *et al.*, 2008). A similar result was also observed by Wei *et al.* (2009) who showed that *Gns5* plays an important role in callose decomposition thus affecting phloem transportation. This study also showed that some specific β-1,3-glucanases are active callose-decomposing enzymes, induced by BPH activity and responsible for the susceptibility of the susceptible rice plant. A recent study showed that increased expression of *PR2*, a gene that encodes 1,3-β-glucanase, activates callose hydrolysis in susceptible phloem cells during BPH attack (Jannoey *et al.*, 2017).

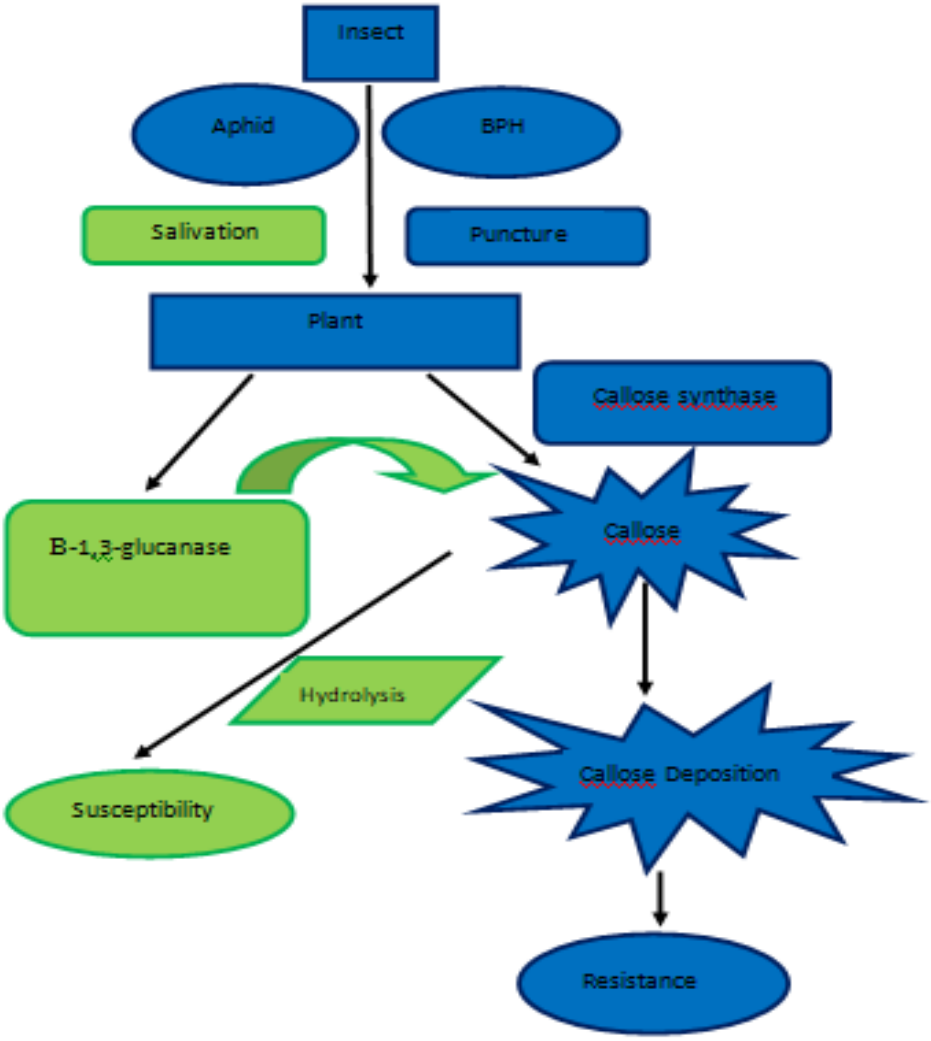


Figure 1.6 Schematic diagram showing the role of beta-1,3-glucanase in plant susceptibility (Shoala, 2012).

1.12 Aim and objectives of the study

The overall aim of this project is to better understand the molecular response of rice to its major insect pest *Nilaparvata lugens* (Brown planthopper; BPH) to inform future rice breeding programmes.

Hypothesis 1: Silencing of β-1,3-glucanase (*Gns5*) in the susceptible rice line TN1 confers enhanced resistance to BPH.

Hypothesis 2: The BPH-resistant rice cultivar IR70 exhibits greater expression of defence-related TFs under both a single stress (N) and dual stress (N and BPH) then the BPH-susceptible cultivar TN1.

The specific objectives of the study are to:

(i) Verify the role of β-1,3-glucanase (*Gns5*) in susceptibility of the rice cultivar TN1 to BPH using RNAi technology (Chapter 2).

(ii) Investigate the physiological response of two rice cultivars (TN1, susceptible to BPH; and IR70, resistant to BPH) under different levels of N stress (Chapter 3).

(iii) Investigate the link between N stress and BPH infestation, focussing on specific TFs which play important roles in the induced defence response in both TN1 (BPH-susceptible) and IR70 (BPH-resistance) rice cultivars (Chapter 4).

(iv) Identify TFs that are involved in the combination of both biotic (BPH infestation) and abiotic (N stress) in the TN1 and IR70 rice cultivars (Chapter 5).

Chapter 2. Knockdown of β-1,3-glucanase 5 in rice using RNA interference confers resistance to rice brown planthopper

Abstract

Brown planthopper (BPH) is one of the most serious rice pests in rice production, causing significant yield loss globally. Developing BPH resistant rice varieties is one of the most effective and environmentally friendly strategies for protecting the rice crop. This study demonstrated that knockdown of β-1,3-glucanase 5 (*Gns5*) in a susceptible rice cultivar, TN1 enhanced resistance to BPH. Two RNAi rice genotypes IR463, each using different constitutive promoters IR463 (CaM35S) and IR462 (maize Ubiquitin) were generated, as were their respective empty vector controls. Both RNAi lines significantly ($p<0.05$) decreased BPH survival and significantly ($p<0.05$) decreased the rate of development. Survival of insects on the RNAi lines decreased approximately to 50% as compared to their respective transformed empty vector lines and TN1, which were used as controls, with approximately only 30% BPH reaching adulthood on the RNAi lines compared to 75%-80% in the control lines. Microscopic examination showed that callose deposition increased significantly in response to BPH infestation in the sieve plates of the leaf and stem tissues of the IR463 and IR462 lines. In contrast, callose deposition in the control lines was rapidly reduced in response to BPH infestation. Our findings further revealed that BPH showed a significantly shorter duration of phloem ingestion and higher non-penetration and pathway occurrences in the RNAi lines using EPG studies. The results clearly showed that BPH feeding was inhibited on the RNAi plants expressing antisense *Gns5*. Taken together, the down-regulation of *Gns5* prevents callose breakdown. Therefore, increased callose deposition was observed in the sieve plates of the RNAi plants which kept the sieve plates occluded. This is an important defence mechanism that prevents the BPH from ingesting the phloem sap of the RNAi lines. In summary, the IR463 and IR462 RNAi lines expressing significantly reduced levels of *Gns5* enhanced resistance to BPH, so providing a sound strategy for breeding resistance rice varieties against BPH.

Keywords: BPH, *Gns5*, callose deposition, survival, phloem ingestion

2.1 Introduction

Rice brown planthopper (BPH; *Nilaparvata lugens*) is the most damaging insect pest in major rice growing countries, causing a significant reduction in global crop production which, in turn has a significant negative economic impact. Currently, the application of pesticides to control the BPH population in rice fields has not only led to environmental pollution but also kills the natural enemies of the target pest and causes the development of BPH populations that are resistant/tolerant to insecticides (Hu *et al*., 2016). Therefore, development of insect-resistant rice cultivars is the most economical and effective control means to overcome this problem (Liu *et al.*, 2017).

An important form of induced plant defence to phloem-sucking insects is callose deposition in the sieve plates of the plant (Yang *et al.*, 2018). Callose, as previously described in Chapter 1 of this study, plays an important role in response to abiotic and biotic stresses in plants. Callose is produced by callose synthases and is degraded by β-1,3-glucanases (Chen and Kim, 2009). Hao *et al.* (2008) reported that the β-1,3-glucanase encoding gene, *Gns5*, from the defence-related subfamily A was highly induced upon BPH infestation and is involved in callose decomposition in susceptible rice cultivar. Therefore, these authors suggested that *Gns5* plays a role in breaking down callose with subsequent consequences for resistance to BPH.

A previous study conducted by Shoala (2012) revealed that *Gns5* was the only gene which showed a differential expression profile in response to BPH infestation in the susceptible TN1, moderately resistant IR64 and resistant IR70 compared to other β-1,3-glucanase genes such as *Gns1*, *Gns2* and *Gns3*. This author also reported that *Gns5* was up-regulated in the infested susceptible TN1 rice cultivar to 2-fold and 2.8 fold at 3 h and 48 h respectively, after BPH infestation compared to the non-infested control plants. This pattern of gene expression was different compared to the moderately resistant IR64 and the resistant IR70 cultivar, where *Gns5* was down-regulated in IR64 in response to BPH feeding at most of all time points taken. Similarly, *Gns5* was down-regulated in the resistant IR70 in response to BPH feeding at 6 h, 12 h, 24 h and 36 h post infestation. In addition, the other β-1,3-glucanase genes such as *Gns1*, *Gns2* and *Gns3* were not expressed in IR64 and IR70 irrespective of whether plants had been infested or not by BPH. In contrast, the *Gns1* and *Gns2* gene were up-regulated in both the infested and non-infested susceptible

TN1 rice cultivar. In contrast, *Gns3* was not expressed in this non-infested and infested rice cultivar.

These initial findings from Shoala (2012) were important aspects to further investigate the role of *Gns5* knockdown in response to BPH infestation in the susceptible TN1 rice cultivar. Previous studies have demonstrated that transgenic plants expressing dsRNA targeting key genes have great potential for crop protection. For example, RNAi-induced gene silencing is a promising and effective tool to develop pathogen-resistant plants. This approach has the potential to be highly species specific. Therefore, RNAi technology can be very effective in creating crops with resistance against economically important pests and diseases (Younis *et al.*, 2014).

Shoala (2012) successfully demonstrated the construction of an RNAi vector system using vector IR462 (pCAMBIA 1300int-Ubi-hpRNAi) and IR463 (pCAMBIA 1300int-35S-hpRNAi), which may potentially reduce the expression of *Gns5* in the susceptible TN1 rice cultivar in response to BPH infestation.

2.2 Aim and objectives of the study

This study aims to demonstrate the role of the β-1,3-glucanase 5 (*Gns5*) in BPH susceptibility in TN1 using antisense RNA interference (RNAi) technology with two different constitutive promoters IR462 (pCAMBIA 1300int-Ubi-hpRNAi) and IR463 (pCAMBIA 1300int-35S-hpRNAi).

Hypothesis:

Knockdown of *Gns5* gene confers resistance to BPH in the susceptible TN1 rice cultivar.

Objectives of study:

(i) To investigate the role of *Gns5* in callose decomposition and subsequent effects for BPH resistance in the RNAi lines.
(ii) To investigate the effects of *Gns5* knockdown in the susceptible TN1 rice cultivar in response to BPH infestation.

2.3 Material and methods

2.3.1 *Plant materials and growth condition*

The RNAi plants expressing an antisense fragment to the *Gns5* gene (IR463 and IR462) and transformed plants expressing the empty vectors were used in this study. The transformation and regeneration of the transformed lines containing the antisense construct was carried out by the Chinese Academy of Agricultural Sciences, Beijing. The constructs of the RNAi vector systems are the binary plasmid vector pCAMBIA containing the 290 bp coding region of the *Gns5* gene with two different constitutive promoters, maize Ubiquitin promoter (IR463) and CaMV 35S promoter (IR462) together with the hygromycin phosphotransferase (*hpt*) gene. Full details of all constructs are provided in (Shoala, 2012).

RNAi plants of the T2 generation were grown for approximately four months and seeds were collected for future experimental studies. Seeds from this "bulk-up" were labelled as the T3 generation plants. The parental TN1 (susceptible to BPH) was used as the control throughout, whilst the IR70 rice cultivar (resistant to BPH) was used as the resistant control in studies investigating the callose deposition and EPG analysis. All experiments in this study were conducted using the fifth to sixth leaf stage of rice genotypes. The seeds of these rice genotypes were germinated as described in 3.3.1 of Chapter 3. The seedlings were grown in compost in the growth room at 28°C during the day and 17°C at night, 16h:8h day night length (photoperiod) and 280-330 µmol $m^{-2}s^{-1}$ illumination.

Table 2.1 Rice genotypes used in this study and their descriptions

Rice plants	Description
463-T3-8	Transformed RNAi plants expressing antisense fragment to *Gns5* (CaMV 35S promoter)
463-T3-26	
463-T3-41	
463-T3-22	
462-T3-16	Transformed RNAi plants expressing antisense fragment to *Gns5* gene (maize Ubiquitin promoter)
462-T3-18	
463-4	Transformed RNAi plants expressing the empty vector (empty vector controls)
462-20	
462-33	
TN1	Non-transformed parent BPH-susceptible rice cultivars
IR70	BPH-resistant rice cultivars

2.3.2 *Insect survival bioassay (long-term)*

For the long term survival bioassay, ten first instar BPH nymphs were introduced to each rice plant covered with a clear perforated bag which was clipped at the top and sealed at the bottom around the pots. Six replicates were set up for all RNAi plants, transformed plants with empty vector and the parental TN1 plants as described in Table 2.1. Nymph survival was monitored every day for 22 days. The overall fecundity of the insect was determined by allowing the surviving nymphs to mate at random and total nymphs produced from the egg laid were counted after a 40 day interval (by which time no new nymphs were emerging). The method described above was carried out according to Rao *et al.* (1998). The bioassay was conducted separately for IR462 and IR463 lines with the parental TN1 control and their respective empty vector lines.

2.3.3 *Short term bioassay*

A similar experimental setup as described in 2.3.2 was carried out in short term bioassays. Twenty-five late first instars (between first to second instar nymphs) were released onto each plant and the experiment was continued for 13 days. The survival, development and total insect biomass were determined as suggested by Rao *et al.* (1998).

2.3.4 *DNA extraction*

Genomic DNA extraction from the leaves of the rice genotypes was performed using the DNeasy Plant Mini Kit (Qiagen). Leaves of rice plants (fifth to sixth leaf stage) were harvested and immediately transferred into liquid nitrogen. Samples were freeze-dried for 48 h and stored at -80°C for future experiments. Extraction was carried out as recommended in the manufacturer's instruction and the eluted DNA samples were stored at -80°C. The quality and quantity of DNA were confirmed by using a NanoDrop® Spectrophotometer (ND-1000). The concentration of DNA (ng/µl), and 260/280 and 260/230 absorbance ratios were recorded. Samples with 260/280 and 260/230 absorbance ratio values of between 1.8-1.9 were selected for further analysis.

2.3.5 *RNA extraction*

All leaf samples used in this study were freeze-dried (ModulyoD Freeze Dryer, Thermo, Electron Corporation) for 48 hours before proceeding to RNA extraction. Approximately 100 mg of freeze dried leaf material was homogenized using the Tissue Lyser II (QIAGEN). Samples were then lysed using Trizol® Reagent (PureLink) and RNA extraction was performed according to the manufacturer's instruction (PureLink RNA Mini Kit, Life Technologies). Samples were replicated in triplicate for each of the biological treatment, in additional an extra replicate was used as an extraction control. The extraction control was included to ensure that there was no contamination in any of the extraction reagents used in this experiment. The quality and quantity of extracted RNA were confirmed by using the NanoDrop® Spectrophotometer (ND-1000). The concentration of RNA (ng/µl), 260/280 and 260/230 absorbance ratios were recorded. The 260/280 absorbance ratio corresponds to the presence of any protein, phenol, and contamination whereas the 260/230 ratio indicates the total genomic RNA present in the sample. Samples ranged from 1.9 to 2.0 of 260/230 absorbance ratio were selected for cDNA synthesis analysis. The RNA samples were immediately transferred and stored at -80°C

2.3.6 *PCR amplification for detection of hygromycin phosphotransferase (hpt) gene*

The 241 bp fragment specific to the *hpt* gene was amplified using the following primer pairs: Reverse-5’ CGGGGATTCCCAATACGAGG and Forward-5’-ATTTGTGTACGC CCGACAGT. PCR was carried out in a T100™ Thermal Cycler (BIO RAD) using the PCR Master Mix (2X) (ThermoFisher Scientific) as described in Table 5.2. The PCR conditions for *hpt* gene detection were as follows: initial denaturation at 95°C for 2 min followed by 35 cycles of denaturation at 95°C for 30s, annealing at 57°C for 30s, elongation at 72°C for 1 minute and a final elongation step at 72°C for 10 minutes. The PCR product was analyzed by 2% agarose gel electrophoresis as described in 4.3.9 of Chapter 4.

Table 2.2 PCR Mastermix reaction for detection of *hpt* gene.

PCR components	Volume per Reaction (µl)
PCR Master Mix	25.0
hpt Forward primer (10µM)	2.0
hpt Reverse primer (10µM)	2.0
DNA template (10ng/µl)	2.0
Nuclease Free Water	19.0
Total volume	50.0

2.3.7 *PCR for detection of antisense fragment of Gns5 transgene in IR463 and IR462 RNAi rice lines*

PCR was carried out as in 2.3.6 The Gns5-1 primers were designed using the Primer 3 programme (http://primer3.ut.ee) to detect the presence of the *Gns5* transgene in the IR463 and IR462 RNAi lines. PCR was conducted using primers Gns5-1(Reverse 5-TTGTAGAGCTGCACGACG and Forward 5'-AGGATAGTGGGATTGTGCGT) generate a 1079 bp antisense *Gns5* transgene fragment. The PCR cycling conditions for the detection of antisense *Gns5* in both the RNAi lines was as follows: initial denaturation at 95°C for 2 min followed by 35 cycles of denaturation at 95°C for 30s, annealing at 58°C for 30s, elongation at 72°C for 1 minute and a final elongation step at 72°C for 10 minutes. The PCR product was analyzed by 2% agarose gel electrophoresis as described in 4.3.9 of Chapter 4.

Table 2.3 PCR Mastermix reaction for detection of *Gns5*.

PCR components	Volume per Reaction (µl)
PCR Master Mix	25.0
Gns5-1 Forward primer (10µM)	2.0
Gns5-1Reverse primer (10µM)	2.0
DNA template (10ng/µl)	2.0
Nuclease Free Water	19.0
Total volume	50.0

2.3.8 *cDNA synthesis*

First-strand cDNA was synthesized using 1µg of total RNA according to the BIOLINE SensiFast™ cDNA Synthesis kit protocol. cDNA synthesis carried out in a T100™ Thermal Cycler (BIO RAD) and was subjected to primer annealing at 25ºC for 10 min, reverse transcription at 42°C for 15 min and inactivation at 85°C for 5 min. The samples were held at 4°C and kept in -20°C for long term storage.

Table 2.4 Mastermix preparation for cDNA synthesis.

PCR components	Volume per Reaction (µl)
Total RNA (1 µg)	n
5x TransAmp Buffer	4.0
Reverse Transcriptase	1.0
DNase/ RNase Free-Water	Up to 20 µl

2.3.9 *Quantitative PCR Analysis (qPCR)*

The qPCR experiments were conducted using the 2X SensiFAST ™ SYBR ® No-ROX Kit Master Mix (Bioline), gene-specific primers and *Actin* as an endogenous control (Table 4.3.4) with three biological replicates. Each biological replicate was then divided into three technical replicates. A two-step cycling qPCR was performed using the Rotor-Gene Q (QIAGEN) real-time PCR system. PCR conditions were 2 minutes of polymerase activation at 95°C, followed by 40 cycles of 95°C for 5 s for denaturation and 60°C for 15 seconds of annealing/extension. Increasing temperature (0.5°C $10s^{-1}$) from 60°C to 95°C was used for melt curve analysis to ensure that the products amplified were specific. Un-transcribed RNA was used as a negative control in each run. The primers used for detection of *Gns5* knockdown effect in RNAi plants were Reverse 5'- TGGTGAGGGCGATGCTTG and Forward 5'-TTGCGGCCATTCCTACAGT which produced a 185 bp fragment (Shoala, 2012). *Actin* (Wang *et al.*, 2012) was used as the endogenous gene for normalization of the qPCR data. PCR efficiency of primers to the antisense *Gns5* was also carried out as described in 4.3.7 of Chapter 4.

Table 2.5 Mastermix reaction for qPCR analysis.

Reagent	**Volume per reaction (µl)**
2x SensiFAST SYBR ® No ROX Mix	25.0
10µM forward primer	1.0
10µM reverse primer	1.0
Template (cDNA)	3.0
H_2O	20.0
Final volume	50.0

2.3.10 *Data analysis*

The relative differences in expression for each sample in individual experiments were determined by normalizing the C_T value for each gene against the C_T value of endogenous gene, *Actin*. The average of three biological replicates was used to obtain the expression value of each sample. These expression data were then normalized by subtracting the mean reference gene C_T value from their C_T value (ΔC_T).The fold change value was calculated using the expression $2^{-\Delta\Delta C}{}_T$, where $\Delta\Delta C_T$ represents ΔC_T condition of interest - ΔC_T control. The relative transcription levels were presented graphically on the log $_2$ scale (Caldana *et al.*, 2007). The standard deviation was calculated from the $\Delta\Delta C_T$ value of the gene in all the samples analyzed and presented as error bars in the gene expression graphs. A one-way ANOVA was applied to determine differences in gene expression between the parental TN1 control line, transformed empty vector lines and RNAi lines which are indicated by a single asterisk ($p<0.05$, Tukey-Post Hoc test).

2.3.11 *Microscopic examination of callose deposition in IR463 and IR462 RNAi lines*

The staining method of callose deposition using methyl blue was modified from Scalschi *et al.* (2015). The BPH-infested and non-infested RNAi plants, resistant IR70, susceptible parental TN1 and transformed empty vector lines were examined for callose deposition. All leaf and stem tissue of the infested plants were collected from the 13 day bioassay. The infested and non-infested IR70 were treated exactly as the other rice genotypes. IR70 was used as a resistant control to ensure the staining process was effective. In order to produce reliable results, the infested and non-infested rice genotypes examined were at the same leaf stage and growing conditions. The leaves of these rice genotypes were placed in 50 ml falcon tubes containing 96% ethanol for chlorophyll removal. The samples were then destained approximately for 2-3 days until they appear completely white. The 96% ethanol can be replaced if necessary for saturated destained samples. The samples were rehydrated in phosphate buffer (0.007 M, pH=9) for 40 minutes and then transferred to freshly prepared 0.05% methyl blue solution and incubated for a further 40 minutes, after which time they were again transferred to freshly prepared 0.5% methyl blue solution then placed in a vacuum desiccator for 2 minutes and were incubated for 72 h in the dark. For microscopic examination, samples were mounted

on slides with the adaxial surface up using freshly prepared 0.05% methyl blue. Stained callose appears as fluorescent blue spots under the fluorescence microscope (Leica DMRB) and bright white fluorescence under a confocal microscope (Zeiss LSM 880 with Airyscan) at 10x magnification.

Stem tissues were cross-sectioned and stained using methyl blue using the same procedure as the leaves. However ,the staining time was reduced to 5 minutes for each stage. No further overnight incubation was required. The samples were examined under the confocal microscope with 40x magnification. Callose deposition was observed as bright white fluorescence in the vascular bundle of the stem tissues.

The relative intensity of the leaf and stem tissues examined under the confocal microscope was quantified using the Image J (Fiji) software (http://imagej.net./Fiji/Downloads). The intensity of fluorescence of the images was calculated as mean intensity per unit area. For the leaf samples, mean intensity of three areas within a leaf was calculated. There were four replicates of each rice genotypes tested. The intensity per unit area measurement was carried out by calculating the average of the intensity of the four replicates. A similar calculation was performed for the stem tissues and there were three to four replicates for quantification of each stem tissue. Significant differences in the relative callose intensity between non-infested and infested rice genotypes were analysed using one way ANOVA with a Tukey Post-Hoc test (Minitab 17 Statistical Software).

2.3.12 *Electric Penetration Graph (EPG) Technique*

BPH feeding behaviour was recorded and classified using an eight-channel Giga-8d EPG recording system which was developed by Tjallinggi (1978) and used previously to investigate whitefly feeding behaviour on tomato by McDaniel *et al.* (2016) and Tosh and Brogan (2014). This study used adult brachypterous female at the fourth to fifth stage instar reared on TN1. The BPH was cooled at -20°C for 1 minute and then carefully connected to a 3cm length of 18.5µm diameter gold wire (EPG system Wageningen University) with conductive silver glue on their dorsum. The BPH were placed on the stem area of each rice plant and connected to the EPG apparatus (Ghaffar *et al.*, 2011). The experiment was conducted in the insectary at 28°C with a photoperiod 16h day:8h night. Probing behaviour was recorded for 12 h continuously. At least 20 replicates per rice genotype were used. These studies were carried out

using 463-T3-18 and 463-T3-26 RNAi lines, 463-4 transformed empty vector lines, parental TN1 and resistant IR70. The resistant IR70 was used as a resistant control as the EPG findings of this resistant rice genotype has been previously published in many studies.

The different waveforms are produced by the completion of a partial electrical circuit between the plant and the BPH's stylet, when the BPH probes the plant the waveforms correspond to different feeding behaviours of this species. Previous studies from Ghaffar *et al*. (2011) and Zhang *et al*. (2015) were used to identify the waveforms obtained in this EPG study. The raw data from the waveform analysis were exported and analysed using the spreadsheet devised by EPG ana Stylet a+. EPG waveform characterization namely NP (non-penetration), C (pathway), E1 (sieve element salivation), E2 (phloem ingestion) and G (xylem ingestion) were identified as described in previous studies using the Stylet a+ EPG_ ParProc (Systems EPG Analysis and Data Processing) (McDaniel *et al.*, 2016). The normality test and equal variances statistical analysis was carried out to test nonparametric samples. Analysis of variance (ANOVA) with p-value <0.05 was used to analyze the mean frequency and distribution of each waveform.

2.4 Results

2.4.1 *Detection of hpt gene in IR463 and IR462 RNAi lines*

PCR analysis using *hpt* primers was carried out to analyze segregation of hygromycin resistance in all the selected T3 homozygous RNAi lines expressing the antisense fragment of the *Gns5* gene and rice plants transformed with empty vectors. PCR analysis revealed that all the RNAi lines (IR463 and IR462) including plants transformed with empty vectors showed a specific amplified band (241 bp) of the *hpt* gene (Figure: 2.1 and 2.2). The parental TN1 rice plants, used for transformation, did not show any amplification in the PCR analysis.

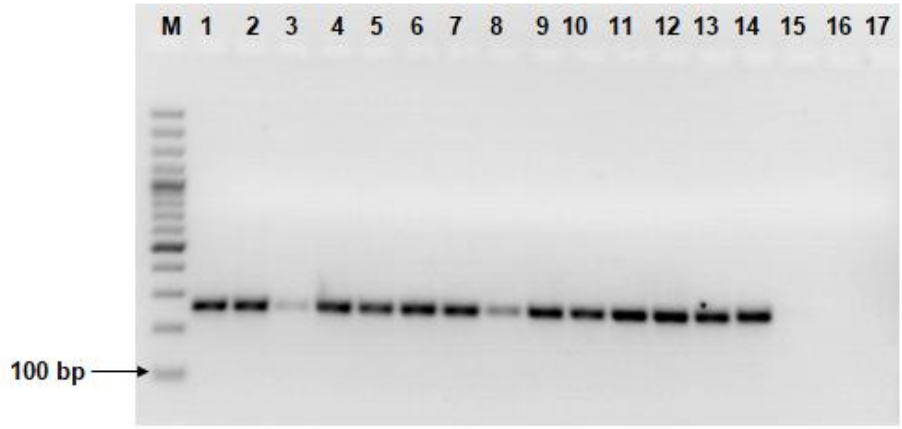


Figure 2.1 PCR analysis for the selection of the *hpt* gene from leaves of IR463 RNAi lines (241 bp). Lane M: 100 bp DNA Marker, 1-3: 463-T3-8, 4-6: 463-T3-22, 7-9: 463-T3-26, 10-12: 463-T3-41, 13-14: 463-4, 15-16: TN1, 17: PCR Negative control.

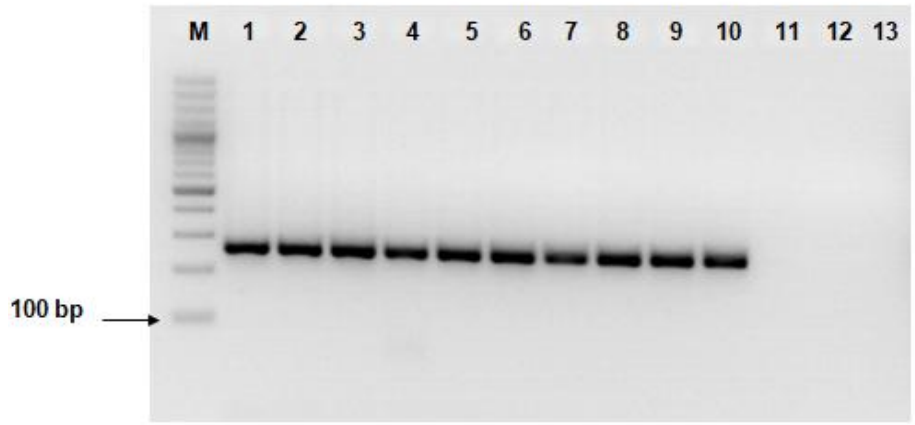


Figure 2.2 PCR analysis for the selection of the *hpt* gene from leaves of IR462 RNAi lines (241 bp). Lane M: 100 bp DNA Marker, 1-3: 462-T3-16, 4-6: 462-T3-18, 7-8: 462-20, 9-10: 462-33, 11-12: TN1, 13: PCR Negative control.

2.4.2 *Detection of antisense fragment Gns5 transgene in the IR463 and IR462 RNAi lines*

In order to demonstrate the presence of the antisense fragment of the *Gns5* transgene in the transformed RNAi lines, PCR analysis was conducted with the Gns5-1 primers. The PCR results showed that only the transformed RNAi lines expressing the antisense fragment of *Gns5* gene were amplified at 1079 kb in both the IR463 and IR462 RNAi lines while, no amplified band was observed in the lines

transformed with the empty vectors (463-4, 462-20 and 462-33) or the parental TN1 rice plants (Figure 2.3 and 2.4).

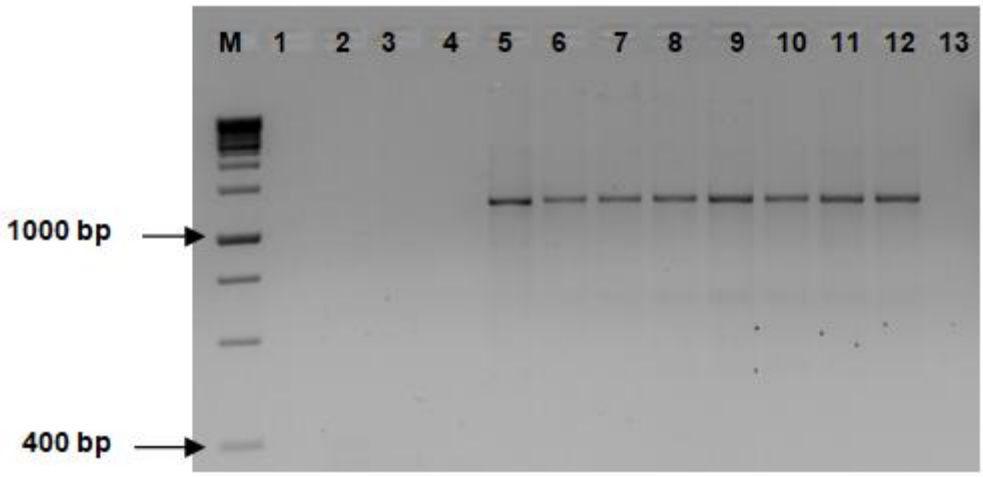


Figure 2.3 PCR analysis to detect the presences of *Gns5* in IR463 RNAi lines (1079 kb). Lane M: 1 kb DNA Marker, 1-2: 463-4, 3-4: TN1, 5-6: 463-T3-8, 7-8: 463-T3-22, 9-10: 463-T3-26, 11-12: 463-T3-41, 13: PCR Negative control.

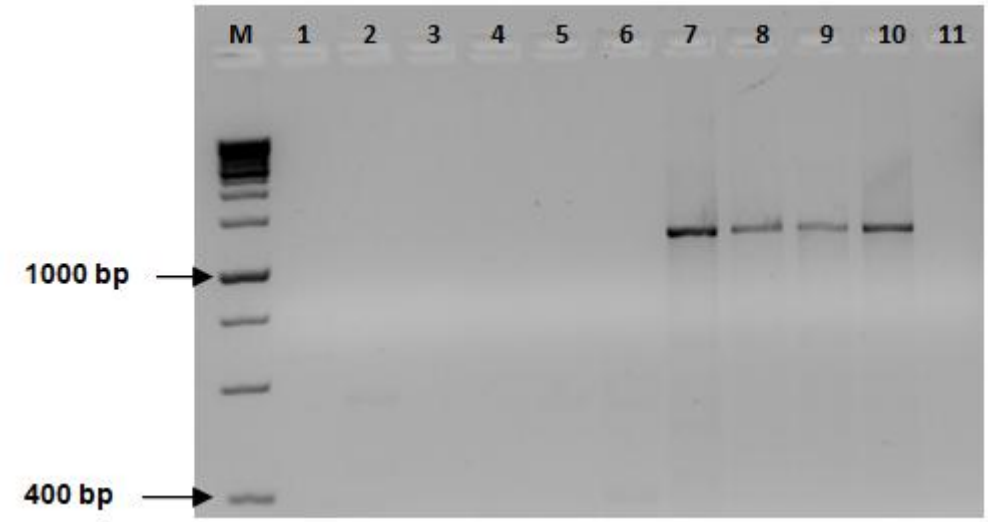


Figure 2.4 PCR analysis to detect the presences of Gns5 in IR462 RNAi lines (1079 kb). Lane M: 1 kb DNA Marker, 1-2: 462-20, 3-4: 462-33, 5-6: TN1: 7-8: 462-T3-16, 9-10: 462-T3-18, 11: PCR Negative control.

2.4.3 *Expression of Gns5 Knockdown in the IR463 and IR462 RNAi rice lines*

A qPCR efficiency test showed that *Gns5* demonstrated high PCR efficiency with E= 92.7% and the R^2 value of 0.997 which indicates reliable gene expression results. A qPCR analysis was carried out to analyse the efficiency of IR462 (pCAMBIA1300int-Ubi-hpRNAi) and IR463 (pCAMBIA1300int-35S-hpRNAi) RNAi lines in knocking down the expression of the *Gns5* gene. Results of the 3 biological replicates showed that the expression level of *Gns5* was significantly down-regulated in all IR463 RNAi lines tested (Figure 2.5). The transformed empty vector lines, 463-4, remained unchanged (~ 1 fold up-regulation) as did the parental TN1. *Gns5* in 463-T3-22 and 463-T3-41 was significantly ($p<0.05$) down-regulated by 2-fold respectively compared to the parental, TN1. The expression profile also indicated that *Gns5* in 463-T3-8 and 463-T3-26 was significantly ($p<0.05$) down-regulated to a greater fold change of ~ 3 fold in both the lines compared to the parental TN1.

Two further IR462 RNAi lines, 462-T3-16 and 462-T3-18, also exhibited a significant ($p<0.05$) down-regulation of *Gns5* by 4-fold compared to the parental TN1 (Figure: 2.6). The transformed empty vectors lines, 462-20 and 462-33, as expected, showed no down-regulation and remained (~ 1 fold up-regulation) the same as TN1. Interestingly, the IR462 RNAi lines (462-T3-16 and 462-T3-18) demonstrated a lower magnitude of gene expression compared to all the 463 RNAi lines expressing *Gns5*. This result indicates that the *Gns5* was successfully knocked down in both the IR463 and IR462 lines but to a greater extent in the IR462 RNAi lines.

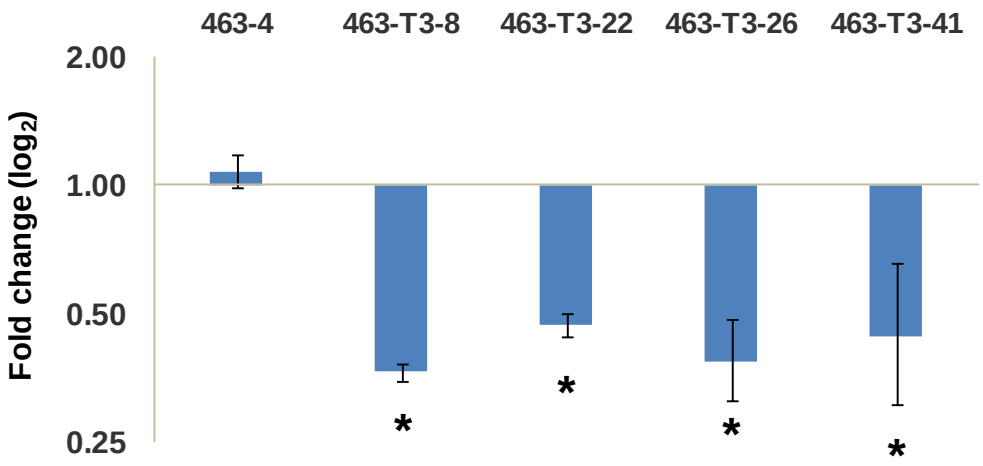


Figure 2.5 Gene expression of *Gns5* in IR463 RNAi lines and transformed empty vector line 463-4 compared to the TN1 control line which was set at 1.0. Significant differences between the control and samples were shown by a single asterisk *, $p<0.05$ according to one way ANOVA followed by Tukey Post-Hoc Test. The error bars represent the ± SD of the three biological replicates.

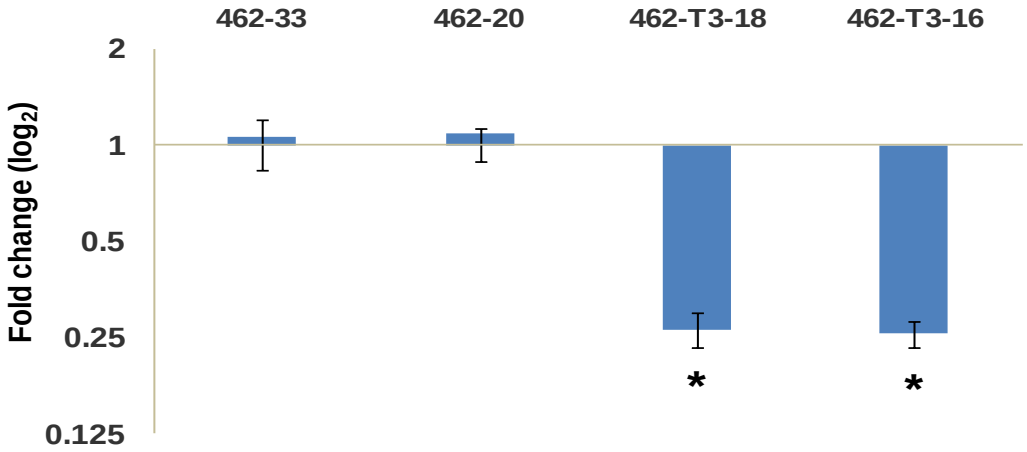


Figure 2.6 Gene expression of *Gns5* in IR462 RNAi lines and transformed empty vector lines 462-20 and 462-33 compared to the TN1 control line which was set at 1.0. Significant differences between control and samples were shown by a single asterisk *, $p<0.05$ according to one-way ANOVA followed by Tukey Post-Hoc Test. The error bars represent the ± SD of the three biological replicates.

2.4.4 *Survival of BPH on IR463 and IR462 RNAi lines*

In order to investigate the effects of *Gns5* on the survival and fecundity of BPH, a 22-day bioassay was carried out using homozygous RNAi plants, their corresponding transformed empty vector lines and the parental TN1 rice plants. The present study demonstrates that the expression of *Gns5* in the IR463 and IR462 RNAi lines significantly decreases the survival of BPH.

In this study, the survival on the control plants declined from 10 insects per plant at day 0 to an average of 5.7 and 6.3 insects per plant on the transformed empty vector line, 463-4 and parental TN1 plants respectively over the 22-day bioassay (Figure 2.7). However, in contrast, a rapid decline was observed on the IR463 RNAi lines where *Gns5* had been knocked down. A significant ($p<0.001$) decrease in the survival was observed in all the IR463 RNAi lines compared to the transformed empty vector line, 463-4 and the TN1. Survival of BPH constantly decreased through the assay from 10 insects per plant to an average of 2.7, 3.0, 2.3 and 2.0 insects per plant on 463-T3-41, 463-T3-26, 463-T3-22 and 463-T3-8 respectively, by the end of the bioassay (day 22). Therefore, the BPH survival was significantly decreased by 57.0%, 52.3%, 63.4% and 68.2% on the 463-T3-41, 463-T3-26, 463-T3-22 and 463-T3-8 respectively as compared to the parental TN1. There were no significant differences observed between the survival of insects on TN1 and the transformed empty vector line 463-4.

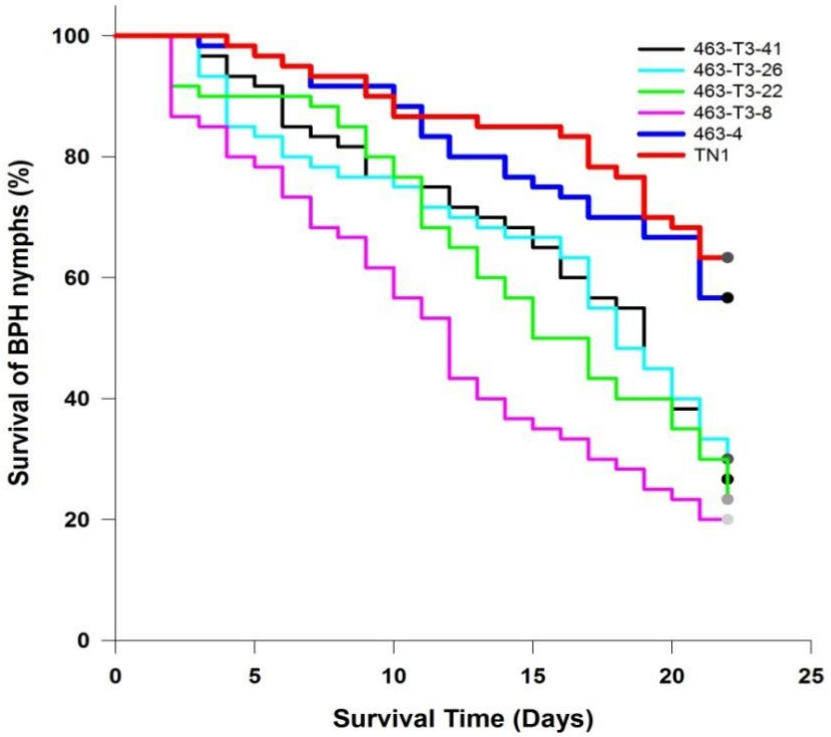


Figure 2.7 BPH survival bioassay showed that the survival of BPH on IR463 RNAi lines was significantly reduced compared to the controls (TN1; transformed empty vector line 463-4) according to Kaplan-Meier survival analysis Log Rank Test, $p<0.001$.

The IR462 RNAi lines (462-T3-18 and 462-T3-16) also showed similar reduced BPH survival in the 22 day bioassay (Figure 2.8). The results of the bioassay showed that BPH survival in the different control plants declined to an average of 6.2, 6.3 and 6.5 insects per plant on the 462-20, 462-33 and parental TN1 respectively at the end of the 22 days bioassay. BPH survival in the IR462 RNAi lines significantly declined from 10 insects per plant to an average of 3 insects per plant on 462-T3-16 and 2.7 insects per plant on the 462-T3-18 plants. Therefore, the survival of BPH has significantly reduced ($p<0.001$) by 53.8% on 462-T3-16 and 58.4% on the 462-T3-18 of both IR462 RNAi lines compared to the parental TN1. As observed in the IR463 RNAi lines, there were no significant differences observed between the survival rate of TN1 and the transferred empty vector lines (462-20 and 462-33).

The 22-day bioassay demonstrated that both the IR463 and IR462 RNAi lines expressing antisense fragment of *Gns5* showed a significant decline in the survival of BPH compared to their respective controls. The survival bioassay showed that both the IR463 and IR462 RNAI lines demonstrated similar patterns of BPH survival.

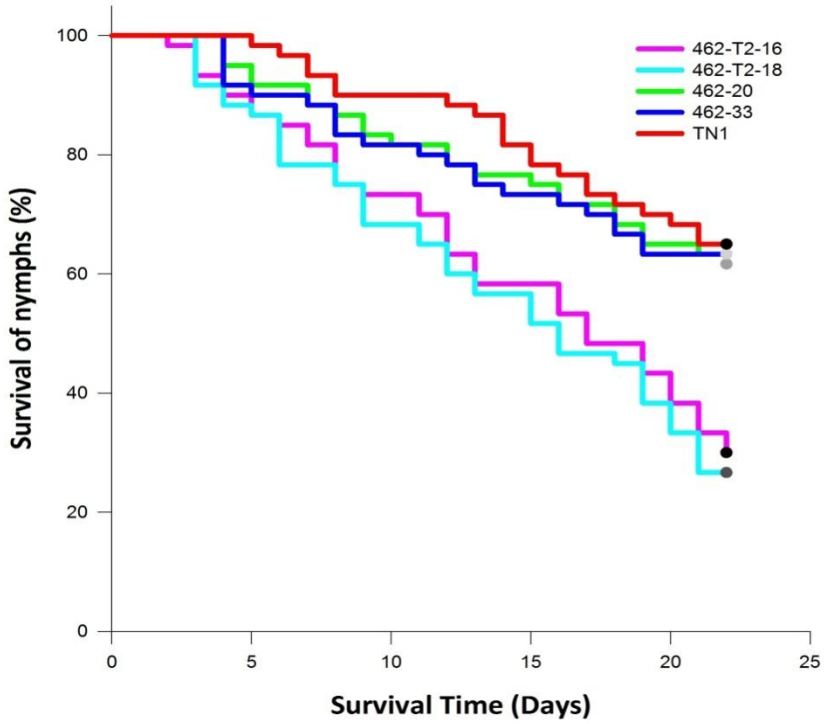


Figure 2.8 BPH survival bioassay showed that the survival of BPH on IR462 RNAi lines was significantly reduced compared to the controls (TN1; transformed empty vector 462-20 and 462-33) according to Kaplan-Meier survival analysis Log Rank Test, $p<0.001$.

2.4.5 *Egg production on the IR463 and IR462 RNAi lines*

After the 22-day assay (2.4.4) the number of eggs produced from random mating in all rice genotypes was recorded. The number of eggs produced was counted on the stems and the leaves of the plants. The IR463 RNAi lines produced an average of 32.8, 34.3, 33.0 and 35.0 eggs per plant respectively on the 463-T3-8, 463-T3-22, 463-T3-41 and 463-T3-26 lines, whilst the control plants produced 73.0 and 70.0 eggs per plant on the parental TN1 and transformed empty vector line 463-4 respectively (Figure 2.9). The egg production on all IR463 RNAi lines was significantly ($p<0.05$) reduced by ~ 50% compared to the TN1 control plant. The egg production per plant on 463-T3-8, 463-T3-41, 463-T3-22 and 463-T3-26 was reduced by 55.1%, 54.8%, 53.0% and 52.1% respectively compared to TN1. There were no significant differences observed between the number of eggs produced per plant on the TN1 and the empty vector line, 463-4. Interestingly, there were no significant differences observed in the mean of eggs produced by per adult between the TN1, the transformed empty vector line 463-4 and all the IR463 RNAi lines suggesting that reduced egg production on the RNAi lines was due to reduced adult survival.

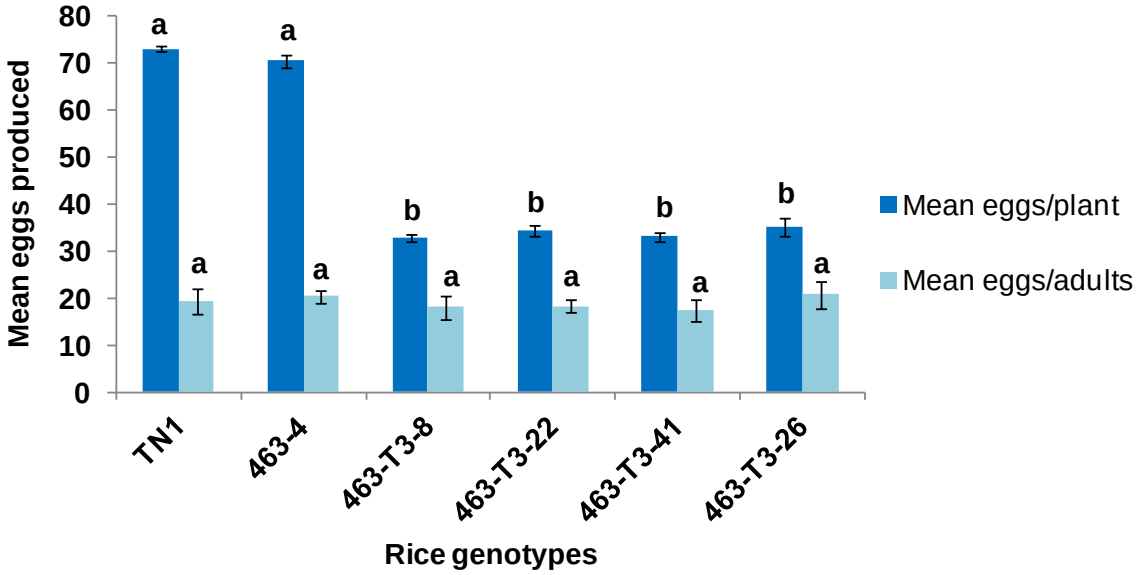


Figure 2.9 Mean eggs produced by adult BPH in IR463 RNAi lines compared to parental TN1 and transformed empty vector line 463-4. Statistical analysis was conducted for mean eggs per plant and mean eggs per adults. Bars with different letter are significant at $p<0.05$ using one-way ANOVA followed by Tukey Post-Hoc Test. The error bars represent the ± SD of the mean values (n=6).

A similar trend of egg production was also observed in the IR462 RNAi lines (Figure 2.10). This study also demonstrated an average of 80.0 eggs per plant on the parental TN1, 81.0 and 85.3 eggs per plant respectively on the transformed empty vector lines 462-20 and 462-33. The average eggs per plant on the 462-T3-16 and 462-T3-18 RNAi lines were reduced significantly ($p<0.05$) by 54.8% (36.2 eggs per plant) and 53.4% (37.3 eggs per plant) respectively compared to the parental TN1 control. These results indicated that the TN1 and the transformed empty vector lines (462-20 and 463-33) showed significantly ($p<0.05$) higher average egg production per plant compared to the IR462 RNAi lines where the *Gns5* gene had been knocked down. The susceptible TN1 and the transformed empty vector lines did not exhibit any significant differences between each other in the mean number of eggs poduced by per plant.

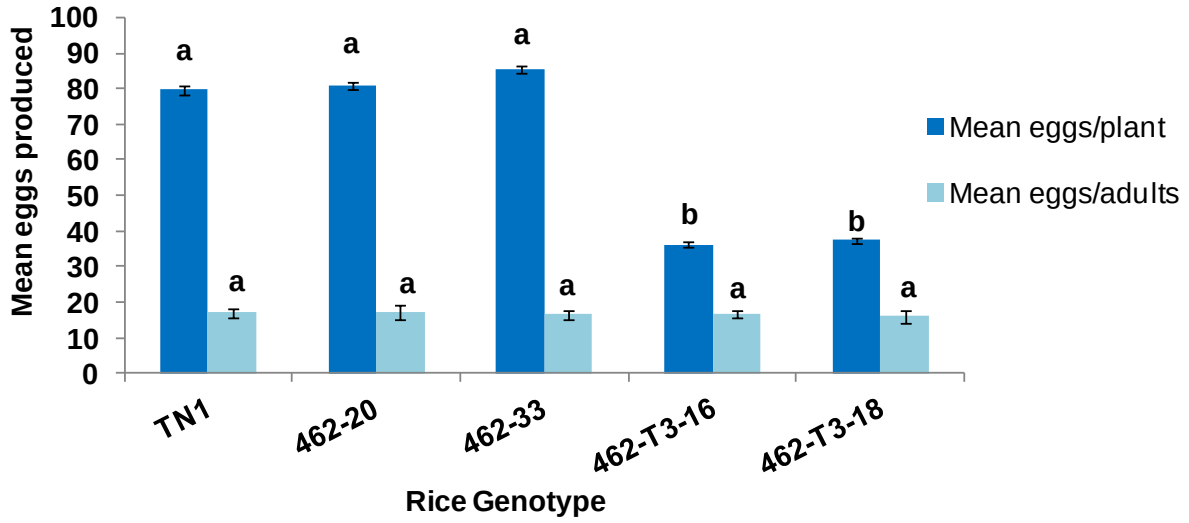


Figure 2.10 Mean eggs produced by adult BPH in IR462 RNAi lines compared to parental TN1 and transformed empty vector lines 462-20 and 462-33. Statistical analysis was conducted for mean eggs per plant and mean eggs per adults Bars with different letter are significant at $p<0.05$ using one-way ANOVA followed by Tukey Post-Hoc Test. The error bars represent the ± SD of the mean values (n=6).

The difference in the number of eggs produced on the IR463 and IR462 assay is influenced by the number of insects that survived from the 22-day assay. There were no significant differences observed in the number of eggs produced between each line in the IR463 and IR462 RNAi lines respectively. Similar to the results produced by IR463, the IR462 RNAi lines also did not show any significant differences ($p<0.05$) in the average eggs produced per adult BPH in both the RNAi lines and the control plants (462-20 and 462-33). Therefore, *Gns5* does not have a direct effect on fecundity in any of the rice genotypes tested but does have a significant effect on insect survival.

2.4.6 *Effect of Gns5 on nymph production in the IR463 and IR462 RNAi lines*

Effect of *Gns5* on BPH fecundity in the IR462 and IR463 RNAi lines was also assessed by the total number of nymphs produced per adult BPH from the survival assay. Over a 40-day assay period, the IR463 RNAi lines showed a significant ($p <0.05$) reduction in the average number of nymphs produced compared to the parental TN1 and the transformed empty vector line, 463-4 (Figure 2.11). The average number of nymphs recorded on the 463-T3-8, 463-T3-22, 463-T3-41 and 463-T3-26 RNAi lines were 31.0, 32.5, 30.5 and 33.0 nymphs per plant, respectively.

The number of nymphs on TN1 was 70.8 nymphs per plant and 67.7 nymphs per plant on the transformed empty vector line 463-4. This assay revealed that there were reductions of 56.2%, 54.0%, 56.9% and 53.4% on the 463-T3-8, 463-T3-22, 463-T3-41 and 463-T3-26, respectively in the fecundity rate of the IR463 RNAi lines compared to the parental TN1 control.

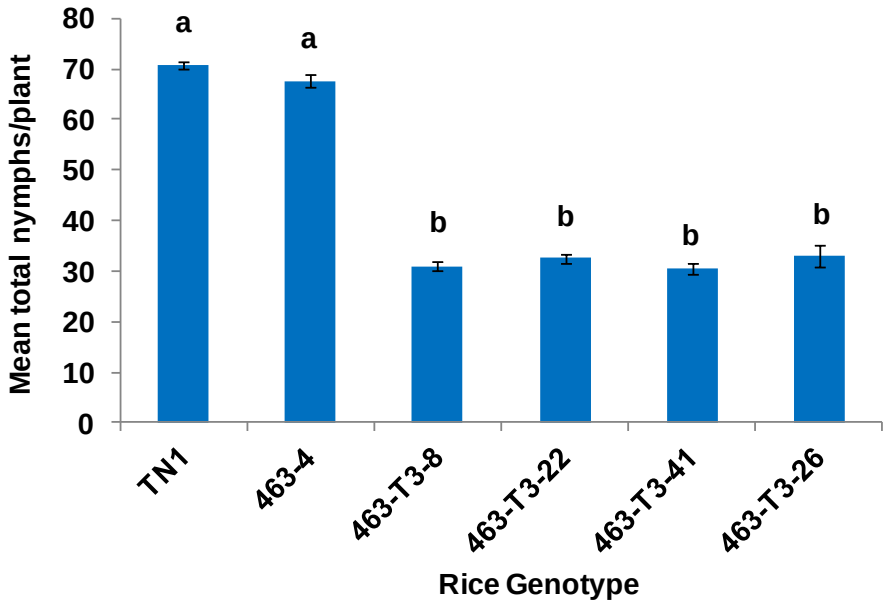


Figure 2.11 BPH fecundity assay demonstrating total nymph production in IR463 RNAi lines compared to controls (TN1; 463-4 transformed empty vector lines). Bars with different letter are significant at $p<0.05$ using one-way ANOVA followed by Tukey Post-Hoc Test. The error bars represent the ± SD of the mean values (n=6).

In the IR462 RNAi lines, the number of BPH nymphs produced on 462-T3-16 and 463-T3-18 was 31.5 and 34.3 per plant respectively (Figure 2.12). The IR462 RNAi lines also demonstrated a significant ($p<0.05$) decline in the nymph production by 57.7% and 53.9% on the 462-T3-16 and 462-T3-18 RNAi lines respectively, compared to the parental TN1 control. This fecundity assay explains that both RNAi lines IR463 and IR462 expressing significantly reduced levels of *Gns5* showed a reduced number of nymphs produced in these lines compared to TN1 and the 462 and 463 transformed empty vector lines. This demonstrates that there were no significant differences between the fecundity rates of the parental TN1 and their respective transformed empty vector lines.

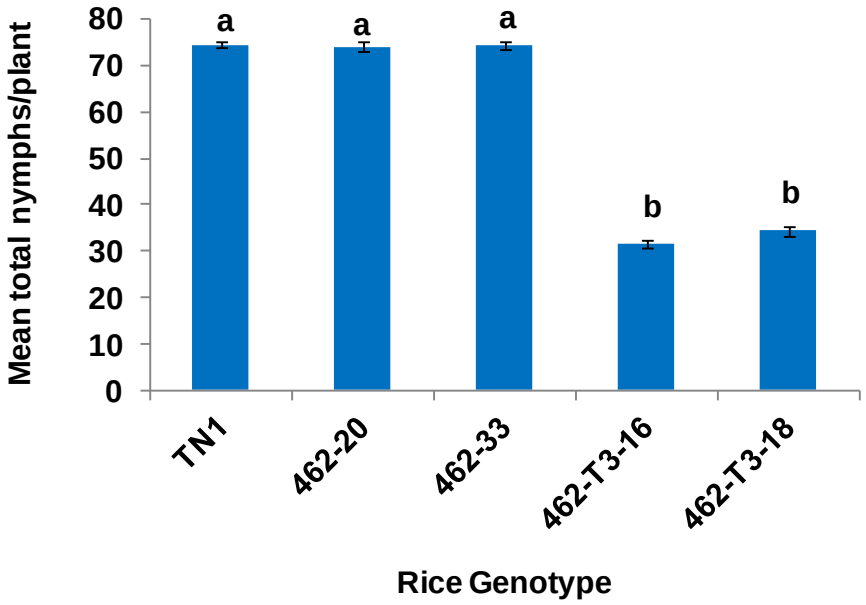


Figure 2.12 BPH fecundity assay demonstrating total nymph production in IR462 RNAi lines compared to controls (TN1; 462-20 and 462-33 transformed empty vector lines). Bars with different letter are significant at p <0.05 using one-way ANOVA followed by Tukey Post-Hoc Test. The error bars show the ± SD of the mean values (n=6).

Figure 2.13 Effect of BPH on plant resilience. (A) Parental TN1, (B) Transformed empty vector line 463-4 and (C) 463-T3-22 RNAi line after 40 days of BPH infestation.

2.4.7 *Effect of Gns5 on BPH development in IR463 and IR462 RNAi lines*

The BPH development assay revealed that BPH survival on the IR463 RNAi lines was reduced from 25 first-instar BPH nymphs per plant to an average of 19.5, 19.2, 12.0, 12.0, 10.0 and 13.0 insects per plant on the parental TN1, 463-4 transformed empty vector line and the RNAi lines, 463-T3-41, 463-T3-22, 463-T3-26 and 463-T3-8 respectively by the end of the 13-day bioassay (Figure 2.14). These data showed that there were differences observed in the development of the insects in the IR463 RNAi expressing reduced levels of *Gns5* compared to TN1 and the transformed empty vector line 463-4. The present study categorized the development of the BPH into adults, nymphs (third to fifth instars) and underdeveloped nymphs (first to second instars). Data from Figure 2.14 show that there were a larger number of adults in the control lines compared to the IR463 RNAi lines. Overall, the average number of adults on TN1 and the empty vector line 463-4, were 76% (14.8 adults per plant) and 75% (14.3 adults per plant) respectively, compared to 27% (3.3 adults per plant), 25% (3.0 adults per plant), 31% (3.2 adults per plant) and 35% (4.3 adults per plant) on the RNAi lines 463-T3-41, 463-T3-22, 463-T3-26 and 463-T3-8, respectively. On the other hand, there were more third to fifth instar nymphs on the IR463 RNAi lines compared to the control lines (TN1 and 463-4). The assay recorded 55%, 57%, 50% and 51% of third to fifth instar nymphs on the 463-T3-41, 463-T3-22, 463-T3-26 and 463-T3-8 respectively compared to 24% on the parental TN1 and 23% on the transformed empty vector, 463-4 lines. Interestingly this study also found that there was an average of two underdeveloped nymphs per plant in all IR463 RNAi lines which was not observed in the control lines, TN1 and 463-4. These data suggest that expression of *Gns5* supports BPH development and that when this gene is knocked down, development is retarded.

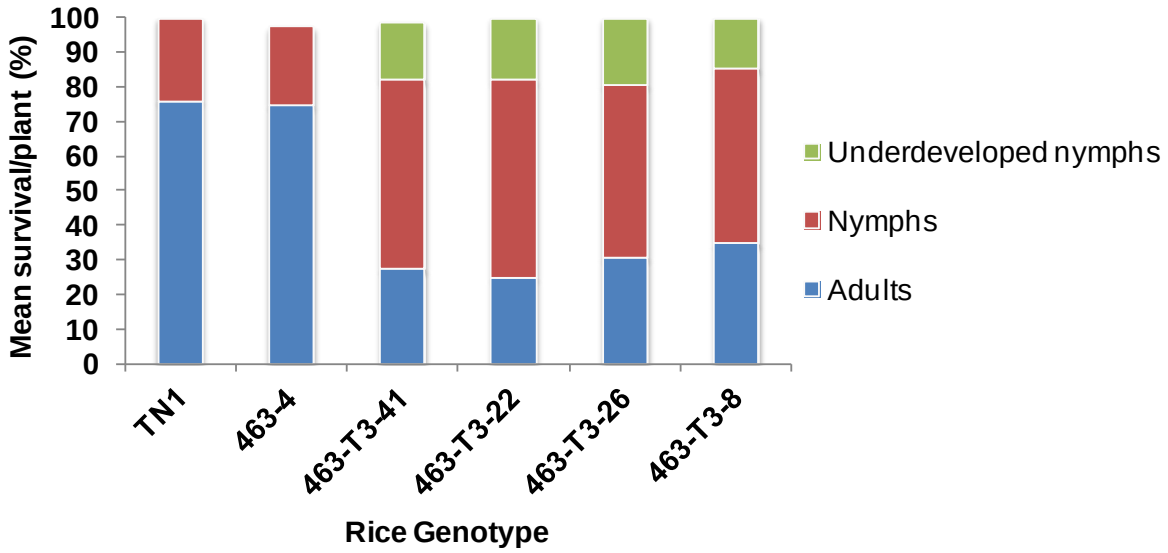


Figure 2.14 Mean survival of BPH after 13 days on IR463 RNAi lines compared to TN1 and 463-4 transformed empty vector control lines in the BPH development assay; n=6.

The IR462 RNAi lines also demonstrated a similar trend of BPH development during the 13 day bioassay (Figure 2.15). The average number of insects decreased from 25 first instar nymphs to 13.0 and 13.2 insects per plant on the RNAi lines 462-T3-16 and 462-T3-18, respectively, compared to 19.2 insects per plant on TN1 and 462-33, and 19.0 insects per plant on the 462-20 respectively. As observed in the IR463 RNAi lines, the IR462 RNAi lines also recorded 33% (4.3 adults per plant) and 34% (4.5 adults per plant) on the 463-T3-16 and 463-T3-18 respectively compared to 84% (16.2 adults per plant) on TN1 and the empty vector line 462-33 and 81% (15.3 adults per plant) on the empty vector line 462-20. As for the IR463 lines, BPH development on IR462 lines demonstrated a higher number of third to the fifth instar nymphs per plant compared to the control lines. The 462-T3-16 recorded an average of 6.5 nymphs per plant (35%) and 7.3 nymphs per plant (34%) on the 463-T3-18 compared to 3.0 (16%) 3.7 (19%), and 3.2 (17%) nymphs per plant on TN1 and the transformed empty vector lines, 462-20 and 462-33 respectively. Underdeveloped nymphs were only observed in the IR462 RNAi lines. There were 2.2 nymphs per plant in both the 462-T3-16 and 462-T3-18 RNAi lines. Both the IR462 and IR463 RNAi lines demonstrated a similar pattern of development and therefore it was difficult to conclude which vector system was more efficient at suppressing BPH development. Overall, the development assay showed that both the IR462 and IR463 RNAi lines expressing significantly reduced levels of *Gns5* affected the development of BPH from the nymph stage to the adulthood of the insect.

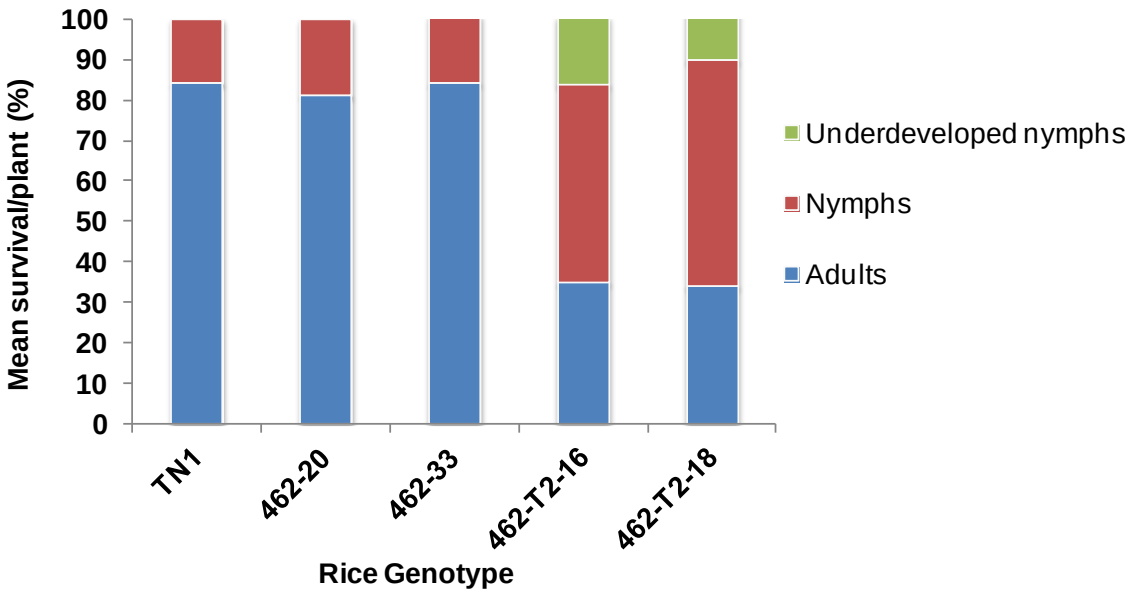


Figure 2.15 BPH development assay showing the mean survival of BPH after 13 days on IR463 RNAi lines compared to TN1 and 463-4 transformed empty vector lines; n=6.

2.4.8 *BPH biomass on IR463 and IR462 RNAi lines*

The delay in the development of BPH nymphs on the IR462 and IR463 RNAi plants was also reflected in the decrease of the mean insect biomass per plant. The mean insect biomass per plant after 13 days on the IR463 RNAi lines was 9.8 mg, 10.2 mg, 9.0 mg and 12.3 mg on the 463-T3-41, 463-T3-22, 463-T3-26 and 463-T3-8 respectively compared to 29.3 mg on the parental TN1 and 28.4 mg on the transformed empty vector line 463-4 (Figure 2.16). Statistical analysis revealed that the mean insect biomass per plant on TN1 did not significantly differ from the 463-4 transformed empty vector line. However, a significant reduction ($p<0.05$) in the insect biomass per plant was recorded on all IR463 RNAi lines tested compared to the control lines (TN1 and 463-4).

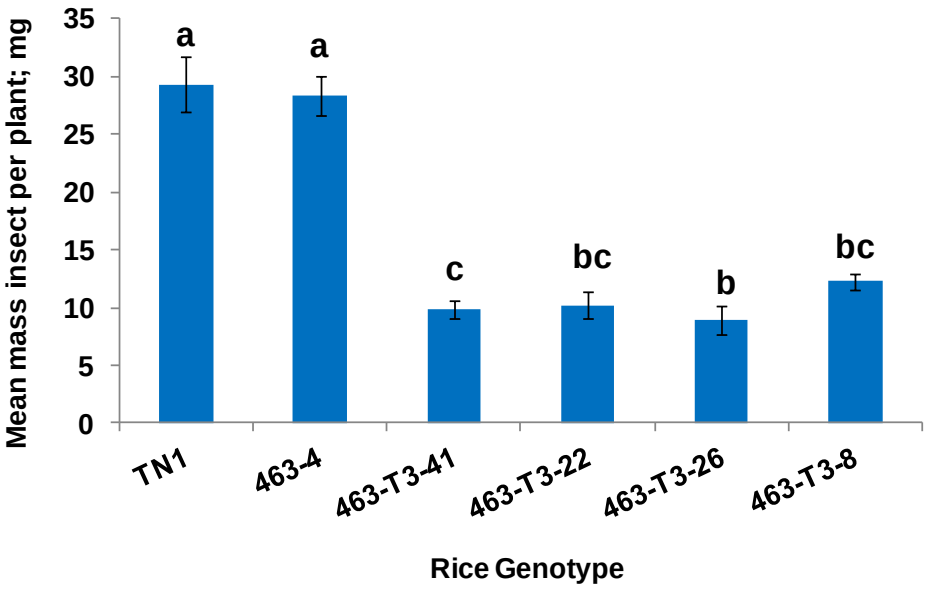


Figure 2.16 Short term bioassay showing BPH biomass in IR463 RNAi lines compared to controls (TN1; 463-4 transformed empty vector lines). Bars with different letter are significant at $p<0.05$ using one-way ANOVA followed by Tukey Post-Hoc Test. The error bars represent the ± SD of the mean values (n=6).

The IR462 RNAi lines also showed a decrease in the mean insect biomass per plant compared to the TN1 control and the transformed empty vector lines (462-20 and 462-33) (Figure 2.17). The mean insect biomass on 462-T3-16 and 462-T3-18 was 12.3 mg and 13.5 mg respectively compared to the parental TN1 (30.9 mg), and the empty vector lines 462-20 (29.6 mg) and 462-33 (29.7 mg). There were no significant differences in the insect biomass between these different controls. However, the insect biomass in all the 462 RNAi lines significantly ($p<0.05$) differed from those of the parental TN1 and the empty vector lines. Overall the IR462 and IR463 RNAi lines expressing significantly reduced levels of *Gns5* showed a reduction in BPH biomass compared to their respective controls.

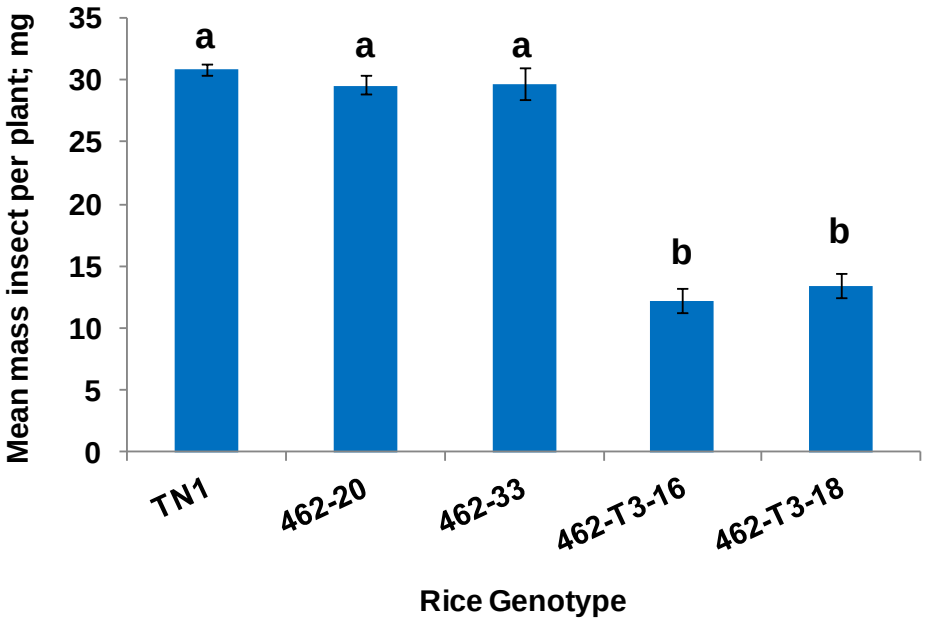


Figure 2.17 Short term bioassay showing BPH biomass in IR462 RNAi lines compared to controls (TN1; 462-20 and 462-33 transformed empty vector lines). Bars with different letter are significant at $p<0.05$) using one-way ANOVA followed by Tukey Post-Hoc Test. The error bars represent the ± SD of the mean values (n=6).

2.4.9 *Callose deposition in the leaves of IR463 and IR462 RNAi lines*

A preliminary examination using fluorescence microscopy showed that the non-infested wild type TN1 leaves showed a higher level of callose deposition compared to the plants infested with BPH. Callose deposition in the leaves was shown as bright blue fluorescence by methyl blue staining. The leaves of transformed empty vector lines (463-4, 462-20 and 463-33) also demonstrated a similar fluorescent effect as the TN1 in both the infested and non-infested plants. In contrast to this finding, the IR463 and IR462 RNAi lines showed a higher level of blue fluorescence intensity on the infected lines compared to their non-infested counterparts (Appendix A). In order to ensure the microscopic examination using methyl blue was reliable, IR70 leaf samples, which were treated exactly as the RNAi leaves, were used as a positive control for the resistant lines. Fluorescence microscopic examination exhibited very strong fluorescent signals on the IR70 leaves infested with BPH compared to the non-infested IR70 leaf samples. This preliminary examination revealed that RNAi lines exhibiting reduced expression of *Gns5* have increased levels of callose deposition in response to BPH infestation. However, the 10x magnification images were not very clear and therefore the intensity of the fluorescence was not measured from these images.

In order to further investigate the effect of *Gns5* and its role in callose deposition, all the leaf samples were analyzed using confocal microscopy. The confocal microscopic examination demonstrated clear images of callose deposition in all rice genotypes tested. As expected, the results obtained from the preliminary examination matched the findings of the confocal imaging of all leaf samples. Induced callose deposition on the sieve plates of the leaves has clearly shown the effect of BPH feeding on the IR463 and IR462 RNAi expressing reduced levels of *Gns5*, compared to the transformed empty vector and the parental TN1 control rice plants (Figure 2.18). The non-infested TN1 and transformed empty vector lines (463-4, 462-20 and 463-33) showed a brighter signal of fluorescence compared to the plants exposed to BPH infestation for 13 days. All these leaf samples showed very faint fluorescent emission after BPH infestation. The differences in the fluorescence emission of callose deposition between the infested and non-infested control samples were determined by measuring the relative callose intensity.

This data showed that callose deposition was higher in the non-infested TN1, 463-4, 462-20 and 462-33 controls compared to their respective infested control samples. The relative intensity (mean intensity per unit area) was recorded as 528.7, 566.4, 386.4 and 584.8 in the TN1, 463-4, 462-33 and 462-20, respectively (Figure 2.19). However, the relative intensity decreased to 47%, 64.1%, 44% and 45.8% in the infested TN1, 463-4, 462-33 and 462-20 compared to their respective non-infested controls. Therefore, the level of callose intensity in all the control rice lines (TN1, transformed empty vector lines, 463-4, 462-20 and 462-33) decreased in response to BPH infestation.

Interestingly, the results of callose deposition in the IR463 and IR462 RNAi lines contrasted with the results of the control plants, showing an increased level in the fluorescent intensity on the leaves after BPH infestation. Callose deposition post BPH infestation was clearly observed on the sieve plates of the IR463 and IR462 RNAi lines leaves. The callose intensity results showed that callose deposition in the IR463 RNAi lines leaves increased to 720.38 (51%), 798.33 (54.4%), 733.93 (32.3%) and 889.79 (46.6%) in the 463-T3-22, 463-T3-26, 463-T3-41 and 463-T3-8 infested lines respectively, compared to their non-infested controls (Figure 2.19).

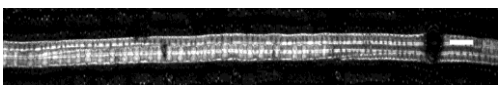
A similar pattern of fluorescence intensity was observed for the IR462 RNAi lines. These RNAi lines demonstrated a higher level of fluorescence in both 463-T3-18 and

463-T3-16 RNAi lines infested with BPH. The relative callose deposition in the 462-T3-16 and 462-T3-18 infested RNAi lines increased to 26% (746.90) and 53% (864.55) compared to their respective infested control lines. The explanation for why 462-T3-16 only showed an increase of 26% in relative callose deposition was because this line showed higher callose intensity on its non-infested lines.

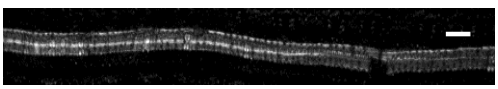
Overall, the non-infested rice genotypes tested, including TN1, the transformed empty vector control lines (463-4 and 462-20), IR463, IR462 and IR70 showed a range of between 520 to 600 callose intensity per unit area except for 462-33 which showed a lower value (386.43 intensity per unit area). One-way ANOVA analysis showed that there were no significant differences observed in the relative callose deposition between all non-infested rice genotypes tested. However, the callose intensity decreased to approximately 200 to 300 callose intensity per unit area on the infested control plants (TN1, 463-4, 462-20 and 462-33) but in the IR463 and IR462 RNAi lines increased to between 700 - 800 intensity per unit area in response to BPH.

The resistant IR70 (used as a positive resistant control) exhibited an increased callose deposition from 601.69 to 918.36 intensity per unit area which indicated an increase of 52.6% on the infested IR70 plants. The infested IR463, IR462 and IR70 exhibited significantly ($p<0.05$) higher levels of callose accumulation in the sieve plates of the leaves as compared to the level of callose in the infested susceptible TN1. The strong fluorescence emission in the sieve tubes of the IR463 and IR462 RNAi leaves of the infested lines showed that *Gns5* plays an important role in callose deposition, will decrease *Gns5* expressing resulting in increased callose. The decrease in callose deposition in TN1 and the transformed empty vector lines revealed that these control plants were susceptible to BPH infestation.

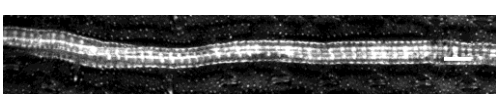
1(a) TN1 non-infested control



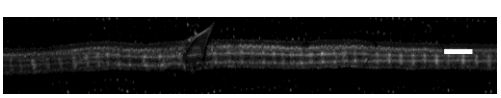
1(b) TN1 infested with BPH



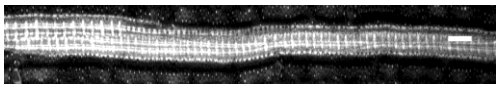
2(a) 463-4 non-infested control



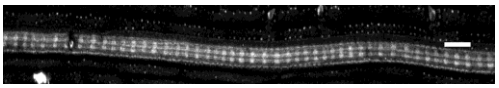
2 (b) 463-4 infested with BPH



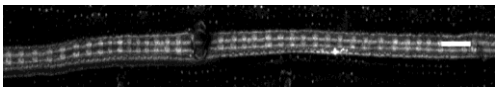
3 (a) 462-20 non-infested control



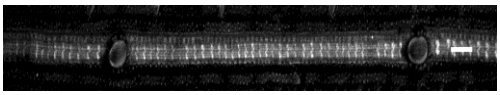
3(a) 462-20 infested with BPH



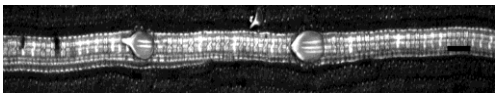
4(a) 462-33 non-infested control



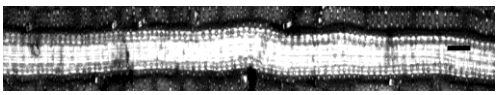
4(b) 462-33 infested with BPH



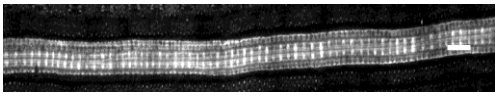
5(a) 463-T3-26 non-infested control



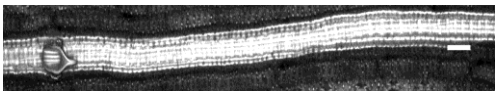
5(b) 463-T3-26 infested with BPH



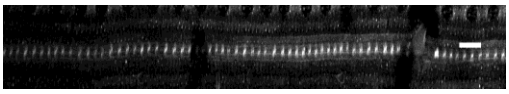
6 (a) 463-T3-41 non-infested control



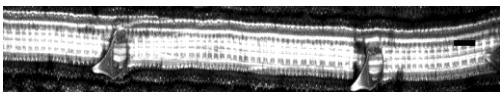
6(b) 463-T3-41 infested with BPH



7(a) 463-T3-22 non-infested control



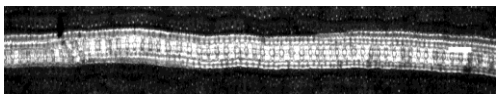
7(b) 463-T3-22 infested with BPH



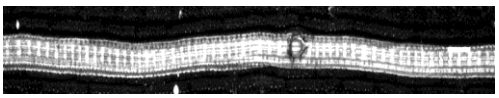
8(a) 463-T3-8 non-infested control

8(b) 463-T3-8 infested with BPH

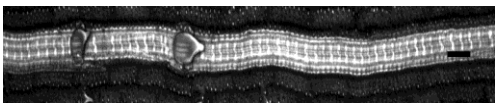
9(a) 462-T3-16 non-infested control



9(b) 462-T3-16 infested with BPH

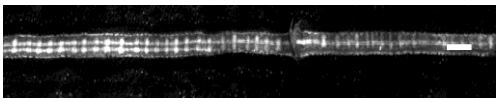


10(a) 462-T3-18 non-infested control



10(b) 462-T3-18 infested with BPH

11(a) IR70 non-infested control



11(b) IR70 infested with BPH

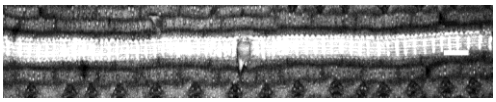


Figure 2.18 Callose deposition in rice leaf sheath tissues 1(a)(b) and 11 (a)(b) obtained by confocal microscopy at 10x magnification. Induced callose (with bright white fluorescence) deposited on the sieve plates in non-infested and BPH infested rice genotypes. Scale bar = 40µm.

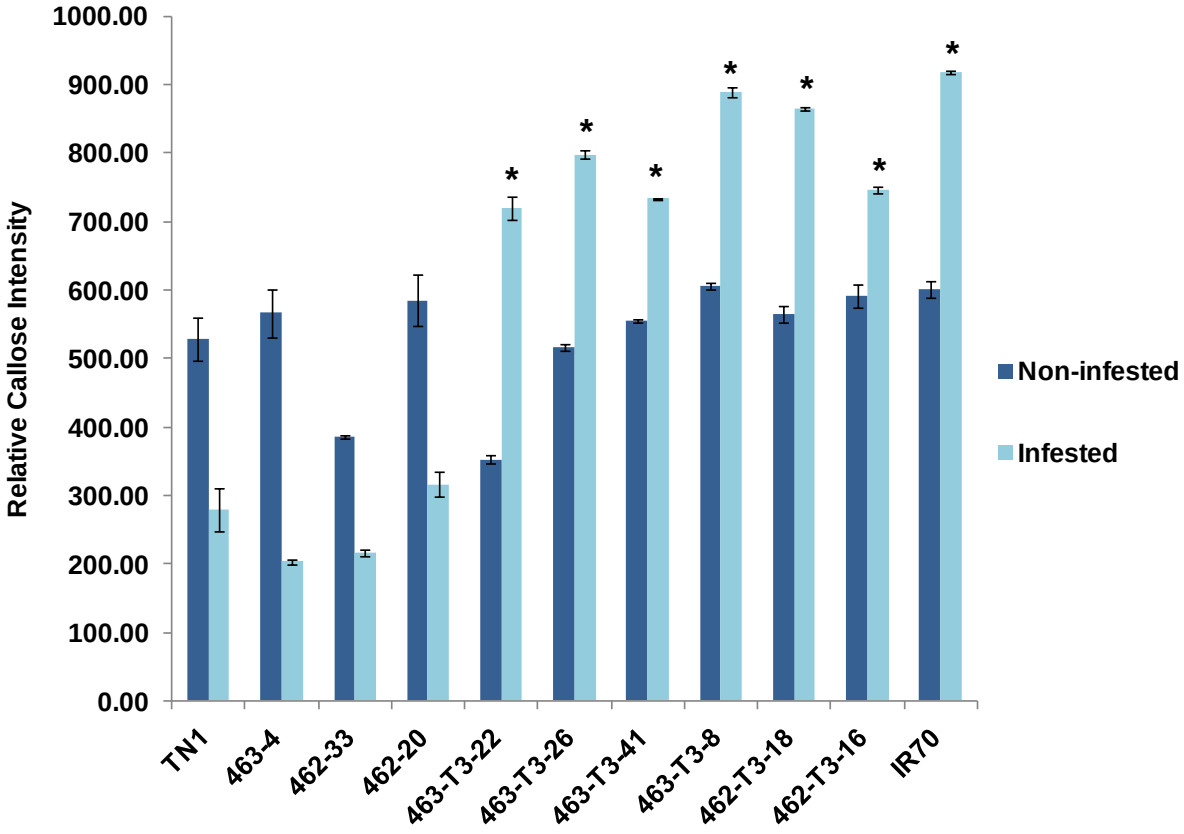


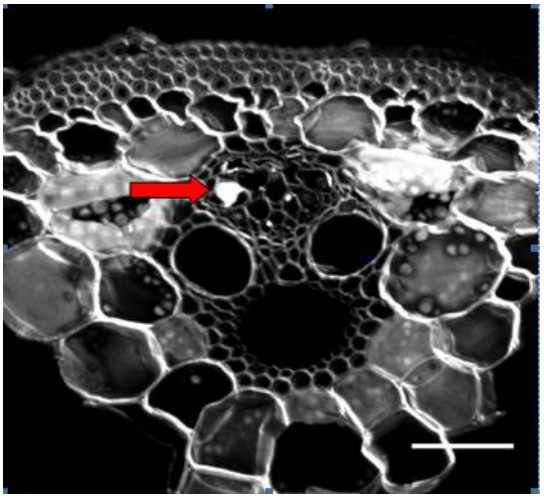
Figure 2.19 Relative callose deposition in rice leaves of IR463 and IR462 RNAi rice lines by the confocal microscopy. Bars with an asterisk * is significant at $p<0.05$) using one-way ANOVA followed by Tukey Post-Hoc Test. The error bars represent the ± SD of the mean values (n=4).

2.4.10 *Callose deposition in the stems of IR463 and IR462 RNAi lines*

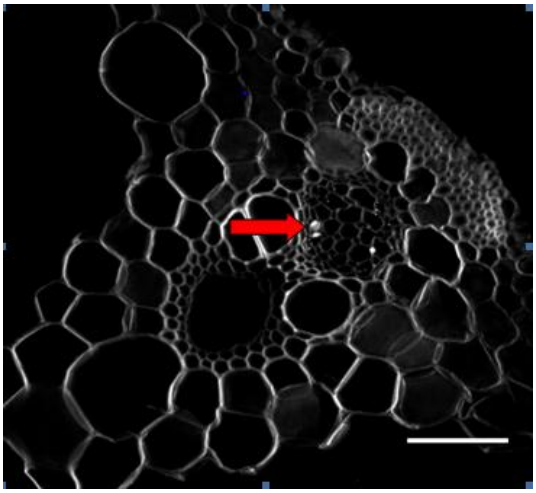
To further confirm our findings on callose deposition and its link to *Gns5* expression, the stems of the control plants, and the RNAi lines IR463 and IR462 were cross-sectioned and examined at 40x magnification using a confocal microscope (Figure 2.20). Four lines were selected to perform the cross-section of stem tissues which included the parental TN1, the transformed empty vector line 462-20 and the RNAi

lines 463-T3-26 and 462-T3-18. Stem tissues from the same plants as used for the leaf samples were examined. The results from this study matched with the results obtained from the epifluoresence microscopy analysis of callose in the leaves as presented above (2.4.9). Autofluorescence of other tissues was examined in the unstained leaves with the same setting of fluorescence and magnification as the samples tested. Callose deposition was clearly distinguished by the methyl blue staining and observed as a bright white spot in the sieve plates within the phloem located at the vascular bundle of the stem tissue. The callose intensity per unit area of the non-infested TN1 and 462-20 lines was 118.32 and 112.64 respectively and reduced to 74.31 and 93.98 per unit area on the infested lines. In contrast, the callose intensity of the RNAi line 463-T3-26 in the non infested plants was 125.47 per unit area, and increased to 145.93 per unit area in the infested plants. The highest level of relative intensity was observed on the 462-T3-18 lines whereby the intensity per unit area on the non-infested plants was 95.84 increasing to 154.45 in the infested lines. Figure 2.21 shows that there were no significant differences in the relative callose intensity between the non-infested lines control lines and the RNAi lines. However, significant differences ($p<0.05$) were observed in the infested 462-T3-18 RNAi line as compared to the infested TN1. The infested 463-T3-26 line showed an increase in callose deposition but was not significantly higher than the non-infested 463-T3-26 control line. Therefore, the IR463 and IR462 expressing reduced levels of *Gns5* showed a higher level of callose deposition post BPH infestation, supporting the hypothesis that *Gns5* plays an important role in callose deposition and increases resistance against BPH.

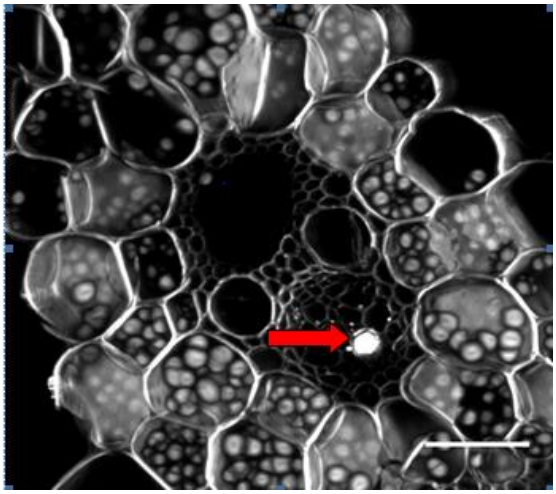
1(a) TN1 non-infested control



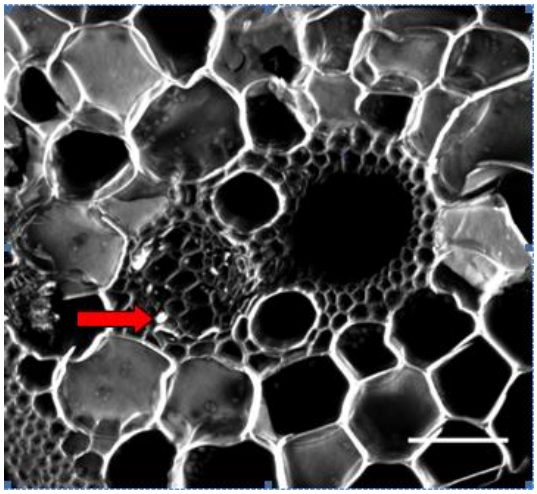
1(b) TN1 infested with BPH



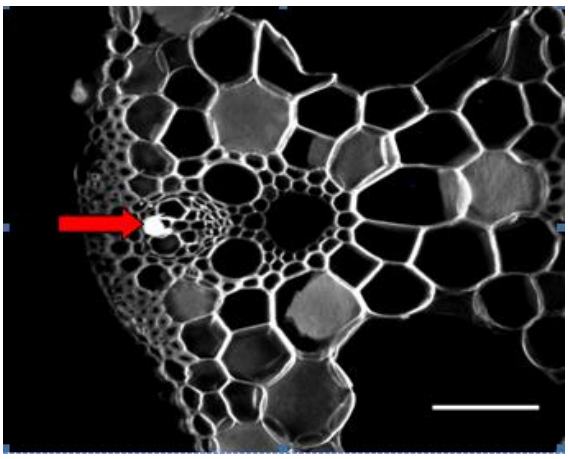
2(a) 462-20 non-infested



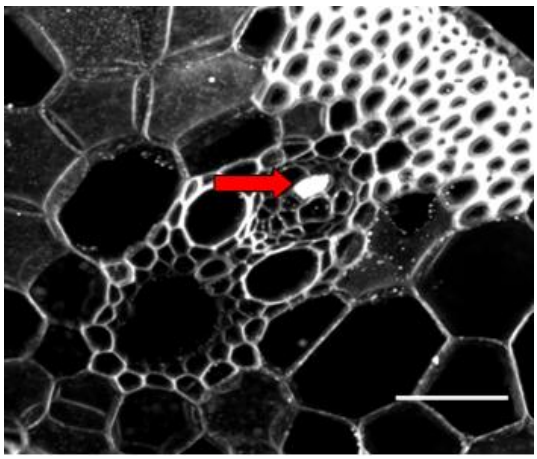
2(b) 462-20 infested with BPH



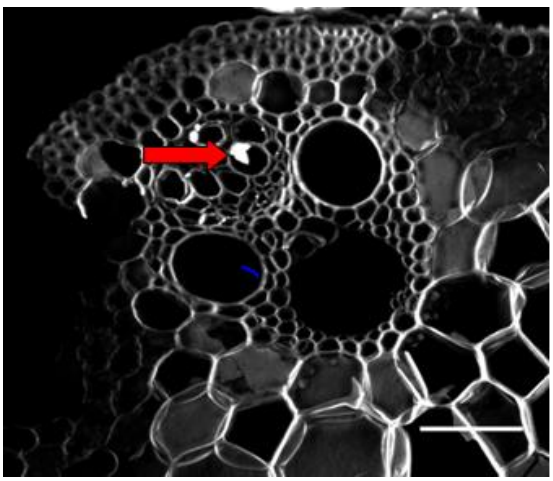
3(a) 463-T3-26 non-infested



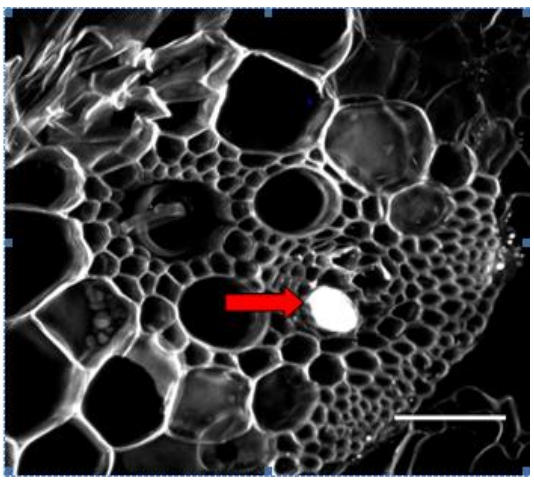
3(b) 463-T3-26 infested with BPH



4(a) 462-T3-18 non-infested



4(b) 462-T3-18 infested with BPH



5. Unstained section of stem tissue (control)

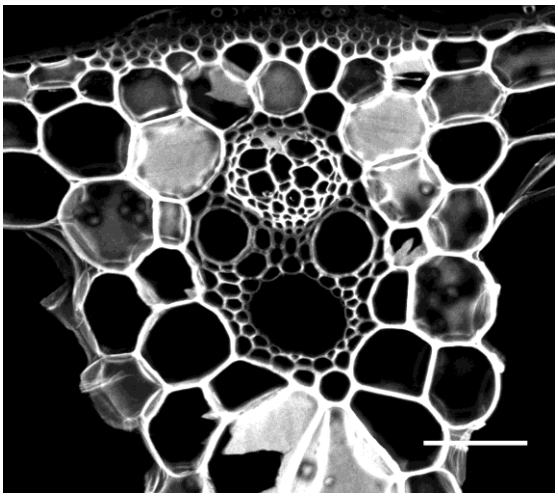


Figure 2.20 Callose deposition in rice stem tissues was visualised by confocal microscopy at 40x magnification. Red arrows show induced callose (with bright white fluorescence) deposited on the sieve plates in non-infested and BPH infested rice genotypes. Scale bar = 40µm.

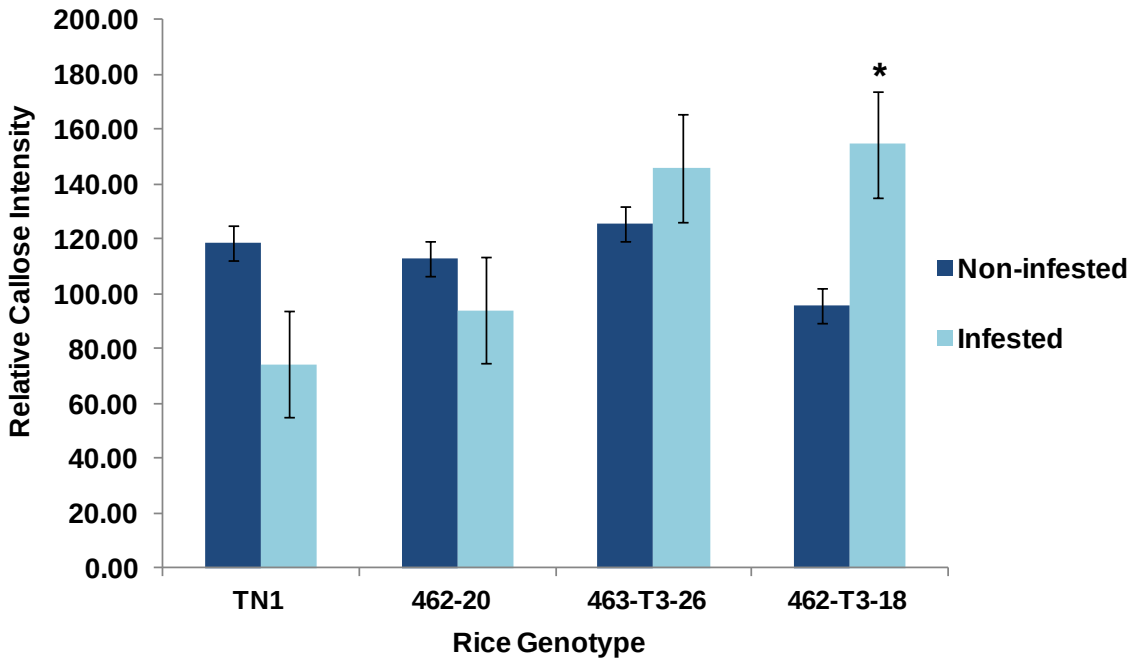


Figure 2.21 Relative callose deposition in rice leaves of IR463 and IR462 RNAi lines under confocal microscopy. Bars with an asterisk * significant at p <0.05) using one-way ANOVA followed by Tukey Post-Hoc Test. The error bars represent the ± SD of the mean values (n=4).

2.4.11 *EPG studies to determine effects of gene knockdown in RNAi lines on BPH feeding behaviour*

An EPG study was conducted in order to assess the impact on BPH feeding behaviour on the RNAi lines expressing reduced levels of *Gns5*. The present study demonstrated that all the IR463 and IR462 lines supported reduced survival and growth rate of BPH, and induced callose deposition in response to BPH infestation. Therefore, four rice genotypes namely 463-T3-26 and 462-T3-18 RNAi lines, parental TN1 and an empty vector line 464-4 were selected for EPG studies. The resistant IR70 line was also included in this study as a resistant control which made the identification of waveforms in the other rice genotypes easier. This study classified the EPG signals obtained into five types of waveform according to the relative voltage levels seen in the total EPG overview chart. These waveforms included NP (non-penetration/non-probing of stylets), C (pathway activities), E1 (sieve element salivation), E2 (phloem ingestion) and G (xylem ingestion) (https://www.epgsystems.eu/). The NP waveform was correlated with the absence of feeding activity and this waveform showed straight lines with nearly zero voltage. The C waveform occurs when the BPH start to insert their stylets into cell wall tissues and attempted to penetrate the plant sieve elements, which produce irregular waveforms with increasing amplitudes. This study combined the N1(penetration initiation), N2 (stylet movement) and N3 (extracellular activities) waveforms as described by Zhang *et al*. (2015) and Ghaffar *et al*. (2011), into one type of waveform, the pathway waveform. The N1, N2 and N3 are irregular waveforms and are difficult to distinguish from one another and usually appear after the NP waveform. During the pathway periods, there were potential drops occurring in the rice lines tested. However, it was very occasional and did not occur in all replicates examined. Therefore, this study did consider the potential drops as one of the waveform parameters.

This study identified a similar pattern of G, E1 and E2 as previously reported in many EPG studies involving the feeding behaviour of BPH in rice. The G, E1 and E2 are represented as N4-a, N4-b and N5 as reported by Ghaffar *et al*. (2011). The E1, E2 and G waveforms were clearly distinguished from the other waveforms whereby these waveforms were very important parameters to provide evidence on the resistance mechanism of the rice genotypes tested. Most of the waveforms in this study showed that the E1 waveform appears before the E2 waveform. There were differences observed in the duration of E1 and E2 waveform in the control lines,

IR463, IR462 and IR70 rice lines. The G waveform has a consistent shape at higher amplitude compared to the E2 waveform in the rice genotypes tested and occurred during the C waveform.

Twenty two parameters of insect feeding were assessed (Table 2.6), including parameters measuring both pre- and post-phloem penetration behaviour. Differences in these parameters between the rice genotypes were detected using ANOVA tests for each parameter and Tukey Post-Hoc tests. Of the 22 parameters outlined in Table 2.5 at least one genotype differed significantly from the others, according to the tests mentioned above. The seven parameters which showed no significant difference between genotypes were Average non-probing, Average C, Average G duration, Time to 1st probe, Time to 1st E1, Time to First E12 probe and Time to 1st E2.

The pre-phloem parameters which differed significantly are as follows: the RNAi line 462-T3-18, which showed significantly more bouts of non-probing than the other genotypes (Figure 4.22), and 463-T3-26, which had a significantly longer total duration of non-probing behaviour than the TN1 and 463-4 control genotypes, with no significant differences between genotypes detected by Tukey Post Hoc test for average non-probing duration despite an ANOVA *p*-value <0.05. The average duration of non-probing recorded within the 12 hours of EPG analysis was 450 sec, 347 sec, 2441 sec, 5636 sec and 3000s in TN1, transformed empty vector line 463-4, resistant R70, and the RNAi lines 463-T3-26 and 462-T3-18, respectively.

A significantly ($p<0.05$) higher total number of probes and number of brief probes occurred when BPH fed upon 462-T3-18 compared to any other genotype, but the average probe length was significantly longer on TN1 than on the other genotypes. The RNAi lines 463-T3-26 and 462-T3-18 exhibited a longer duration of average probes compared to the control lines. The number of C probes was significantly higher on both the 463-T3-26 and 462-T3-18 RNAi lines than the wild type TN1 and the 463-4 empty vector line (Figure 4.22), but no significant differences were observed for average C duration. The number of feeding bouts from the xylem (G probes) was significantly higher on the 463-T3-26 line than either TN1 or the 463-4 empty vector line, but no significant differences were observed between any plant genotype for an average duration of xylem feeding.

The phloem-located parameters are as follows. Both the number of E1 probes without E2, and the total number of all E1 probes (Figure 2.22), were significantly higher on 462-T3-18 compared to the susceptible control TN1 and 463-4 lines. The average E1 duration, however, was significantly longer on the resistant IR70 line compared with any of the other lines. Significantly more probes consisting of both E1 and E2 phases, and more probes of E2 alone (Figure 2.23), were detected in the 462-T3-18 line when compared with the IR70, TN1 or 463-4 lines, however the mean duration of E1+E2 probes, and E2 probes alone, were significantly longer in the parental TN1 and 463-4 empty vector plants than the remaining three lines. BPH made a significantly longer initial probe on the 463-4 empty vector line compared with the RNAi 462-T3-18 line, but exhibited no significant differences in the time to make the first E2 probe between the genotypes according to the Tukey Post-Hoc test, despite a significant ANOVA result.

Thus, the EPG data shows that the penetration was significantly longer and the duration of phloem ingestion was clearly shorter on the RNAi lines compared to the transformed empty vector 463-4 and TN1 lines. There were also more occurrences of non-probing and pathway activities observed in the RNAi lines and resistant IR70 than in the susceptible control lines. Therefore, these results demonstrated that BPH feeding was inhibited on the RNAi lines expressing reduced levels of *Gns5* and on the resistant line IR70, compared to susceptible lines.

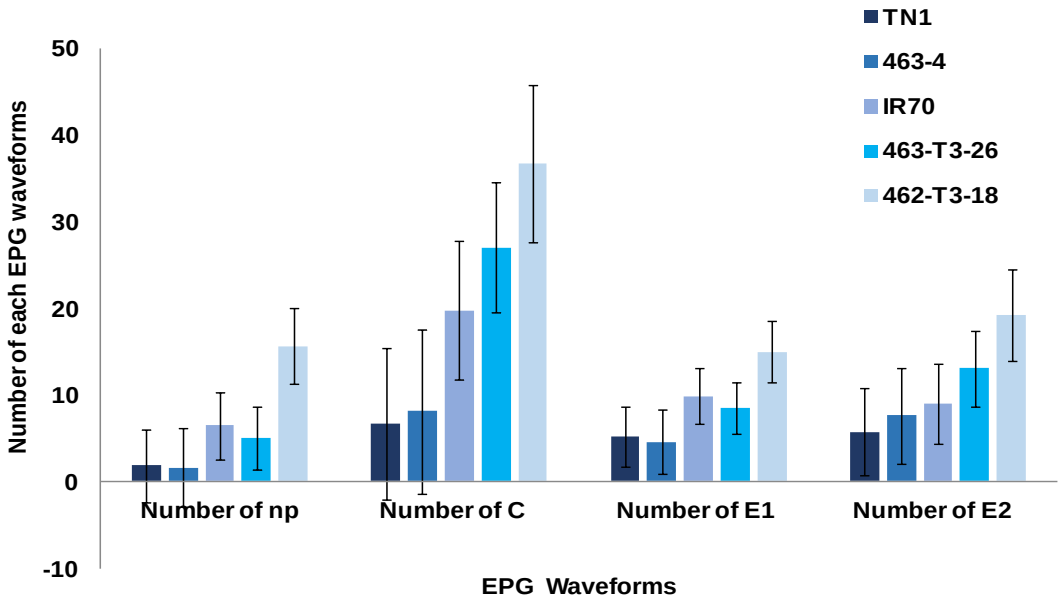


Figure 2.22 Four key phloem feeding parameters of BPH on each of five rice lines collected using the Electrical Penetration Graph technique. Parameters are number of non-probing periods, number of C probes, number of E1 probes and number of E2 probes, with 95% confidence intervals.

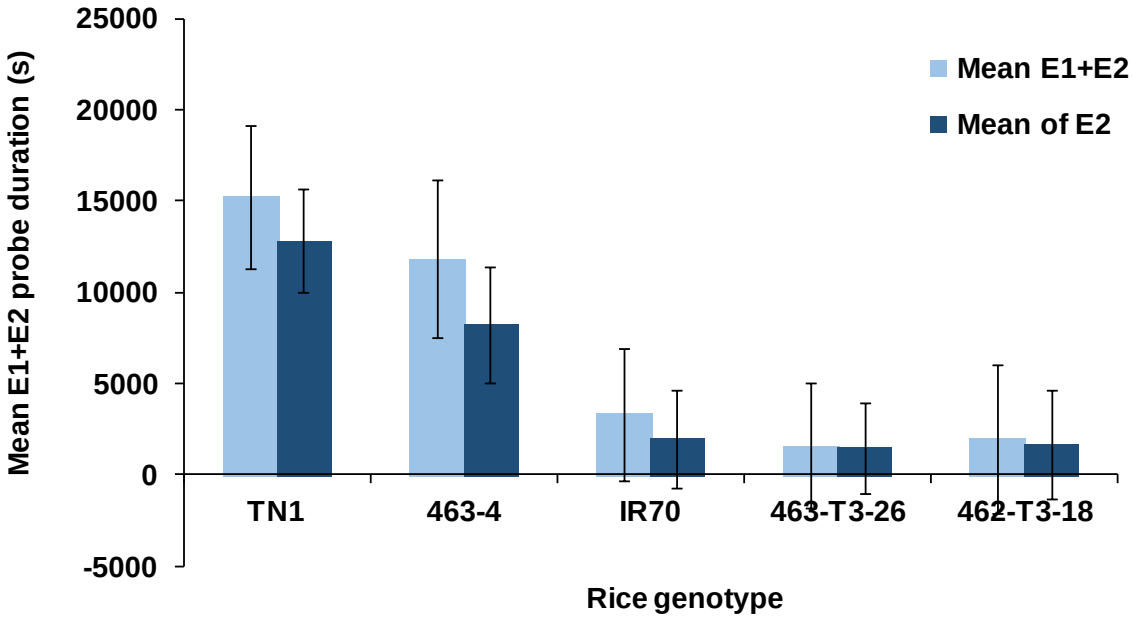


Figure 2.23 Two key phloem feeding parameter of BPH: mean E1 and E2 probe duration and mean of E2 on each of the five rice lines, collected using the Electrical Penetration Graph technique. Measurements are presented with 95% confidence intervals.

Table 2.6 Mean values ± 95% confidence intervals for 22 parameters measured during an Electrical Penetration Graph (EPG) studies to assess the resistance of a range of rice plant genotypes to BPH infestation. Differences in EPG parameters were analyzed using an ANOVA with Tukey Post Hoc test; p-values and N values are displayed for each genotype, and genotypes which differed from each other for each parameter are indicated with letters. Genotypes which have different letters for each parameter differ significantly.

Waveforms	TN1		463-4		IR70		463-T3		462-T3		*p* value
	Mean ± 95% CIs	*N*	Mean ± 95% CIs	*N*	Mean ± 95% CIs	*N*	Mean ± 95% CIs	*N*	Mean ± 95% CIs	*N*	
Pre-phloem parameters											
Number of non probing periods	1.83 ± 4.16 ***b***	12	1.60 ± 4.55 ***b***	10	6.43 ± 3.85 ***b***	14	5.00 ± 3.60 ***b***	16	15.6 ± 4.34 ***a***	11	0.000
Average non probing duration	450 ± 3312 ***a***	8	347 ± 3123 ***a***	9	2441 ± 2598 ***a***	13	5636 ± 2342 ***a***	16	3000 ± 2962 ***a***	10	0.045
Sum of non probing (s)	1298 ± 9606 ***b***	12	677 ± 10523 ***b***	10	13926 ± 8894 ***ab***	14	24527 ± 8319 ***a***	16	17447 ± 10033 ***ab***	11	0.002
Total number of probes	2.75 ± 4.14 ***b***	12	2.60 ± 4.53 ***b***	10	7.14 ± 3.83 ***b***	14	5.31 ± 3.58 ***b***	16	16.36 ± 4.32 ***a***	11	0.000
Number of brief probes (< 180 s)	0.167 ± 2.00 ***b***	12	0.20 ± 2.19 ***b***	10	1.14 ± 1.85 ***b***	14	1.13 ± 1.73 ***b***	16	6.36 ± 2.09 ***a***	11	0.000
Average probe (s)	31486 ± 9625 ***a***	12	25319 ± 10544 ***ab***	10	14204 ± 8911 ***ab***	14	11817 ± 8335 ***b***	16	10360 ± 10052 ***b***	11	0.009
Number of C probes	6.67 ± 8.67 ***b***	12	8.10 ± 9.50 ***b***	10	19.8 ± 8.02 ***ab***	14	27.00 ± 7.51 ***a***	16	36.7 ± 9.05 ***a***	11	0.000
Average C (s)	535 ± 369 ***a***	12	702 ± 405 ***a***	10	650 ± 342 ***a***	14	1131 ± 320 ***a***	16	477.9 ± 385.9 ***a***	11	0.066
Number of G probes	1.92 ± 2.46 ***b***	12	2.80 ± 2.69 ***b***	10	4.79 ± 2.27 ***ab***	14	8.75 ± 2.13 ***a***	16	5.00 ± 2.57 ***ab***	11	0.001
Average G (s)	658 ± 1327 ***a***	12	590 ± 1455 ***a***	10	2952 ± 1229 ***a***	14	889 ± 1150 ***a***	16	1596 ± 1386 ***a***	11	0.057
Phloem parameters											
Number of E1 probes without E2	2.67 ± 2.74 ***b***	12	2.10 ± 3.00 ***b***	10	5.50 ± 2.53 ***ab***	14	5.25 ± 2.37 ***ab***	16	9.27 ± 2.86 ***a***	11	0.007
Total number of E1	5.17 ± 3.45 ***b***	12	4.60 ± 3.78 ***b***	10	9.86 ± 3.19 ***ab***	14	8.50 ± 2.99 ***ab***	16	15.00 ± 3.60 ***a***	11	0.001
Average E1 (s)	317.5 ± 454.2 ***b***	12	141.9 ± 497.6 ***b***	10	1643 ± 421 ***a***	14	350.5 ± 393.4 ***b***	16	283.2 ± 474.5 ***b***	11	0.000
Number of E1+2 phloem probes	4.75 ± 4.78 ***b***	12	6.60 ± 5.23 ***b***	10	7.86 ± 4.42 ***b***	14	12.4 ± 4.13 ***ab***	16	17.8 ± 4.99 ***a***	11	0.002
Average E1+2 probe (s)	15252 ± 3912 ***a***	12	11842 ± 4285 ***a***	10	3336 ± 3622 ***b***	14	1608 ± 3388 ***b***	16	1939 ± 4086 ***b***	11	0.000
Number of E2 probes	5.75 ± 5.02 ***b***	12	7.60 ± 5.50 ***b***	10	9.00 ± 4.65 ***b***	14	13.1 ± 4.35 ***ab***	16	19.2 ± 5.25 ***a***	11	0.004
Average E2 (s)	12837 ± 2874 ***a***	12	8224 ± 3148 ***a***	10	1968 ± 2661 ***b***	14	1447 ± 2489 ***b***	16	1650 ± 3001 ***b***	11	0.000
Time to 1st probe (s)	636 ± 633 ***a***	12	177 ± 693 ***a***	10	757 ± 586 ***a***	14	737 ± 548 ***a***	16	192 ± 661 ***a***	11	0.503
Duration of 1st probe (s)	7752 ± 9007 ***ab***	12	26123 ± 9866 ***a***	10	8065 ± 8338 ***ab***	14	11170 ± 7800 ***ab***	16	806 ± 9407 ***b***	11	0.009
Time to 1st E1 (s)	464 ± 963 ***a***	12	908 ± 1056 ***a***	10	1646 ± 892 ***a***	14	1107 ± 834 ***a***	16	815 ± 1006 ***a***	11	0.476
Time to 1st E1+2 probe (s)	1739 ± 1883 ***a***	12	2542 ± 2063 ***a***	10	4623 ± 1744 ***a***	14	4651 ± 1631 ***a***	16	2624 ± 1967 ***a***	11	0.081
Time to 1st E2 (s)	1777 ± 1937 ***a***	12	2637 ± 2123 ***a***	10	5187 ± 1794 ***a***	14	5221 ± 1678 ***a***	16	2663 ± 2024 ***a***	11	0.025

2.5 Discussion

2.5.1 *Expression of hpt and Gns5 genes in IR463 and IR462 RNAi lines*

PCR screening has clearly distinguished the transformed RNAi lines (IR463 and IR462) from the transformed empty vector lines (463-4, 462-20 and 462-33) and the parental TN1 plants. The expressed *hpt* gene under two different constitutive promoters, CaMV 35S and maize Ubiquitin, indicated the presence of the *hpt* gene in the subsequent T3 generation of the RNAi lines and the transformed empty vectors. Thus, it was demonstrated that expression of *hpt* gene in the homozygous RNAi lines could be stably inherited through the T3 generation.

A specific pair of primers was constructed to check the presence of the antisense *Gns5* fragment transgene in the transformed IR463 and IR462 RNAi lines. PCR analysis revealed that this antisense gene fragment was only present in the RNAi lines and not in the transformed empty vector lines or TN1. This PCR study was conducted to ensure that the RNAi lines contain the antisense *Gns5* transgene and most importantly that the antisense *Gns5* transgene was absent from all of the empty vectors used in this analysis (462-20, 462-33 and 463-4). Therefore, the results of this study strongly indicate the role of *Gns5* in BPH susceptibility and the consequences of knockdown of this gene in the RNAi lines for BPH resistance.

Previous studies reported that the expression of introduced genes (transgenes) in transgenic rice are stably inherited at least through to the T6 generation (Sun *et al*., 2002). This could be an important aspect of this study, as future studies carried out with different generations of RNAi lines can be compared to the findings of the present study. Other research also showed that homozygous RNAi lines with enhanced resistance to *Rice stripe virus* obtained after T3 generation was stably inherited to the T5 generation (Li *et al.*, 2016). This study demonstrated that the *hpt* and antisense *Gns5* genes in the IR463 and IR462 RNAi lines were stably inherited through to the T3 generation. All experiments in this study were carried out using the T3 generation RNAi lines and transformed empty vector lines.

2.5.2 *Knockdown of Gns5 in IR463 and IR462 RNAi lines*

The qPCR analysis showed that expression of the *Gns5* in IR463 and IR462 RNAi lines was significantly down-regulated compared to their respective parental TN1 controls. As expected, *Gns5* expression in the transformed empty vector lines which do not contain the antisense, *Gns5* remains at a similar level as in the TN1 control. This analysis also revealed that *Gns5* was down-regulated in IR462 to a greater magnitude (~ 4 fold) compared to the IR463 RNAi lines. The result from this expression profile shows that *Gns5* expression is significantly reduced in both the RNAi lines but to a greater extent in the IR462 RNAi lines. The dsRNA-mediated RNAi knock down efficacy was not 100% for the GNS5 gene, however substantial down-regulation of gene expression between 50% to 75% in both IR463 and IR462 RNAi lines was sufficient to achieve significant effect on the survival and development of BPH.

Shoala (2012) demonstrated that *Gns5* was up-regulated in the susceptible TN1 and down-regulated (~ 2 fold) in the resistant IR70 upon BPH infestation. A similar finding by Kumari *et al.* (2016) revealed that *OsGNS5* was up-regulated ~ 4 fold in a susceptible line, Pusa 1121, than the resistant line Vandana, upon infection by the root-knot nematode (RKN), *Meloidogyne graminicola*. These authors concluded that lignin and callose-mediated plant basal defence may inhibit the penetration and delay the development of RKNs in the root of resistant varieties. An earlier report by Hao *et al.* (2008) showed that *Gns5* was up-regulated in the susceptible TN1 and resistant B5 rice variety but to a higher expression level (~ 9 fold) in the susceptible rice plants. In support of the findings of Shoala (2012), this study showed that knocking down of the *Gns5* gene in the susceptible TN1 rice using RNAi resulted in a similar expression pattern of down-regulation as he reported for the resistant IR70 rice cultivar.

2.5.3 *Survival and fecundity of BPH on IR463 and IR462 RNAi lines*

The long term bioassay carried out on different events of both the RNAi lines, namely 463-T3-8, 463-T3-22, 463-T3-26, 463-T3-41, 462-T3-16 and 462-T3-18, showed significant resistance against BPH, with minimal plant damage. This study also showed that the parental TN1 line and transformed empty vector lines (463-4, 462-20 and 462-33) exhibited severe damage upon BPH infestation as compared to the IR463 and IR462 RNAi lines. These studies clearly showed a significant reduction in insect survival of 52% to 63% in all the RNAi lines as compared to the control lines. This decline in insect survival is attributable to the significantly reduced expression of *Gns5* in both the IR463 and IR462 RNAi lines.

A similar pattern of BPH survival was reported by Du *et al.* (2009) who reported that in *Bph14* -transgenic plants showing increased callose deposition, the growth rate of BPH was reduced to one-fifth of that on the wild type rice variety. Furthermore, there was a rapid decrease in the survival rate of BPH on these *Bph14* -transgenic plants. The wild type plants also showed symptoms of stem chlorosis and death of the whole plant after infestation by BPH whereas the *Bph14*-transgenic plants remained healthy, similar to symptoms exhibited by the RNAi lines, wild type TN1 and empty vector lines in this present study. Interestingly, the expression of *Bph14* in the transgenic lines showed abundant deposition of callose upon BPH infestation and

conferred resistance to BPH in rice crops, thus demonstrating similar effects to those observed in the RNAi lines of this study.

The present study indicates that there were significant differences observed in the number of eggs produced by BPH on the RNAi lines compared to the parental TN1 and transformed empty vector lines. The average eggs produced by BPH per plant was reduced by approximately 52% to 55% on the IR463 and IR462 RNAi lines compared to their respective transformed empty vector lines and TN1. This finding may be contributed to by the number of surviving adults in the 22 day bioassay. However, interestingly there were no significant differences in the number of eggs produced by per adult BPH in any of the lines tested. Hence this finding shows that knockdown in the expression of *Gns5* in the IR463 and IR462 RNAi lines did not show any significant effect on the fitness of the surviving insects in terms of fecundity.

Fecundity tests conducted on the IR463 and IR462 RNAi lines also explained that reduced expression of *Gns5* also decreased the fecundity of the insects besides reducing their survival. The number of nymphs produced after forty days in the long term bioassay showed that the fecundity in the IR463 and IR462 RNAi lines was significantly reduced by approximately 54% to 58% in both lines compared to TN1 and their respective transformed empty vector lines.

Du *et al.* (2009) reported that the host choice tests showed no significant differences in the number of BPH nymphs and eggs produced between the *Bph14*-transgenic lines and the wild type plants. This observation was confirmed by Zhang *et al.* (2017) who suggested that the defence responses in rice can only be induced by BPH oviposition and not by BPH feeding. Therefore, the results above support the finding of the present study that knocking down of the *Gns5* in the RNAi rice lines does not affect the fitness of the surviving BPH. In summary, the IR463 and IR462 RNAi lines showed significant differences in the survival and fecundity of the insect compared to the controls (TN1 and transformed empty vector lines).

2.5.4 *Effect of Gns5 on BPH development in IR463 and IR462 RNAi lines*

The 13-day development assay carried out with late first instar nymphs on both the IR463 and IR462 RNAi lines, parental TN1 and transformed empty vector lines (463-4, 462-20 and 462-33) recorded significant differences in the development of BPH in

all the rice genotypes tested. Nymphs fed on the IR463 and IR462 RNAi with reduced *Gns5* expression showed a significant delay in reaching adulthood compared to insects fed on their respective control plants (TN1 and transformed empty vector lines). Among the survivors, only an average of 30% and 34% reached adulthood on the IR463 and IR462 RNAi lines respectively, when compared to 75% to 84% for TN1 and their respective transformed empty vector lines.

The present study also revealed that there was an average of 53% nymphs between the third to fifth instar nymphs in both the RNAi lines as compared to 17% to 24% in the control lines. The undeveloped nymphs (16% to 19%) were found in the RNAi lines and none in the control lines. These development studies clearly show that reduced expression of *Gns5* in the RNAi lines caused a deleterious effect on the development of BPH through to reach adulthood.

The poor development of BPH nymphs on the IR463 and IR462 RNAi lines plants was also reflected by the insect biomass. The mean insect biomass per plant significantly decreased by approximately 58% and 60% respectively in the IR462 and IR463 RNAi lines compared to TN1 and their respective empty vector controls after 13 days. These results demonstrated that the RNAi lines significantly retarded BPH development to adulthood and therefore affected the insect biomass.

The present study represents the first study to demonstrate that reduced expression of *Gns5* caused an effect on the development of the BPH nymphs to adulthood. However, an earlier report by Du *et al.* (2009) showed that the expression of the *Bph14* gene (which increases callose deposition) in the rice variety also reduced the fecundity and longevity of BPH. Other similar findings such as expression of snowdrop lectin [*Galanthus nivalis* agglutinin (GNA)] delayed the development of whitebacked planthopper (*Sorgatella furcifera*) nymphs to adulthood on the transgenic rice (Nagadhara *et al.*, 2004). Rao *et al.* (1998) also demonstrated that expression of GNA in rice reduced the insect biomass and retarded the development of BPH nymphs in the transgenic rice variety. Both studies demonstrated that expression of GNA conferred resistance to the whitebacked planthopper and BPH. Therefore, this study also showed a similar pattern of results in the survival, fecundity and development of BPH in the RNAi lines where knockdown of *Gns5* expression conferred high levels of resistance to BPH.

2.5.5 *Callose deposition in RNAi lines expressing reduced levels of Gns5*

Microscopic examination revealed that RNAi lines with reduced levels of *Gns5* transcripts have increased callose deposition in leaves and stems in response to BPH infestation. The IR70 leaf samples were used as BPH-resistant controls. The sieve plates deposited with callose in the phloem located at the vascular bundle of all the IR463 and IR462 RNAi lines including the resistant IR70 was obviously thickened and emitted strong fluorescence in the infested plants as compared to the infested control lines (TN1, 463-4, 462-20 and 462-33). Quantification of fluorescence indicated that callose was also more abundantly accumulated in the sieve plates of IR463 and IR462 leaves and stems infested by BPH than in the non-infested tissue samples. This observation indicates RNAi lines expressing reduced *Gns5* mediate the constitutive accumulation of callose and its stronger deposition in response to BPH infestation.

The results of the current study are consistent with those of Hao *et al.* (2008) who reported that the susceptible TN1 variety showed little or no callose deposition on the sieve plate in the leaf sheaths of non-infested plants. When the plants were infested with BPH, more callose deposition was observed. However, the resistant B5 (carrying *Bph14* and *Bph15* gene) rice variety showed more callose deposition after BPH infestation compared to the infested TN1. Callose deposition increased during the first 3 days of infestation on the resistant B5 (13.7 callosic sieve plates in 50 sections) compared to the infested TN1 (5.8 callosic sieve plates in 50 sections). Prolonged BPH feeding in the susceptible TN1 rapidly decreased the callose deposition in this plant to 2.4 callosic sieve plates in 50 sections after 4 days but a high level of callose deposition was observed in the resistant B5 plants with BPH infestation. Further analysis carried out by Hao *et al.* (2008) revealed that there were strong fluorescence signals from callose in almost all the sieve tubes penetrated by stylet sheaths in the resistant B5 plants. In contrast, the majority of target sieve tubes did not have bright callose deposition in the susceptible TN1 plants. The results from Hao *et al.* (2008) and the present study showed that both genes (*Bph15* and *Gns5*) are involved in the same defence mechanism, which exhibits induced callose deposition upon BPH attack.

Wei *et al.* (2009) also demonstrated increased expression of *Gns5* in the susceptible TN1 but not in the resistant rice lines (carrying *Bph15*). These finding are consistent

with the findings of both Hao *et al.* (2008) and those from the present study and lead these authors to conclude that *Gns5* gene is clearly induced when rice plants are exposed to BPH infestation and is likely to play an important role in callose deposition which allows the ingestion of phloem sap by BPH in the susceptible rice plants. Therefore, absence or suppression of this gene allows sieve tube occlusion to be maintained in the resistant rice plants.

The defence mechanisms against BPH in rice plants have also been investigated by Cheng *et al*. (2013) who reported that sealing of the sieve tubes is an important mechanism against phloem feeders. Callose deposition through sieve tube sealing is useful to quantify plant immunity to insects and pathogens including phloem feeders such as BPH. Rice plants with the BPH resistance gene such as *Bph14* allow callose deposition on the sieve plates which occludes the sieve tubes. Occlusion of the sieve tubes prevents BPH from ingesting the sap phloem. In contrast, plants which are able to produce increased levels of β, 1-3-glucanase genes (*Gns5*) in response to BPH attack promote callose hydrolysis in the phloem cells of the susceptible rice plants and therefore allow continuous feeding of BPH.

The findings above are consistent with and support the findings of the current study. Higher levels of callose deposition observed in the RNAi lines expressing antisense *Gns5* enhanced their resistance to BPH by the occlusion of phloem sap flow. Therefore, this evidence shows that *Gns5* plays an important role in the defence mechanism of the rice plants and provides evidence that callose deposition was induced by *Gns5*-mediated resistance.

2.5.6 *EPG analysis to study feeding behaviour of BPH to Different Rice Genotypes*

In this study, we characterized BPH feeding behaviour using the EPG technique and identified differences in EPG waveforms for feeding on the different rice genotypes tested. Three different genetically transformed rice lines, and two rice varieties were used to analyse the effect of enhanced callose accumulation on BPH resistance. The rice lines used were the parental TN1 (a rice variety susceptible to BPH), IR70 (a rice variety resistant to BPH), 462-T3-18 and 463-T3-26 (two RNAi lines formed from transformation of TN1 with vectors carrying antisense *Gns5* gene conferring enhanced wound-induced callose accumulation), and 463-4 (TN1 variety transformed

in the same manner as above but with an empty vector inserted). There were five typical waveforms of BPH feeding identified in this present study, which showed almost consistent findings as described in previous studies by Ghaffar *et al*. (2011), He *et al.* (2011) and Zhang *et al*. (2015).

Overall, the IR463, IR462 and IR70 demonstrated a higher number of occurrences of NP (non-penetration of stylet), C (pathway activities), E1 (sieve element salivation), E2 (phloem ingestion) and G (xylem ingestion) waveforms in the EPG analysis compared to the controls (TN1 and transformed empty vector line, 463-4). This finding is supported by the EPG results obtained by Hao *et al.* (2008) and Zhang *et al*. (2015) whereby both the authors reported that all of the above waveforms were higher in the resistant rice variety tested compared to the susceptible TN1. These findings indicated that the insect spent more time moving around, probing more frequently and that the feeding was interrupted more often on the RNAi and the resistant IR70, compared to the susceptible lines.

The resistance of the rice plants to BPH is closely associated to the phloem related waveforms (Hao *et al.*, 2008). Interestingly the present study demonstrated that there were no significant differences in the mean number of E1 between the IR463, IR462, TN1 and 463-4. However, on the resistant line IR70, BPH exhibit significantly longer duration of salivation. This finding is different from these report by Ghaffar *et al*. (2011) whereby the authors reported that that the pattern of sieve element salivation waveform did not significantly differ between the susceptible, moderately resistant and resistant rice genotypes.

The present study revealed that BPH ingestion of the phloem sap was significantly longer in the control lines (TN1; 463-4) compared to the RNAi lines or the resistant IR70. This result indicated that BPH could reach the sieve element region of both RNAi lines, resistant IR70 and susceptible TN1 and the transformed empty vector line, but could only ingest for a longer duration without interruption in the susceptible TN1 and 463-4. These results are consistent with the findings of Du *et al.* (2009) and Hao *et al.* (2008), both of which showed that the duration of phloem ingestion in the respective resistant rice genotypes was shorter compared to their susceptible rice genotypes.

As reported by Zhang *et al*. (2015), the present study also showed that there were no significant differences observed in the mean duration of the G waveform in all the rice genotypes tested. As a phloem feeding insect, BPH ingestion in the plant xylem has been observed, but only occasionally, in aphids, the xylem ingestion increased after a period of starvation. Unlike phloem, xylem ingestion does not provide nutrition to the insect but might be useful for compensating for dehydration (water stress) (He *et al.*, 2011). Although no significant differences were observed in terms of the duration of xylem ingestion between genotypes, they showed that the number of xylem ingestion occurrences was higher in the resistant lines compared to the susceptible lines. BPH feeding was inhibited in the resistant lines and caused a higher level of dehydration which may be the potential reason for the higher number of occurrence of xylem ingestion in these rice genotypes.

Similar to the findings of Du *et al.* (2009) and Ghaffar *et al*. (2011), the present study revealed that there were no significant differences observed in the time to the first E1 waveform across all of the genotypes tested. There were also no significant differences observed in the duration of the first E2 in all rice genotypes tested in this study, thus supporting the results obtained by Du *et al.*, 2009. This timing shows that BPH is able to locate the sieve elements across all the rice genotypes but that the ability to sustain phloem sap ingestion was different between the controls (TN1 and 463-4) and the resistant lines (IR70, IR463 and IR462).

The results obtained from the current EPG study, monitoring BPH feeding provided further evidence that enhanced resistance to BPH was due to knockdown of *Gns5* in rice, which was more effective in 462-T3-18 (maize Ubiquitin), than the 463-T3-26 line (CaMV 35S promoter). Evidence for enhanced resistance in the 462-T3-18 RNAi line lies in a large number of parameters that are significantly altered in this genotype compared to the parental TN1 and transformed empty vector line (463-4). These include significantly more non-probing periods, probes of all types, brief probes, pathway phase (C) probes, E1 probes without a subsequent E2, E1 and E2 probes, and E2 probes. They also include a significantly lower mean duration of all probe types, mean E1 probe duration, mean E1 + E2 probing, mean E2 duration, and mean first probe duration when compared to the controls.

These parameters indicate that BPH is less able to exploit the 462-T3-18 RNAi line as a host, with differences in certain parameters indicating different behaviours in the

insect in its attempts to interact with the plant host. More non-probing behaviour, more probes overall, and more brief probes, but reduced duration of all probes generally and first probes specifically, were observed on 462-T3-18, which indicates the insect is encountering difficulties in initiating long term feeding and is traversing the leaf surface and attempting to feed on different leaf parts in order to find a suitable feeding site. More pathway phase probing, more E1 probes, and more E1 probes that do not culminate in a successful E2 all indicate that once feeding commences the insect is successful in navigating the plant tissue but, is unable to successfully settle into phloem extraction. More E1+ E2 probing and more E2 probing overall initially appear to indicate that BPH are able to access the phloem on this rice line, but when combined with the significantly reduced duration of E probing of all types (E1, E1+E2 and E2), these results indicate that the insect is able to reach the phloem as easily as in the susceptible TN1 and transformed empty vector 463-4 line, but is unable to successfully establish a long term feeding association, resulting in more short-lived feeding attempts than on the parental TN1 and transformed empty vector lines.

The results outlined above indicate that the other transformed RNAi line expressing antisense *Gns5*, 463-T3-26, also exhibits enhanced resistance to BPH, but to a lesser extent. Significantly more non-probing behaviour was observed in comparison to the parental TN1 and transformed empty vector line 463-4, again indicating that BPH is struggling to establish a feeding relationship with its host. Additionally, lower numbers of pathway phase probing events and significantly shorter mean probes of all types on the 463-T3-26 line, in comparison to TN1 and 463-4, indicates less successful feeding when an association has been established. Furthermore, a lower mean E1+E2 probing duration, mean E2 probe duration, and xylem feeding events show that phloem feeding and even xylem feeding is less successful on 463-T3-26.

The basis of the resistance observed above is proposed to be in the enhanced wound-induced callose deposition observed in the transgenic rice line 462-T3-18, and to a lesser extent in the 463-T3-26 line. This enhanced resistance is due to the inserted transgene (antisense *Gns5*), and is independent of the vector used to insert the gene, as the empty vector line performed near-identically to the susceptible line, with no significant differences in the insect's feeding behaviour between the TN1 and transformed empty vector line, 463-4.

This result is expected, and is consistent with microscopy studies which show that 462-T3-18 produces a slightly stronger callose deposition response than 463-T3-26 post BPH infestation. As the only difference between these inserts is the constitutive promotor controlling gene expression (463-T3-26 in CaMV 35S and maize Ubiquitin in 462-T3-18), this can only be due to the change in promotor being more efficient at driving gene expression. The fact that resistance to BPH is correlated with the strength of the callose deposition response is further evidence that the resistance observed is due to the transgene insert in 463-T3-26 and 462-T3-18.

The IR70 line is a rice variety that has been shown to be resistant to BPH attack (Jena and Kim, 2010; Shoala, 2012). However, in the present study, only three parameters are significantly different between IR70 and the TN1 and the empty vector lines: significantly increased mean E1 duration (i.e. time spent salivating), and significantly reduced mean E2 duration (time spent ingesting phloem sap), mean E1+E2 probe duration. However, when the two RNAi rice varieties are removed from the ANOVA analysis, and IR70 is compared to the TN1 and empty vector lines alone, the number of parameters for which there is a significant difference increase to ten (analysis not shown), including number of non-probing events, number of probes, number of brief probes, number of C probes, number of xylem probes, number of E1 without an E2, and number of E1 probes in total.

IR70 presents a different profile of parameters that differ significantly to the TN1 and empty vector lines, then those presented by the RNAi lines 462-T3-18 and 463-T3-26. IR70 is the only line to show significantly higher levels of salivation (E1 probing) when compared to the controls. Increased salivation has been correlated with higher host plant resistance in other study systems (McDaniel *et al*. 2016) and may be indicative of an attempt by the BPH to overcome host resistance mechanisms, as occurs in aphids (Will *et al*. 2007) and whiteflies (Jiang and Walker, 2007). Increased salivation, the highest level of callose deposition upon BPH attack, higher non-penetration and longer duration of phloem ingestion in IR70 indicates that this is a resistant rice variety. The IR70 plants might possess different or similar resistance mechanisms as the RNAi lines expressing *Gns5*, but this would require further investigation.

The fact that the transgene insert does not cause greater levels of salivation could be promising for the use of this insert as a means to increase host plant resistance to

BPH: phloem-feeding insect saliva is a major cause of plant virus transfer (Xue *et al.*, 2010), so the fact that no more salivation occurs in the IR463 and IR462 RNAi lines than the control is a promising result, as no more viral transfer will occur than in an insect-susceptible host feeding interaction.

Based on EPG studies, 462-T3-18 was shown to be the most resistant RNAi line compared to line 463-T3-26. However, both the RNAi lines expressing antisense *Gns5* demonstrated enhanced resistance to BPH as compared to the susceptible TN1 and their respective transformed empty vector lines. Results from EPG studies are consistent with all other results obtained in this project.

2.6 Conclusions

The present study explored the role of β-1,3-glucanase 5 (*Gns5*) to better understand the interaction between BPH and its host plant, rice. In this study, RNAi lines expressing antisense *Gns5* with different constitutive promoters were generated. In IR462 maize ubiquitin was the promoter system used whereas in IR463 the CaMV 35S promoter was used. The results of this study revealed that these RNAi lines, with reduced *Gns5* expression, exhibited enhanced resistance to BPH both in terms of significantly reduced survival and retarded development.

As reported, knockdown in the expression of *Gns5* had a significant deleterious effect on the overall fecundity of BPH. However, this effect was most likely due to lower survival of the BPH rather than a decreased number of eggs produced or lowers viability of the eggs. The transformation itself had little effect on the insect as the transformed empty vector lines (463-4, 462-20 and 462-33) clearly exhibited similar results to the susceptible TN1 rice genotype.

The gene expression profile of *Gns5*, which encodes the callose hydrolyzing enzyme, β-1,3-glucanase was down-regulated in the RNAi lines IR463 and IR462 so preventing the breakdown of callose thus keeping the sieve tubes occluded. Taken together, the findings of the present study showed that knockdown of *Gns5* in the susceptible TN1 rice genotype contributes to a significant reduction of BPH feeding through the mediation of callose deposition which enhanced the plant resistance to BPH. Callose was deposited abundantly on sieve plates of the vascular bundle, the site at which BPH feeds, in both of the RNAi lines in response to BPH infestation. Therefore, these results suggest that *Gns5* plays an important role in the defence

mechanism of the plants, and its down-regulation/ knockdown prevents BPH from ingesting the phloem sap of the plant.

The EPG studies were an indicator of BPH feeding behaviour in the RNAi lines expressing antisense *Gns5*. The results strongly correlated with the mode of feeding of the EPG analysis. BPH exhibited rapid occurrences regarding the pre-phloem waveforms such as non-probing (NP) and pathways (C). Most importantly, this study showed that BPH ingested the phloem sap from the RNAi lines for a shorter duration with high occurrences of interruption than on the susceptible controls tested. The shorter duration of phloem ingestion reflected the increased callose accumulation in the RNAi lines, as observed by confocal microscopy.

Although the IR462 RNAi line exhibited higher knockdown of *Gns5*, higher relative callose deposition in the stem tissues and a higher level of resistance in the EPG analysis, both IR463 and IR462 RNAi lines showed significantly enhanced resistance to BPH with no differences between them, despite the different constitutive promoters used.

In summary, this present study confirms the hypothesis and demonstrates that knockdown of *Gns5* in the BPH-susceptible rice cultivar TN1, results in enhanced resistance to this devastating insect pest as a consequence of sieve tube occlusions and increased callose deposition on the phloem sieve plates, thus preventing abstraction of phloem sap by the insect. This study proposes that RNAi is a valuable tool for rice breeding for enhanced resistance to BPH and that *Gns5* represents a viable target. As such, this technology can provide an alternative method to the control of BPH infestation in rice.

Chapter 3. Physiological Response of Rice to Nitrogen Stress

Abstract

Rice, *Oryza sativa* is one of the most important staple foods for more than half of the world population. In order to fulfil the food demand of the growing population, rice production needs to be increased significantly to ~42%, from its current level. Abiotic stress such as nutrient deficiency has been a major constraint in rice growing areas. Nitrogen (N) is one of the most essential macronutrients for the growth of rice cultivars, and its availability is a limiting factor affecting rice crop production across the world. Therefore, rice cultivars with high tolerance to N deficiency, play an important role in increasing rice production globally. This study investigated the response of two rice genotypes (BPH: TN1, susceptible; IR70, resistant) under four different levels of N (1.44 mM NH_4NO_3,1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3, and 0.24 mM NH_4NO_3) and a range of physiological parameters were measured. The most significant difference was seen with shoot height at the lowest N input with mean shoot height of 33.0 ± SD cm and 29.0 ± SD cm for TN1 and IR70, respectively representing a decrease of 26.3% and 27.9% compared to the optimal N level (1.44 mM NH_4NO_3). At 1.44 mM NH_4NO_3 N input there were fewer leaves (7 leaves/ plant) on the susceptible rice plants compared to the resistant IR70 (8 leaves/plant). As expected, plants grown under the optimal N level produced the highest number of tillers (with a mean of 6 tillers/ plant) in both rice cultivars. Similarly, leaf area under 1.44 mM NH_4NO_3 N input was 17.0 cm^2 (TN1) and 16.0 cm^2 (IR70) and decreased significantly ($p<0.05$) with a reduced level of N input. The lowest chlorophyll index was recorded in the TN1 and IR70 rice cultivars with the lowest N input which was 24.0 and 25.0 SPAD units, respectively. The IR70 showed higher relative water content level (89%) compared to TN1 (84.2%) at the highest N input. The shortest root length of both the rice cultivars was in the rice cultivars with the lowest N input at 6.9 ± SD cm (TN1) and 6.8 ± SD cm (IR70) respectively. Changes observed in both genotypes for all physiological parameters measured were directly correlated with N input.

Keywords: rice, Nitrogen, physiological parameters

3.1 Introduction

3.1.1 *The role of Nitrogen in rice growth*

Most plants experience nutrient deficiency due to various abiotic factors which have affected crop productivity and caused enormous economic loss globally. Therefore, many investigations have been extensively carried out to study the impact of nutrients on the physiological process of crops. Nitrogen (N) is one of the most abundant minerals in plant tissues which can be found in the soil (Tabar, 2013). N in the soil is absorbed as ammonium and nitrate and converted to other nitrogenous compounds within the plant (Mostafa and Mazinani, 2013).

N is usually taken up during the early stages of growth and is accumulated in the vegetative parts of the plant which will be used for grain formation (Tabar, 2013). It is also required as an important substrate for starch and protein synthesis during grain development (Jiang *et al.*, 2004). N plays an important role in spikelet production during the early stages of panicle formation stage and contributes to sink size during the late panicle formation stage. A recent study also reported that grain yield, biological yield, panicle weight and primary and secondary branch formation in rice has a significant correlation with available N levels (Singh *et al.*, 2014).

Jing *et al.* (2013) reported that the amount of nutrient absorption varies with the different growth stages of rice. The absorption of N is low at the seedling stage and peaks before the heading stage; absorption then decreases as root activity declines. The highest N uptake occurs during the tillering stage followed by the young panicle developmental stage. N concentration also plays an important role in the photosynthetic rate in the rice crop. There is a positive correlation between photosynthetic rate and leaf N concentration whereby the rice leaf N concentration is a critical factor in rice yield (Turner and Jund, 1994).

N uptake also influences the root characteristics such as root length, density and root weight. Under low N conditions, rice acquires more N by increasing the root surface area which increases the root-to-shoot ratio (Vinod and Heuer, 2012). An insufficient N supply inhibits the growth of rice plants and leads to smaller leaves, lower chlorophyll content, less biomass production and subsequently reduced grain yield and quality (Zhang *et al.*, 2015). In contrast, excessive N uptake stimulates shoot

growth but causes root inhibition, delayed flowering and senescence in the rice crop (Vinod and Heuer, 2012). It may also cause significant biochemical changes in plants leading to nutritional imbalance (Tabar, 2013). Therefore, understanding the physiological response of rice cultivars under different levels of N stress is a promising strategy to develop improved plant breeding strategies.

3.2 Objective of the study

The objective of this study is to investigate the physiological response of N stress (abiotic stress) in two rice cultivars, TN1 (BPH; susceptible) and IR70 (BPH; resistant) under four different levels of nitrogen (1.44 mM NH_4NO_3,1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3).

Hypothesis: Rice cultivars, TN1 and IR70 show reduced growth in terms of shoot height, number of tillers and leaves, leaf area, root length, relative water content and chlorophyll content in response to reduced N.

3.3 Materials and Methods

3.3.1 *Seedling preparation and germination of seeds*

Rice seeds Taichung Native1 (TN1) and IR70 were obtained from the International Rice Research Institute (IRRI). TN1 and IR70 seeds were rinsed with distilled water several times and soaked with distilled water in a 50 ml falcon tube. The seeds were left in the dark for 24 hours at room temperature (20°C) and then placed in sterile petri dishes (90 mm diameter) lined with two Whatman NO.1 filter papers and moistened with approximately 5 ml of distilled water. The petri dishes were sealed with parafilm and incubated at 28°C for 48 hours in the dark. At the initial stage of the experiment, the germination rate of both the rice cultivars, TN1 and IR70 were observed. There were 10 seeds per petri dish and three replicates for each rice cultivar. A seed is considered germinated when the radicle is 1 mm long. The germination rate was determined by counting the average number of seeds germinated in the TN1 and IR70 respectively (Vibhuti *et al.*, 2015).

3.3.2 *Composition of nutrient solution and N stress treatments*

The TN1 and IR70 were grown in Yoshida nutrient solution consisting of 1.44 mM NH_4NO_3, 0.3 mM NaH_2PO_4, 0.5 mM K_2SO_4, 1.0 mM $CaCl_2$, 1.6 mM $MgSO_4$, 0.17 mM $NaSiO_3$, 50 µM Fe-EDTA, 0.06 µM $(NH_4)_6$ MO_7O_{24}, 15 µM H_3BO_3, 8 µM $MnCl_2$, 0.12

µM $CuSO_4$, 0.12 µM $ZnSO_4$, 29 µM $FeCl_3$, 40.5 µM Citric acid, pH 5.5 (Lian *et al.*, 2006). Plants were treated with three different levels of N (1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3) as N stress treatments. TN1 and IR70 rice cultivars grown under 1.44 mM NH_4NO_3 (optimal N level) were used as control plants. A N concentration of 0.24 mM NH_4NO_3 was selected as the most extreme stress treatment, based on studies carried out by (Lian *et al.*, 2006), which showed stress symptoms in the rice plants tested.

3.3.3 *Plant growth conditions*

The germinated seeds were transferred into 3.5-inch black plastic pots containing silica sand. Approximately 30 ml of the control nutrient solution (1.44mM NH_4NO_3) was added to the pots and two seedlings were transferred into each pot. There were six replicates for each N level for both the rice cultivars. Approximately 80 ml of control nutrient solution was supplied to plastic containers placed under each pot. Seedlings were grown under the controlled environmental conditions of 28°C during the day and 17°C at night, 16h:8h day night length (photoperiod) and 280-330 µmol $m^{-2}s^{-1}$ illumination. The nutrient solution was renewed every two days.

After two weeks (the second leaf stage), six replicates of each rice cultivars were treated with different N levels (1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3). The remaining 6 replicates were continued to be grown with the control nutrient solution (1.44 mM NH_4NO_3). The top surface of the pots was covered with aluminium foil to prevent algal growth or growth of other contaminants. Plants were treated with the different N levels for three weeks after which time physiological measurements such as shoot height, number of tillers and leaves, root length, leaf area, chlorophyll content and relative water content were recorded (the 5^{th} week of plant growth).

3.3.4 *Growth measurements*

Shoot height of the rice cultivars was measured from the culm base to the tip of the longest leaf and root length was measured from the root-shoot junction to the tip of the longest root (Shaibur *et al*., 2008). The average shoot height, root length, number of tillers and number of leaves of each of the two rice plants per pot were taken; six readings were taken for each plant.

3.3.5 *Leaf area measurements*

The leaf area was determined when plants were at the 5 th week of plant growth by measuring the width of the leaf blade in the middle position and the length of fully developed leaves (Khan, 2014). The leaf area of two fully developed leaves per plant was calculated using the formula as below:

Leaf Area (cm^2) = W x L X 0.75

where W is the width of the leaf blade and L is the length of the leaf (n=6).

3.3.6 *Chlorophyll Index - Soil-Plant Analysis Development (SPAD value)*

The chlorophyll index of two fully developed leaves per plant was measured using a CCM-200 plus Chlorophyll Content Meter. In each leaf, the chlorophyll index of 5 points was measured and the average was calculated. Mean chlorophyll index of each plant was obtained (n=6) (Shaibur *et al*., 2008).

3.3.7 *Relative Water Content (RWC)*

Six randomly selected leaves from each treatment and control for both cultivars were sampled and a mid-leaf section of about 5-10 cm^2/sample was cut using scissors. Each sample was placed in a pre-weighed airtight (oven proof) vial, kept on ice and taken immediately to the laboratory where they were weighed to obtain the leaf sample weight (FW), after which they were immediately hydrated to full turgidity for 4 h at 10°C. After 4 h the samples were taken out of the water, dried using a filter paper and immediately weighed to obtain the fully turgid weight (TW). Samples were then oven dried at 80°C for 24 h and weighed to determine dry weight (DW). All weighing was done using an analytical balance with a precision of 0.0001 g (Lu *et al.*, 2004). RWC was calculated using the following formula:

RWC (%) = [(FW – DW) / (TW – DW)] x 100

where FW is sample fresh weight, TW is sample turgid weight, and DW is sample dry weight.

3.3.8 *Statistical Analysis*

Data were statistically analyzed using the one way ANOVA to analyze the effect of N stress levels in both TN1 and IR70 compared to optimal N level. Differences between the mean values were tested using the Tukey Post-Hoc test at ($p<0.05$). The experimental design was a randomized complete block design with 6 replicates per treatment per cultivar.

3.4 Results

3.4.1 *Effect of N on the growth of rice cultivars TN1 and IR70*

The effect of different N levels on the growth of TN1 and IR70 was determined by measuring a range of different physiological parameters such as shoot height, root length, leaf area, number of leaves and tillers and relative water content. The results obtained for both the rice cultivars under different N levels (1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3) were compared with those of their respective control plants (1.44 mM NH_4NO_3). Overall, there was a significant reduction of these parameters with decreasing N treatment, irrespective of the cultivar. However, even at the lowest N level, neither cultivar showed any visible symptoms of yellowing and drying of leaves after three weeks of N stress (Figure 3.1 and 3.2).

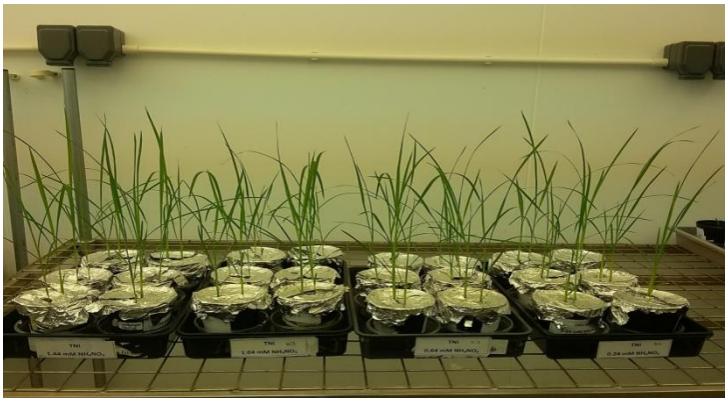


Figure 3.1 TN1 rice cultivar grown under different N levels (from left: 1.44 mM NH_4NO_3, 1.04 mM HN_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3).

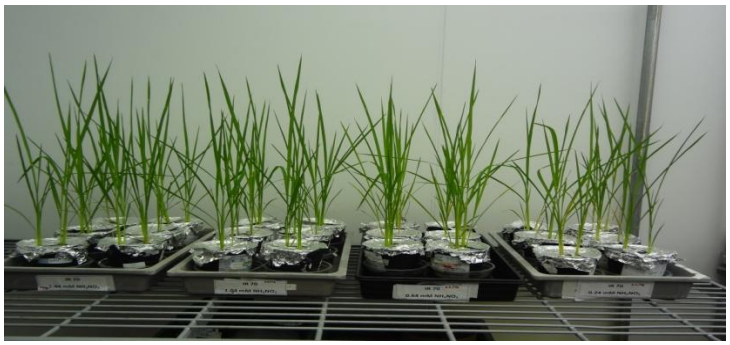


Figure 3.2 IR70 rice cultivar grown under different N levels (from left: 1.44 mM NH_4NO_3, 1.04 mM HN_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3).

3.4.2 *Effects of different N levels on shoot height of TN1 and IR70*

The shoot height of TN1 and IR70 was significantly affected by the application of different N levels. Both rice cultivars showed a decrease in shoot height with decreasing NH_4NO_3 in the nutrient solution. Under optimal nutrient levels (1.44 mM NH_4NO_3) the mean shoot height of TN1 and IR70 cultivars was 44.8 ± SD cm and 40.2 ± SD cm respectively. The mean shoot height decreased to 33.0 ± SD cm in TN1 and 29.0 ± SD cm in IR70 under the lowest N level (0.24 mM NH_4NO_3). Both the TN1 and IR70 rice cultivars showed a significant reduction ($p<0.05$) in shoot height with the decrease in N levels compared to the control rice cultivars (Figure 3.3). The shoot height of IR70 and TN1 rice cultivars was lower by 27.9% and 26.3% respectively in the lowest N level (0.24 mM NH_4NO_3) compared to the controls (1.44 mM NH_4NO_3). Overall, N stress caused a decrease in growth performance of TN1 and IR70 rice cultivars.

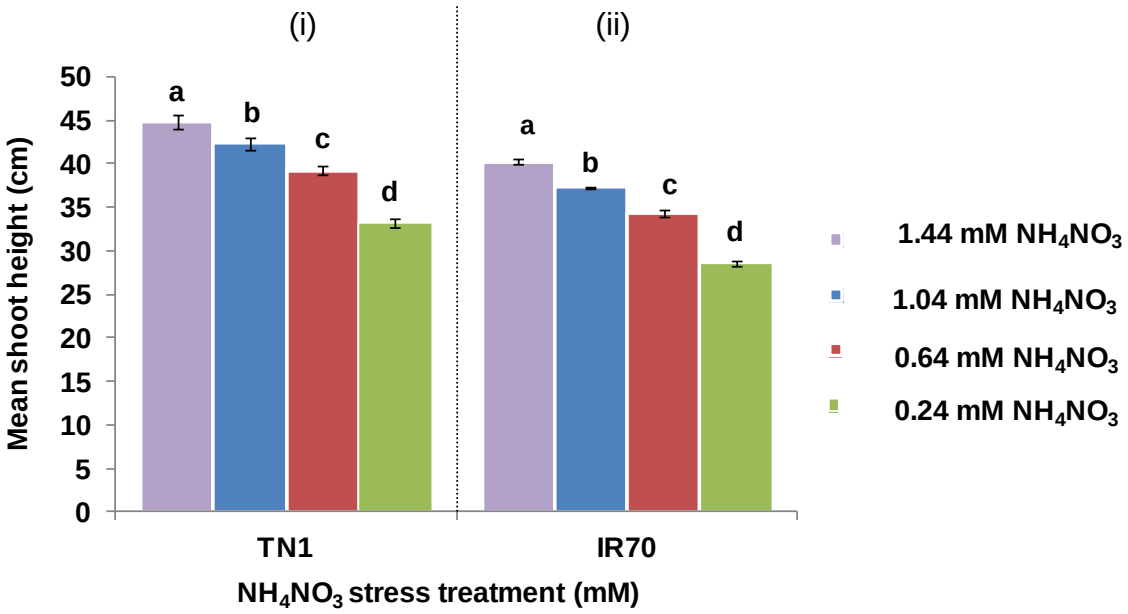


Figure 3.3 Mean shoot height of (i) TN1 and (ii) IR70 rice cultivar grown under different N levels on the 5th week of growth. Data refers to mean ± SD (n=6) and bars not sharing the same letters are significantly different at $p < 0.05$ according to one-way ANOVA followed by Tukey Post-Hoc test within each variety.

3.4.3 *Effect of different N levels on the number of leaves in TN1 and IR70*

The total number of leaves observed at the 5th week of growth of the TN1 and IR70 rice cultivars was significantly affected by the reduced N levels as was the mean number of leaves ($p<0.05$) compared to the control plants (Figure 3.4). TN1 rice cultivar grown under control levels (1.44 mM NH_4NO_3) produced 7 leaves and declined to 5 leaves per plant under lowest N level (0.24 mM NH_4NO_3). The IR70 rice cultivar also showed similar trends in response to different N levels. There were 8 leaves per plant observed in the control IR70 rice cultivar (1.44 mM NH_4NO_3) compared to 6 leaves per plant grown in lowest N level (0.24 mM NH_4NO_3). Neither cultivar showed any significant differences in the number of leaves at the intermediate N levels (1.04 mM NH_4NO_3 and 0.64 mM NH_4NO_3).

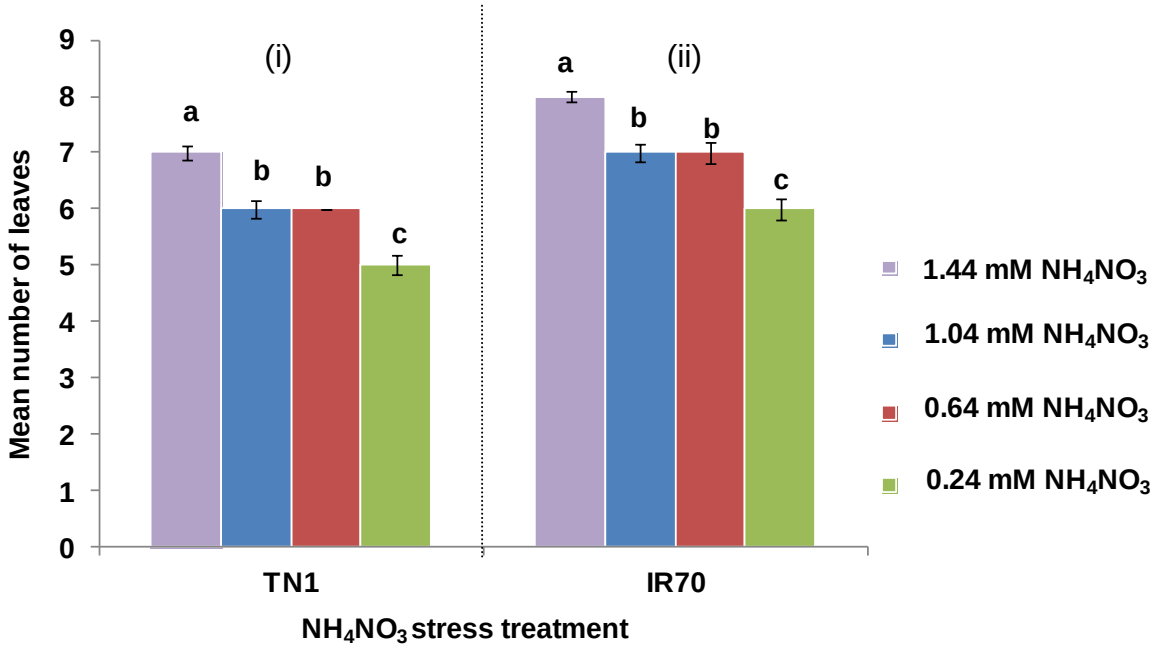


Figure 3.4 Mean number of leaves in (i) TN1 and (ii) IR70 rice cultivar grown under different N levels on the 5th week of growth. Data refers to mean ± SD (n=6) and bars not sharing the same letters are significantly different at $p < 0.05$ according to one-way ANOVA followed by Tukey Post-Hoc test within each variety.

3.4.4 *Effect of different N levels on the number of tillers in TN1 and IR70*

The number of tillers produced in TN1 and IR70 was significantly reduced ($p < 0.05$) under reduced N levels compared to control plants (1.44 mM NH_4NO_3) (Figure 3.5), but was identical between cultivars at each N level. Application of N at 1.44 mM NH_4NO_3 resulted in a maximum number of tillers (6 tillers/plant) with the minimum number of tillers recorded for the lowest N level, 0.24 mM NH_4NO_3 (4 tillers/plant) in both TN1 and IR70, respectively. Overall, the reduction in available N reduced the number of tillers in both the rice cultivars, TN1 and IR70.

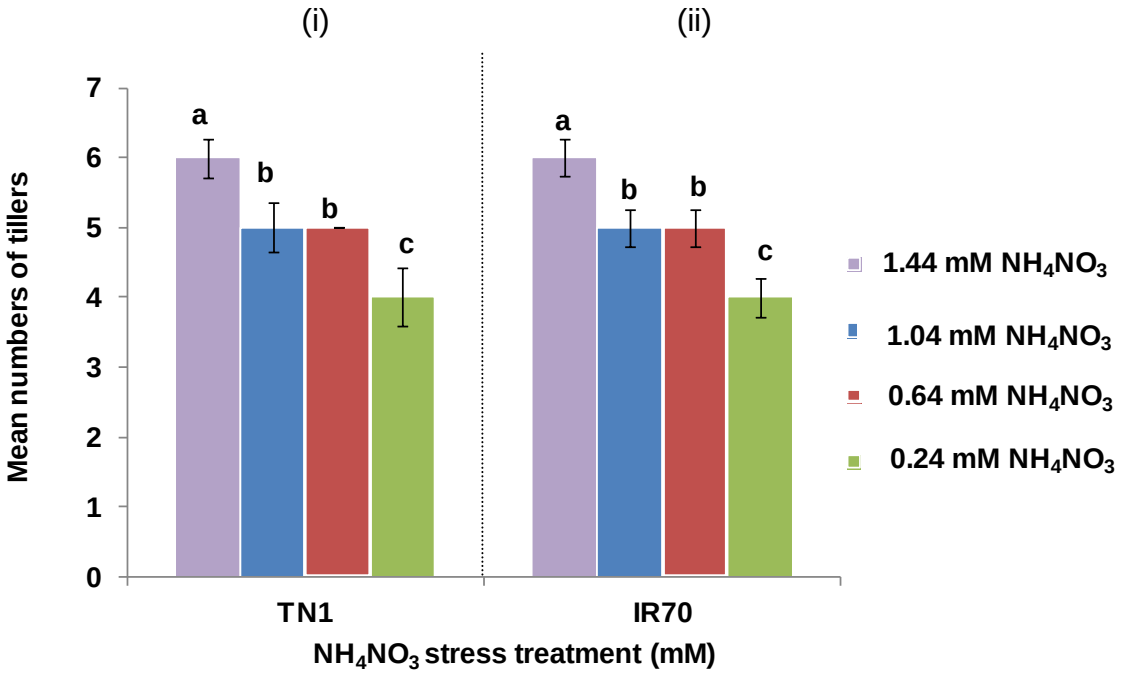


Figure 3.5 Mean number of tillers in (i) TN1 and (ii) IR70 rice cultivar grown under different N levels on the 5th week of growth. Data refers to mean ± SD (n=6) and bars not sharing the same letters are significantly different at $p< 0.05$ according to one-way ANOVA followed by Tukey Post-Hoc test within each variety.

3.4.5 *Effect of different N levels on leaf area of TN1 and IR70*

Different N levels had a significant effect on leaf area. The highest leaf area was recorded in the control plants (1.44mM NH_4NO_3) with 17.0 cm^2 and 16.0 cm^2 for TN1 and IR70 respectively (Figure 3.6). The smallest leaf area was observed in plants grown under the lowest N levels (0.24 mM NH_4NO_3), which was 8.0 cm^2 in TN1 and 7.0 cm^2 in IR70. Both rice cultivars, showed a significant reduction ($p<0.05$) in the leaf area with decreasing N, compared to the controls.

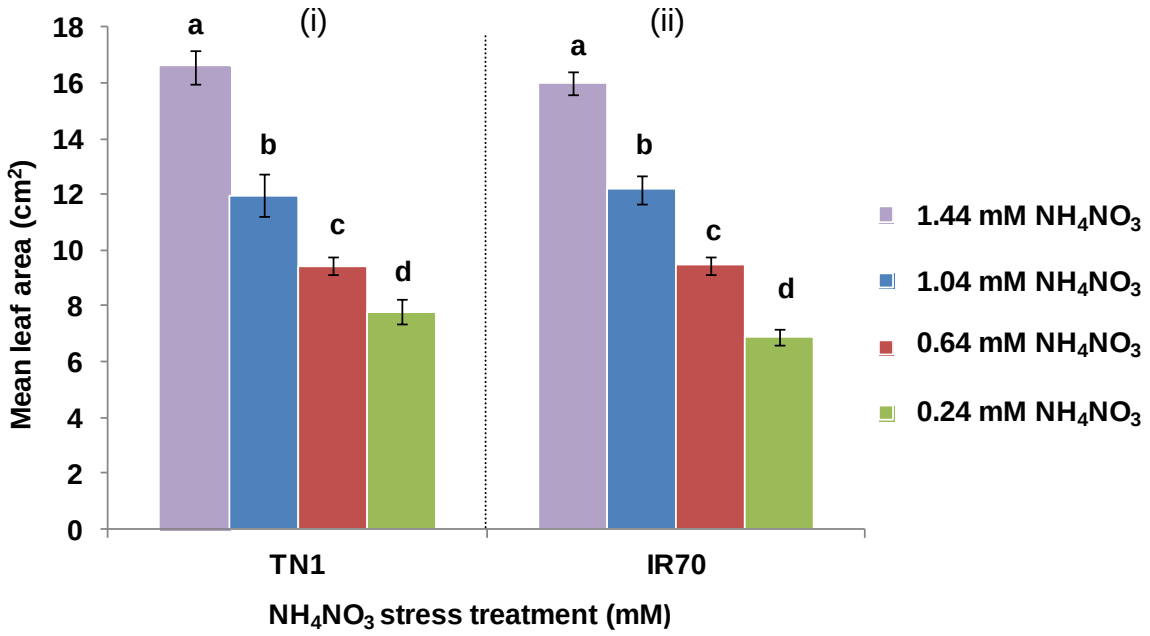


Figure 3.6 Mean value of leaf area of (i) TN1 and (ii) IR70 rice cultivar grown under different N levels on the 5th week of growth. Data refers to mean ± SD (n=6) and bars not sharing the same letters are significantly different at p< 0.05 according to one-way ANOVA followed by Tukey Post-Hoc test within each variety.

3.4.6 *Effect of different N levels on chlorophyll Index (SPAD value)*

This study showed that there was a rapid and significant (p<0.05) decline in the chlorophyll content of both rice cultivars, with decreasing N availability (Figure 3.7). The highest chlorophyll index was observed in the control TN1 and IR70 rice cultivars with SPAD values of 48.4 and 51.3 SPAD, respectively decreasing to 23.6 and 25.3 under the lowest N levels (0.24 mM NH_4NO_3). Both rice cultivars also showed significant differences in the chlorophyll SPAD value between all four different N levels. The results obtained from this study showed that N stress had a great impact on the chlorophyll content of both TN1 and IR70.

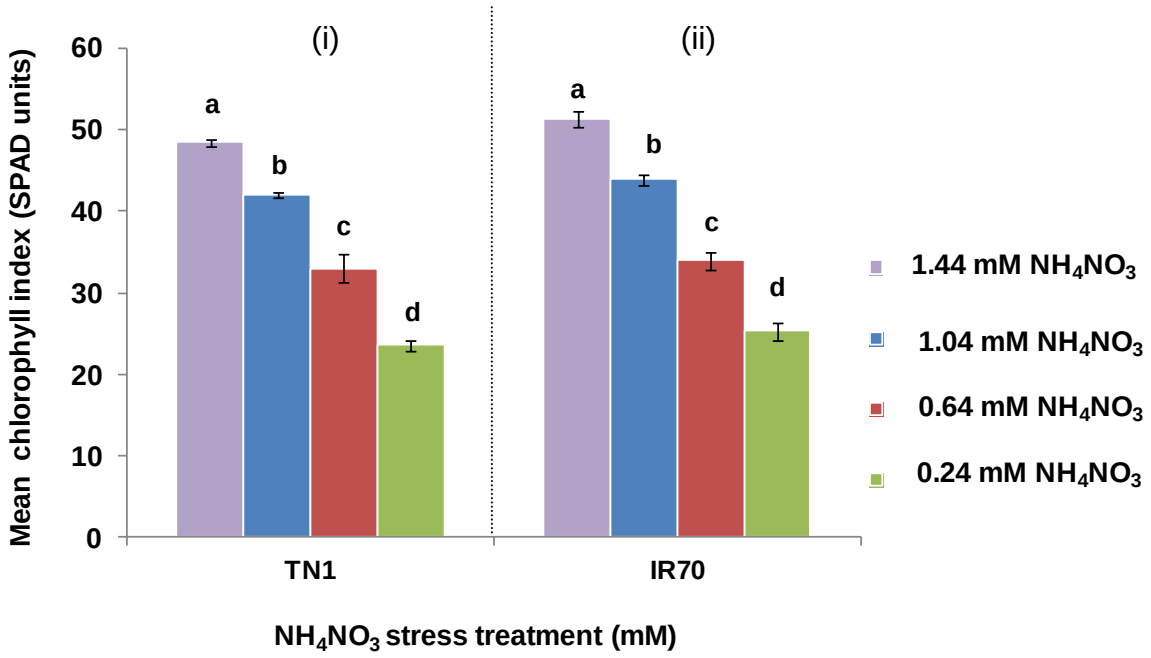


Figure 3.7 Mean value of chlorophyll content in (i) TN1 and (ii) IR70 rice cultivar grown under different N levels on the 5th week of growth. Data refers to mean ± SD (n=6) and bars not sharing the same letters are significantly different at $p< 0.05$ according to one-way ANOVA followed by Tukey Post-Hoc test within each variety.

3.4.7 *Effect of different N levels on RWC in TN1 and IR70*

The relative water content (RWC) in the TN1 rice cultivar was reduced from 84.2% in the control plants (1.44 mM NH_4NO_3) to 61.2%, 57.2% and 46.6% in plants treated with 1.04mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3, respectively (Figure 3.8). A similar trend of reduction in RWC was also observed in IR70. The RWC in the IR70 rice cultivar was 89.0% in the control plants and the percentage declined to 76.1% (1.04mM NH_4NO_3), 63.8% (0.64 mM NH_4NO_3) and 58.9% (0.24 mM NH_4NO_3) with decreasing N. IR70 showed a higher percentage of RWC in the control and treated plants compared to TN1. The results show that a reduction in N availability significantly ($p< 0.05$) reduced the rate of RWC in both the TN1 and IR70 rice cultivars.

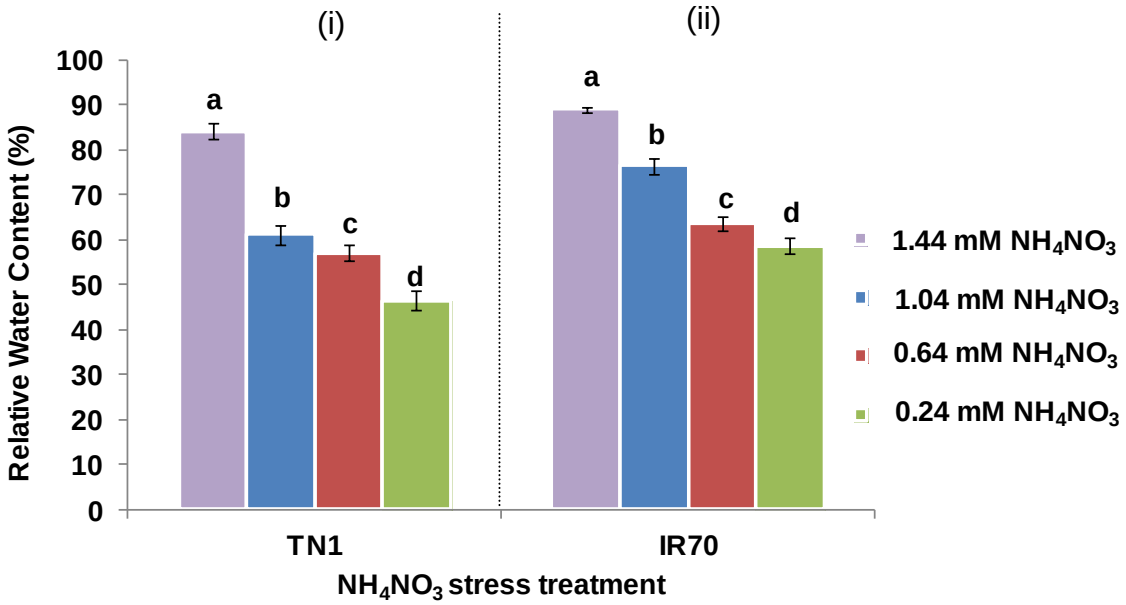


Figure 3.8 Mean value of RWC on (i) TN1 and (ii) IR70 rice cultivar grown under different N levels on the 5th week of growth. Data refers to mean ± SD (n=6) and bars not sharing the same letters are significantly different at $p < 0.05$ according to one-way ANOVA followed by Tukey Post-Hoc test within each variety.

3.4.8 *Effect of different N levels on root length in TN1 and IR70*

Reduced N also significantly reduced root growth in both cultivars (Figure 3.9). The longest mean root length was recorded in the control plants (1.44 mM NH_4NO_3) at 10.7 ± SD cm and 11.2± SD cm in TN1 and IR70, respectively. The shortest mean root length was 35.5% (6.9 ± SD cm) and 39.3% (6.8 ± SD cm) lower than their respective control plants for TN1 and IR70 respectively. This drastic reduction in the root length was observed in the rice cultivars at the lowest N levels (0.24 mM NH_4NO_3). A significant difference ($p<0.05$) in the root length was also observed between different N levels for both cultivars. This study showed that N deficiency affects the growth of roots in the TN1 and IR70 rice cultivars.

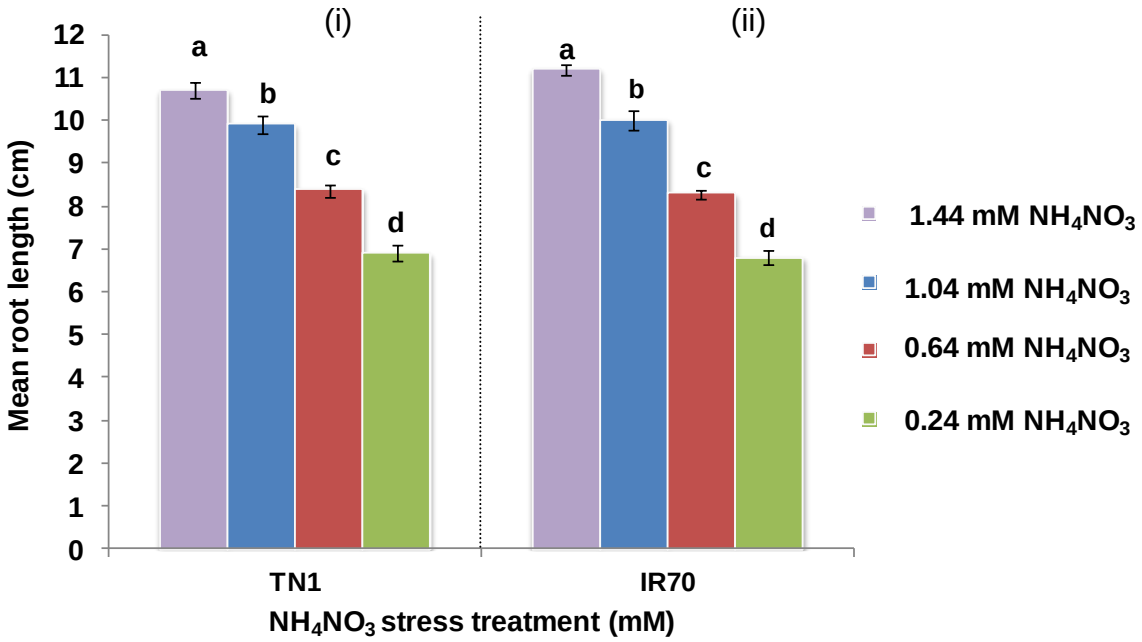


Figure 3.9 Mean of root length in (i) TN1 and (ii) IR70 rice cultivar grown under different N levels on the 5th week of growth. Data refers to mean ± SD (n=6) and bars not sharing the same letters are significantly different at $p< 0.05$ according to one-way ANOVA followed by Tukey Post-Hoc test within each variety.

3.5 Discussion

3.5.1 *Effects of N stress on growth*

Rice cultivars, TN1 and IR70 grown under different N levels demonstrated that low N affected all the physiological parameters measured for both rice cultivars. TN1 and IR70 grown under low N for three weeks showed no visible symptoms such as yellowing of leaves. This result is in contrast to the findings of Lian *et al.* (2006), who showed that there were stress symptoms such as yellowing leaves within 9 days of low N treatment in the Minghui 63 (*Oryza sativa spp.indica*) rice cultivar.

3.5.2 *Response of N stress on the shoot height*

Plant height is an important indicator to show the influence of various nutrients on plant metabolism (Malik *et al.*, 2014). In this study, shoot height was influenced positively by different N levels in a dose-dependent manner. This finding is supported by results obtained by Hach *et al*. (2006) and Singh *et al.* (2014), who demonstrated that increased levels of N significantly increased the plant height of all rice varieties tested. Haque and Haque (2016) also reported that application of higher doses of N

in the soil increased plant height in local varieties of rice in Iran. Many studies have revealed that N is an important macronutrient which is required for growth and development. However, whilst excessive usage of N has been shown to increase plant height, it has also been shown to reduce the grain yield of the rice plants (Tayefe *et al*., 2014).

3.5.3 *Effects of N stress on leaf number*

The morphological characteristics of leaves plays an important role in utilization of light energy (Zhu *et al.*, 2009). The growth of leaves depends on the application of N nutrition in the plants. “Remobilized-N” is crucial to enhance leaf growth under a wide range of N levels in plants (Imai *et al.*, 2005). The present study showed that the number of leaves produced under 1.04 mM NH_4NO_3 and 0.64 mM NH_4NO_3 remains the same in the TN1 rice cultivars. A similar trend was also observed in IR70 whereby there was no decrease in leaf production when grown in 1.04 mM NH_4NO_3 or 0.64 mM NH_4NO_3. This may be due to a short time length of the exposure of the rice cultivars to the N stress. However, application of low N nutrient solution in TN1 and IR70 resulted in a significant reduction in number of leaves compared the control plants. These findings were broadly similar with the results of Singh *et al.* (2014) who reported that the number of leaves increased with increased N application in different genotypes of rice and that the number of leaves increased gradually during the active tillering to the reproductive stage. Therefore the number of leaves in a rice cultivar depends on the number of tillers produced in the plant. Furthermore, application of N affects the gibberellin hormone indirectly through cytokinin and increases the growth of young leaves (Azarpour *et al*., 2014).

3.5.4 *Effect of N stress on number of tillers*

N is an important element required by plants during the vegetative stage to enhance the growth and tillering, which determines the potential number of panicles in rice plants (Tayefe *et al*., 2014). The number of tillers produced was significant in response to different N levels in the TN1 and IR70. Data from this study revealed that the application of low N significantly reduced the number of tillers in TN1 and IR70 compared to the respective control plants. Both the rice cultivars showed the same pattern of response to different levels of N whereby there were no changes observed in the number of tillers produced with 1.04 mM NH_4NO_3 and 0.64 mM NH_4NO_3

between TN1 and IR70. The results also showed that there is a direct relationship between the response of N and the production of tillers and leaves in the rice cultivars. This finding is consistent with previous studies which reported that application of high levels of N significantly increased the tiller production (Mannan *et al.*, 2012). Singh *et al.* (2017) also reported that the maximum numbers of tillers was observed at the highest level of N and the minimum was at the low level of N over 4 different genotypes of rice tested. Wang *et al*. (2007) demonstrated a synchronous relationship in the time of appearance and growth between a tiller and leaf. A similar synchrony was observed between leaf emergence rates on the main stem and tillers of the rice plants. The final number of leaves on a rice tiller at a given position is strongly dependent on the main stem leaf number (Jaffuel and Dauzat, 2005).

3.5.5 *Effect of N stress on leaf area*

Leaf area measurement is used as an important growth indicator (Azarpour *et al*., 2014). This study showed leaf area was directly affected by different N levels in the nutrient solutions, decreasing significantly with decreasing doses of N compared to the control plants. These results are supported by the finding of Azarpour *et al*. (2014), Salem *et al.* (2011) and Singh *et al.* (2014) who demonstrated that as the N levels increased the leaf area also increased gradually. The leaf area is higher at the higher N level due to increased translocation of N to the leaves.

Leaf area is observed to increase slightly over time during the early growth stage and increases rapidly during the later stages. The maximum value of leaf area was observed during the flowering stage of the rice cultivars (Azarpour *et al*., 2014). A similar explanation was described by Kumar *et al.* (2015) who reported that the leaf area index increased consistently with increasing N levels in rice genotypes tested. Shieh and Liao (1985) also reported that low N inhibits the leaf area of rice plants supporting the findings of this study. N plays an important role in cell division of a plant. Disruption in cell division decreases the leaf area and loses its potential to produce an adequate yield (Kumar *et al.*, 2015). Leaf area measurement is closely correlated with the biological yield of a crop plant (Azarpour *et al*., 2014). Therefore, higher leaf area is an important physiological trait for achieving higher yield (Kumar *et al.*, 2015).

3.5.6 *Effect of N stress on chlorophyll content*

A SPAD chlorophyll meter is a tool used to evaluate the concentration of N in the leaves of plants (Yang *et al.*, 2014). Chlorophyll is one of the most important compounds in plants and is responsible for the conversion of energy from sunlight into chemical energy through photosynthesis. Chlorophyll content is also an indicator of leaf senescence, which enhances the photosynthesis in the plants. There is a high correlation between photosynthetic capacity and N content in leaves of higher plants. The amount of N applied to the chloroplasts accounts for 70-80% of leaf N content (Imai *et al.*, 2005). Therefore increased application of N increases photosynthesis processes. Approximately 50% of leaf N is utilized as photosynthetic proteins in leaves (Xiong *et al.*, 2015). N is also an element that influences the chlorophyll content in the leaf of a crop plant. Therefore, N availability affects the chlorophyll content in the leaf of the crop plants, including rice. Crops with a higher chlorophyll content have a greater leaf photosynthesis capacity under adequate N levels which is an important factor in increasing crop production (Urairi *et al.*, 2016).

As expected, this study showed that the lower SPAD values occurred in TN1 and IR70 plants grown under low N and the highest SPAD values were produced by control plants that received the optimum N level. A similar observation was also reported by Turner and Jund (1994) and Pramanik and Bera (2013) that application of high nitrogen fertilizer resulted in higher value of total chlorophyll content. The plants that received a sufficient amount of N will have dark, blue-green leaves which will enhance the role of photosynthesis (Zahoor *et al.*, 2014). Chen *et al.* (2003) also reported that there is a positive correlation between N content of leaves and photosynthetic capacity. A lower content of all photosynthetic components is usually observed in N deficient leaves. Therefore, N availability affects the chlorophyll content in the leaf of the rice crop which is consistent with the findings of this study.

3.5.7 *Effect of N stress on RWC*

In crop plants, the regulatory function of N to water stress tolerance depends on the intensity of the stress and N level of the plants. Therefore, plants with low uptake of N will be exposed to water stress. Studies have reported that water also affects the photosynthesis process due to the decline in N metabolism of the plants (Zhong *et al.*, 2017). RWC is an estimation of plant water status in terms of cellular hydration

under the possible effect of both leaf water potential and osmotic adjustment (OA) (Lu *et al.*, 2004). This study revealed that the leaf RWC was lower in both rice cultivars grown under low N, decreasing in a dose-dependent manner. These results agree with other studies that found the leaf RWC of rice grown at different stages were significantly correlated with N content (Lu *et al.*, 2004). Zhong *et al.* (2017) also reported that there were significant differences in leaf RWC at different levels of N, with the highest leaf RWC observed in rice plants exposed to the highest N level. Therefore, N deficiency will affect the RWC in rice, which in turn will reduce the yield of rice.

3.5.8 *Effect of N stress to root length*

Roots are one of the vital parts of the plant absorbing water, nutrients and oxygen from the soil (Xu *et al.*, 2013). Root characteristics such as root surface area and diameter of the root play an important role in determining the uptake of N. The root surface area depends on the total length of roots in the plants. Therefore, previous studies have shown that there is a close correlation between morphological attributes and N uptake in plants (Mostafa and Mazinani, 2013).

In this study, the root length of TN1 and IR70 showed significant differences in length under different N conditions. The control plants (under optimal N levels) resulted in the longest root length and the total length of root decreased with the increased N stress in a dose-dependent manner. N deficiency at low N levels recorded the shortest root length in both the rice cultivars. This finding is supported by results obtained by Abou-khalifa (2012) who showed that the root lengths increased in rice plants with higher N levels. Fageria and Moreira (2011) also reported that more vigorous root systems were produced at higher N rates in crop plants. In addition, Mostafa and Mazinani (2013) and Fan *et al.* (2010) showed that there was a positive correlation between N uptake and total root length in rice cultivars thus supporting the results from the present study.

Under limited water availability, roots induced a signal cascade to the shoots via xylem causing physiological changes which determine the level of adaptation to the stress in the plant (Anjum *et al*., 2011). Root development in rice has been reported to be involved in response to many plant stress particularly drought and mineral deficiency (Allah *et al.*, 2010). This study showed that the leaf relative water content

is an important indicator of water status and the decrease in RWC in response to N stress has also inhibited the growth of roots in the TN1 and IR70 rice cultivars.

Based on the results of this study and the findings of previous studies, it can be inferred that the root length is an important morphological characteristic for efficient N uptake in rice cultivars. Application of N promoted root growth which aids in the extraction of a larger proportion of soil N and enhanced crop growth resulting in higher grain yield (Mannan *et al.*, 2012). The optimum N uptake in rice is essential to increase the yield which contributes to the rice productivity.

3.6 Conclusions

This study investigated the physiological response of two rice cultivars TN1 (susceptible to BPH) and IR70 (resistant to BPH) to the reduced N levels. Overall the findings revealed that both the rice cultivars showed the same trend of response to N stress. The physiological parameters such as shoot height, number of tillers and leaves, leaf area, root length, chlorophyll content and relative water content were measured and all the changes were directly correlated with nitrogen input compared to the respective controls. All physiological parameters demonstrated significant decreases in both the rice cultivars with increasing N stress. These parameters, as compared to the control plants, decreased in response to N stress in a dose-dependent manner. This finding showed that N is a key element which is closely related to the growth of the both TN1 and IR70 rice cultivars. All the physiological traits tested in this study had a close relationship to each other, which contributes towards the increase of the rice yield. For example, the leaf area and chlorophyll content determine the photosynthetic efficiency and enhance yield in rice crop.

Developing rice cultivars that are highly tolerant to N stress is one of the most fundamental approaches to overcome N deficiency, which is a major constraint to rice production. Identification of crop tolerance to abiotic stress such as N stress is an important way to build improved breeding strategies. Selection of more N efficient rice genotypes will increase crop production and, most importantly, minimize the environmental pollutions caused by excessive usage of N fertilizers. Therefore, the findings from this study supported the hypothesis of the study and will contribute towards the selection of traits with high tolerance to N stress in the rice cultivars.

Chapter 4. Identification of transcription factors involved in the response of rice cultivars TN1 and IR70 to nitrogen deficiency stress

Abstract

Nitrogen (N) is a major abiotic stress that causes substantial losses in rice productivity across the world. Being an important staple crop, rice yield is drastically affected by N stress. The present study analyzed the differential expression of 12 transcription factors (TFs) related to brown planthopper (BPH) resistance in response to different levels of N stress (1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3) in two rice cultivars the TN1(susceptible to BPH) and IR70 (resistant to BPH). Gene expression profiling revealed that the TF genes were more responsive to N stress in IR70 compared to TN1. All TFs exhibited the highest level of expression under the lowest N level (0.24 mM NH_4NO_3) in both the TN1 and IR70 rice cultivars. Among the TFs investigated, all 12 TFs were up-regulated in IR70 whilst 10 TFs were up-regulated in the TN1 in response to the reduced N levels compared to the optimal N levels (1.44 mM NH_4NO_3). *Os03g0860100* and *Os01g02145500* were down-regulated in the TN1 in response to N stress which indicated that both the genes might not be responsive to N stress in this rice cultivar. This study identified *Os01g0971800*, *Os01g0108400*, *Os09g0240200*, *Os03g0180800* and *Os07g0410700* which exhibited the highest level of expression in IR70. Our findings highlighted that the TFs which have been linked to resistance to BPH were potentially responsive to N stress in the IR70. The MYB-related TFs, *Os08g0157600* and *Os06g0728700* showed a similar level of expression in response to N stress in the TN1 and IR70 demonstrating a common role in response to N stress. The differential expression of these genes in the TN1 and IR70 indicated its further importance in developing rice cultivars with improved N stress tolerance.

Keywords: rice, nitrogen, transcription factors, up-regulation, down-regulation

4.1 Introduction

4.1.1 *Molecular response of rice to Nitrogen deficiency stress by transcriptional profiling*

Several studies have shown that the application of “Omics” technologies such as transcriptomics and proteomics in plant biotechnology have the potential to discover the complexity of regulatory networks at the transcriptional and post-transcriptional mechanisms of plant responses to N deficiency stress (Ding *et al.*, 2011). As described in Chapter 3 of this study, N plays an important role in the growth and development of the plant. N is involved in several biological processes such as carbon metabolism, amino acid metabolism and protein synthesis (Cai *et al.*, 2012). Previous studies including the findings from this study showed that N deficiency stress has caused drastic changes in growth and physiology of rice genotypes.

It has also been proven that deficiency of one mineral in the plants will affect the uptake of other nutrients within the plant (Cai *et al.*, 2012). When plants are under N deficiency stress, several genes related to nutrient deficiency will be triggered to support the survival of the plants by increasing the chlorophyll synthesis capacity and lignin content, altering the root architecture, enhancing N-assimilation and adjusting the amount of sugar and sugar phosphates (Yang *et al.*, 2015). Genes that are directly involved in N transport, reduction and assimilation such as the nitrate/ammonium transporter (NRT/AMT), nitrate/nitrite reductase (NR/NiR), glutamine synthase (GS), glutamate synthase (GOGAT) and asparagine synthetase (AS) and genes which are indirectly involved such as the gene encoding uroporphyrin III methyltrans-ferase (UPM), ferrodoxin (Fd), ferredoxin-NADP oxidase-ductase (FNR) and glucose-6-phosphate dehydrogenase (G6PDH) has been found to respond to low N condition (Cai *et al*., 2013).

Previous research has shown the molecular basis of plant responses to N deficiency stress and identification of N-responsive genes in order to manipulate the gene expression which will be helpful in using N more efficiently (Bi *et al.*, 2007). N deficiency has been proven to be regulated at the transcriptional level by several TF genes. Most of TFs induced under N deficiency were from the WRKY, ERF and MYB families (Bi *et al.*, 2007). Lian *et al.* (2006) also reported that TFs from the MYB, bHLH, and zinc finger family were involved in low N stress in rice. All these findings

have provided important information on N deficiency stress responses and its relationship to other biological pathways in plants.

A study conducted on *O. sativa* L. ssp. *japonica* cv. Dongjin rice variety identified 1650 genes that were differentially expressed after 12 hours of N-starvation. Data obtained from this research is an important platform to identify N-deficiency-induced genes and the signal transduction pathway of N-utilization (Yang *et al.*, 2015). Another study conducted on 'Minghui 63' rice identified 471 ESTs (Expressed Sequence Taq) responsive under low N-stress in the root tissues of the rice cultivar (Lian *et al.*, 2006).

Cai *et al.* (2012) also identified some genes which play a vital role in N deficiency in rice. Some of these genes were induced or suppressed under N limitation and these changes affected various cellular metabolic pathways including the stress response, primary and secondary metabolic, molecular transport, the regulatory process and organismal development. The findings from this report gave a better understanding of potential targets for Nitrogen-utilization efficiency (NUE) improvement of the rice crop.

Other findings by Kant *et al*. (2010) reported that N-responsive genes were identified using the genome transcriptional profiling technology and was used in NUE by a transgenic approach. The transgenic rice, early nodulin gene (*OsEOD93-1*) was overexpressed under different N stress conditions. This plant showed a significant increase of 10-20% in a number of spikes and spikelet and seed yield under both limiting-N and optimum-N conditions. This plant also had higher shoot dry biomass compared to the wild-type plants under limiting N-conditions. This suggests that identification of genes though a transcriptional response to different N levels is an effective approach for identification of genes that may contribute to the improved genetics of rice crop.

Previous research also revealed that Tolerance of Nitrogen Deficiency 1 (TOND1) conferred tolerance to N deficiency in the Indica rice cultivar, Teqing. These authors identified TOND1, a major QTL which controls tolerance of N deficiency stress. Overexpression of this gene showed a remarkable increase in the N-deficiency tolerance and grain yield of rice plants grown under N-deficient condition. Identifying this gene may significantly decrease the use of N fertilizers, reduce the cost of rice

production and protect the environment (Zhang *et al.*, 2015). Similar findings were also reported by Kurai *et al.* (2011) whereby the author identified that expression of the *ZmDof1* gene (Dot1 rice) enhanced the growth of rice under the N-deficient condition with an increase in the net photosynthesis rate and a decrease in the shoot-to-root dry weight ratio (S/R ratio).

The comprehensive transcriptomic findings above provide a valuable resource to better understand rice in response to N deficiency stress and subsequent improvement of NUE. Improper usage of this nutrient has caused severe implications to the environment which has been described in detail in Chapter 1 of the present study. Previous studies have revealed that rice field with high nitrogenous fertilization became the favourable habitat for many insect herbivores including brown planthopper (BPH) which caused significant losses in rice production worldwide (Lu and Heong, 2009). Therefore, identification of TF genes associated with tolerance to N deficiency and BPH infestation will be a useful breeding strategy to overcome these losses.

This study aims to investigate the correlation between N input and BPH focussing on specific TFs which play important roles in the induced defence response in rice cultivars, TN1 (susceptible to BPH) and IR70 (resistant to BPH). This study used qPCR analysis to identify the candidate gene to improve the understanding of N deficiency stress response in rice cultivars which were involved in BPH resistance. The findings of this study will provide information to understand the molecular mechanism of both TN1 and IR70 rice cultivars in response to N deficiency stress. The entire study on TFs in Chapter 4 and Chapter 5 of this study focused on 12 TF genes which have been potentially involved in BPH resistance in the rice varieties (Wang *et al.*, 2012).

Wang *et al.* (2012) reported that several microarray analyses were carried out on the Sri Lankan rice cultivar, Rathu Heenati (RHT) to identify the potential TF genes involved in BPH resistance. The gene expression profiles were compared to the susceptible rice cultivar Taichung Native 1 (TN1) which was used as a negative control. Hence, through this screening, many TFs from different families were identified that are correlated with resistance to BPH. TFs from families such as AP2/EREBP, MYB, NAC domain-containing and bZIP families were identified to play important role in plant defence response pathways in this research (Table 4.1). The

differential gene expression profiles between the susceptible TN1 and resistant RHT rice cultivars provided significant information on the expression level upon BPH infestation. Hence these authors have identified the induced and constitutive TFs genes related to BPH resistance.

Table 4.1 TF genes analyzed in this study (Wang *et al*., 2012).

TF Gene	Family	Description
Os03g0860100	AP2-EREBP	AP2 domain-containing protein, expressed
Os07g0410700		
Os08g0157600	MYB-related	MYB family transcription factor, putative, expressed
Os06g0728700		
Os01g0971800	G2-like	MYB family transcription factor, putative, expressed
Os09g0439200	Tify	ZIM domain-containing protein, putative, expressed
Os03g0180800		
Os06g0298200	C2C2-CO-like	CCT/B-box zinc finger protein, putative, expressed
Os09g0240200		
Os02g0214500	NAC	no apical meristem protein, putative, expressed
Os01g0108400	bHLH	helix-loop-helix DNA-binding domain containing protein, expressed
Os03g0437200	C2H2	C2H2 zinc finger protein, expressed

4.2 Objectives of this study

Hypothesis

The transcription factors (TFs) (Table 4.1) show greater levels of expression in the resistant IR70 rice cultivar compared to the susceptible TN1 rice cultivar in response to reduced levels of N.

The specific objective of this study is to:

(i) determine the differential expression levels of the 12 TF genes in the susceptible TN1 and resistant IR70 rice cultivars in response to the reduced N levels (1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3)

(ii) identify TF genes which are responsive to N deficiency stress in both the susceptible TN1 and resistant IR70 rice cultivars

4.3 Material and methods

4.3.1 *Plant materials and growth condition*

TN1 and IR70 rice cultivars were germinated and grown as described in 3.3.1 and 3.3.3 of Chapter 3. After five weeks of growth under optimal N level (1.44 mM NH_4NO_3) and reduced levels of N (1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3), the leaves of both susceptible TN1 and resistant IR70 rice cultivars were harvested. The harvested samples were immediately frozen in liquid nitrogen and stored at -80°C until used for further analysis.

4.3.2 *RNA extraction*

RNA was extracted as described in 2.3.5 of Chapter 2.

4.3.3 *cDNA synthesis*

The first-strand cDNA synthesis was performed using the iScript™ Reverse Transcription Supermix for RT-qPCR (BIO-RAD). A total of 1 µg RNA for each sample was used as a template in a reaction volume of 20.0 µl. The polymerase chain reaction (PCR) conditions using the GeneAmp PCR System 9700 thermal cycler (ABI) were as follows: priming 5 min at 25°C, reverse transcription 20 min at 46°C and RT inactivation 1 min at 95°C. The cDNA samples were stored at -20°C prior to qPCR analysis.

Table 4.2 Mastermix preparation for cDNA synthesis.

Reagent	Volume per reaction (µl)
iScript RT supermix	4
RNA template (1µg)	Variable
Nuclease-free water	Variable
Total volume	20

4.3.4 *Primers*

Table 4.3 List of TF gene-specific primers used for qPCR analysis (Wang *et al*., 2012).

Gene Names	Forward Primer (5'-3')	Reverse Primer (5'-3')	Size (bp)
Os03g0860100	CCCAAGCCACCACTCCATCTGATCT	ACCGGATCCACATGTTAACCACTGC	108
Os07g0410700	GTGCAGACAATGCAACACATGCTGG	AAGTTCAGTCCCCGCCCAAAGTACA	116
Os08g0157600	CTTCCCCAAAGCTTTTCTCCTCCGC	CGGCATTCTTGTTGAGGTCAACCGT	106
Os06g0728700	AAGCCAAGCAACAGCGGAGATGAAG	ACCACCAATCCCCGCAAACAAACAA	185
Os01g0108400	GTCAAGTTCCACGACGTCATCACCG	CGGATCACCAATGCCCGTAGTCTCT	176
Os02g0214500	CGTCTTCCCACTGATGATCTCACGGA	ACCCATCACCATTCAACCCCACTGA	130
Os06g2098200	TGTTGCTTTCGGGAGGAGCGATTTG	ACCACCAAAAACACCACAGCAAGGG	145
Os09g0240200	ATAATGTCGCCACCGCAGTTCATGG	TACCTGATGGTCTTCTCGAACCGCC	104
Os09g0439200	CGTCCAAGCAAGCTAACGGTGACAA	AACCACGCATCTCTTCCCCACAGAT	140
Os03g0180800	TCGTCGCATTAACGGCCTTGAGTTG	GACCGTGCTTAATTATACGCCGCGA	124
Os03g0437200	CCGTCAGGAACTTCGACCTCAACCT	ATCCTGAGCTTCTTGACCGGCAGTG	121
Os01g0971800	CAGGTTCCTTCTCCTTACCACCCCC	AAGGAATGGATCAGTGGTTGGCGTG	118
Actin	CCATCGAGCATGGTATCGTCAGCAAC	TGTGGTACGACCACTGGCATACAGAG	239

4.3.5 *Quantitative PCR Analysis (qPCR)*

qPCR analysis was conducted as previously described in 2.3.9 of Chapter 2.

4.3.6 *Primer Optimization Using Temperature Gradient*

Gradient PCR determined the optimal annealing temperature of all the primers analyzed. The optimization was carried out to calculate the optimal annealing temperature of primers which works best at the range between 60°C to 63ºC as recommended in the 2X SensiFAST ™ SYBR ® No-ROX Kit Master Mix (Bioline) reagent which is used for qPCR analysis in this study. PCR reaction using PCR Master Mix (2x) (Thermo Scientific) was carried out following PCR condition as such: initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 30s, annealing at 57°C to 63°C for 30s, extension at 72°C for 30s and final extension 72°C for 15 min.

Table 4.4 PCR Mastermix reaction for primer optimization.

PCR components	1x Reaction (µl)
PCR Master Mix	25.0
Forward primer (10µM)	1.0
Reverse primer (10µM)	1.0
cDNA template (1µg)	2.0
Nuclease-Free Water	21.0
Total volume	50.0

4.3.7 *qPCR efficiency*

cDNA samples were serially diluted 2-fold using RNase Free Water (RFW) to give a total of 7 concentrations. qPCR was carried out as described in 2.3.9 of Chapter 2. Serially diluted cDNA started from a concentration of 1µg/µl, with the lowest concentration used 15.6 ng/µL. In order to determine the efficiency of the qPCR, a standard curve was generated for each gene and the regression correlation coefficient (R^2) and PCR efficiency (E) was calculated.

The amplification efficiency, E is calculated from the slope of the standard curve using the following formula:

Efficiency = $2^{(1/-slope)}$

Amplification efficiency is presented as a percentage of the template that was amplified in each cycle.

% Efficiency = $(2^{(1/-slope)} -1) \times 100$

Studies have suggested that qPCR efficiency should be between 90 – 100% (BIO-RAD, 2006).

4.3.8 *Validation of reference gene*

Validation of the reference gene, Actin was conducted using the qPCR analysis. The C_T value generated from the *Actin* gene was used to determine the stability of gene expression across different N deficiency stressed samples and the control samples in both the TN1 and IR70 rice cultivars. The significance of the C_T value between the entire N deficiency stressed samples and the control samples were analyzed by one-way ANOVA and further evaluation using Tukey Post-Hoc test ($p<0.05$).

4.3.9 *Agarose gel electrophoresis*

The PCR amplified DNA fragment was analyzed by using gel electrophoresis. A 2% agarose gel was (1 g of agarose in 50 ml of 1 x Tris-acetate EDTA, TAE buffer) dissolved by heating in the microwave. The molten agarose was then cooled in running water before adding 1µl of ethidium bromide and swirled to mix well. The agarose was poured into a gel tray with a comb and allowed to set for approximately 20 minutes in the fume cupboard (Lewis, 2001). Agarose gel was placed in the electrophoresis tank filled with 1X TAE buffer. A volume of 12 µl of sample was mixed with 2.5 µl of loading dye (6x Orange DNA Loading Dye, Thermo Scientific). Samples were then loaded into the wells and 5 µl of 100 bp molecular DNA marker (Hyperladder 100bp, Bioline) was filled for each set of samples. The electrophoresis power pack was set at a constant voltage of 100V with 400 mA for 60 minutes. Samples were viewed under the gel documentation system (Gel Doc ™ EZ Imager, Biorad) and images of the gels were captured.

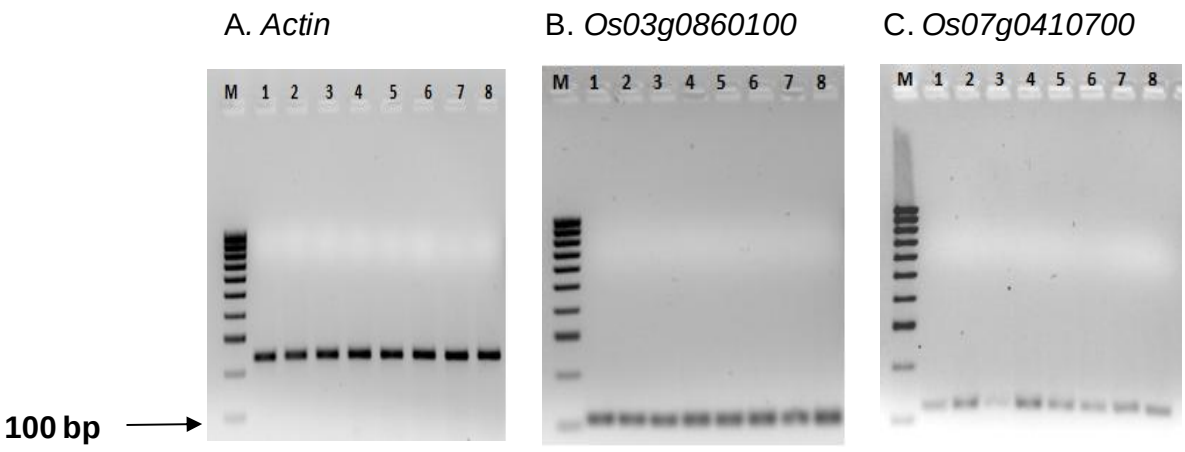
4.3.10 *Data analysis*

qPCR data analysis was carried out as in described in 2.3.10 of Chapter 2. In order to determine the changes in gene expression between the controls and N deficiency stressed samples, the optimal N level (1.44 mM NH_4NO_3) was treated as a baseline control (1.0). The relative transcription levels were presented graphically on the $\log_2$ scale (Caldana *et al.*, 2007). The standard deviation was calculated from the $\Delta\Delta C_T$ value from each gene across all of the N deficiency stressed samples and presented as error bars in the gene expression graphs. A one-way ANOVA was applied to determine differences in gene expression between the control and N deficiency stressed conditions which are indicated by a single asterisk ($p<0.05$, Tukey-Post Hoc test).

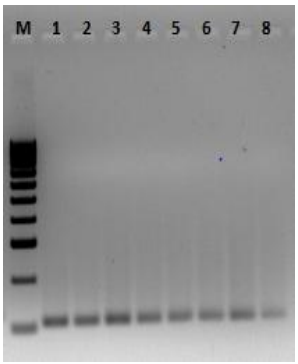
4.4 Results

4.4.1 *PCR Optimization for primers*

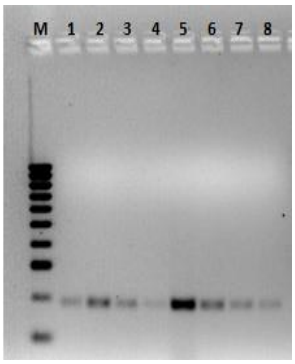
All primers were initially tested to determine the optimal annealing temperature of the primer pairs and to demonstrate that each reaction produced a single amplified product. The gradient PCR showed that 61°C is the optimal annealing temperature for endogenous primer, Actin and all primers tested in this analysis. This optimal annealing temperature was used throughout entire qPCR analysis in this study. This annealing temperature was compatible with the range of temperature recommended by the manufacturer of SensiFAST ™ SYBR ® No-ROX Kit Master Mix (Bioline).



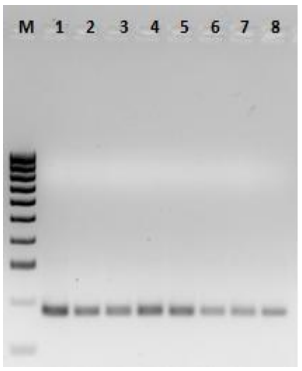
D. *Os08g0157600*



E. *Os06g0728700*

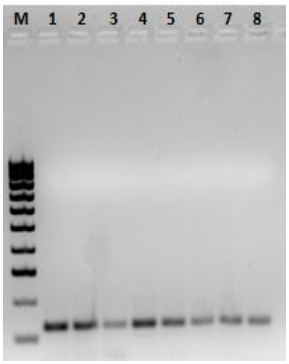


F. *Os01g0108400*

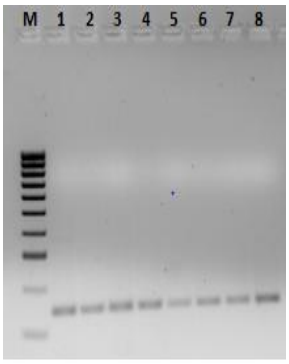


100 bp →

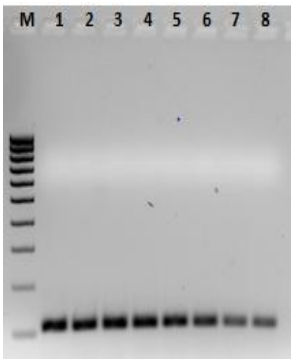
G. *Os02g0214500*



H. *Os06g2098200*

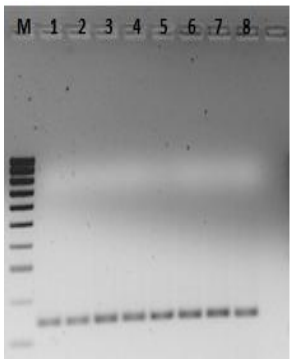


I. *Os09g0240200*

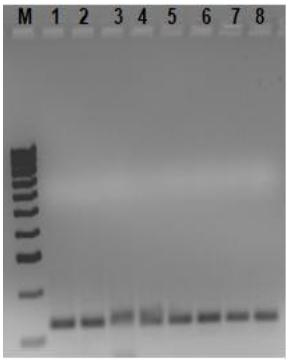


100 bp →

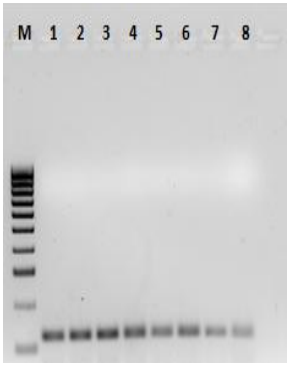
J. *Os09g0439200*



K. *Os03g0180800*



L. *Os03g0437200*



100 bp →

M. *Os01g0971800*

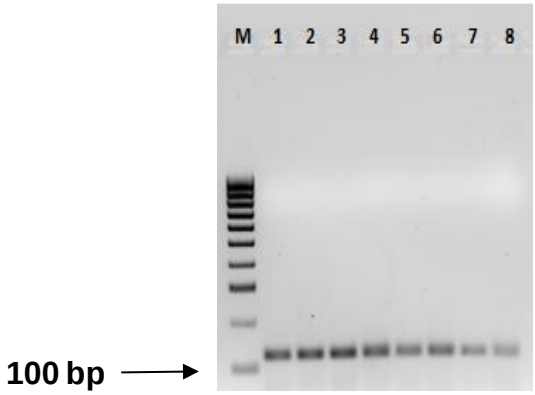


Figure 4.1 Gradient PCR performed on the endogenous gene (A) *Actin* and all TFs tested (B) to (M). Lane M: 100 bp DNA Marker (which was used in all the gels, A-M), Lane 1: 57.0°C, Lane 2: 57.4°C, Lane 3: 58.2°C, Lane 4: 59.3°C, Lane 5: 60.7°C, Lane 6: 61.8°C, Lane 7: 62.6°C and Lane 8: 63.0°C.

4.4.2 *qPCR efficiency test*

In order to determine whether the qPCR assay is optimized, a serial dilution was run and the results obtained were used to generate a standard curve. The standard curve is constituted by plotting the $\log_2$ of the relative concentration of templates against the C_T value obtained during amplification of each dilution. Only C_T values <40 were used for calculation of the qPCR efficiency. All the genes displayed PCR efficiency (E) values between 93.3% and 100% and showed R^2 values greater than 0.97. For each primer pair, specific amplification was confirmed by a single peak in the melt-curve analysis. The qPCR efficiency graphs are as attached in Appendix B.

Table 4.5 qPCR Efficiency of TFs analyzed in this study.

Gene	Efficiency (%)	R^2
Os03g0860100	97.9	0.978
Os07g0410700	96.1	0.988
Os08g0157600	98.9	0.988
Os06g0728700	100.0	0.986
Os01g0108400	99.0	0.988
Os02g0214500	93.3	0.994
Os06g0298200	97.8	0.995
Os09g0240200	96.4	0.990
Os09g0439200	95.9	0.991
Os03g0180800	95.8	0.970
Os03g0437200	93.9	0.981
Os01g0971800	98.0	0.982
Actin	99.5	0.997

4.4.3 *Validation of reference gene*

Actin which is used as the endogenous gene in this study showed no significant differences in the C_T values between all the four N levels tested in the TN1 (ANOVA, p=0.459) and IR70 (ANOVA, p=0.665) rice cultivars. The variation in expression of Actin between different N conditions was very low. Therefore, *Actin* demonstrated to be a suitable endogenous gene for normalizing gene expression in this study.

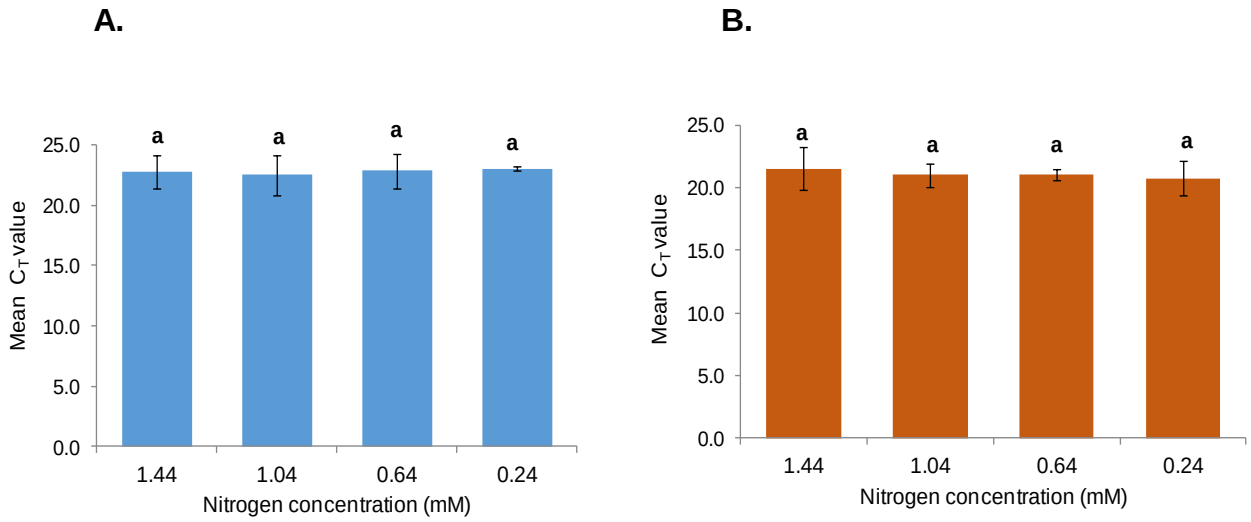


Figure 4.2 Expression of *Actin* across different N concentrations in (A) TN1 and (B) IR70 rice cultivars. Data refers to mean ±SD (n=3) and p<0.05 according to one-way ANOVA followed by Tukey Post-Hoc-test for each rice variety. Bars with the same letter are not significantly different.

4.4.4 *Expression profiles of TFs in response to N stress*

A qPCR analysis was conducted on the susceptible TN1 and resistant IR70 to investigate the differential expression pattern of all 12 TF genes related to BPH resistance namely *Os03g0860100*, *Os07g0410700*, *Os08g0157600*, *Os06g0728700*, *Os01g0108400*, *Os02g0214500*, *Os06g0298200*, *Os09g0240200*, *Os09g0439200*, *Os03g0180800*, *Os03g0437200* and *Os01g0971800* in response to the reduced levels of N. These TF genes showed differential expression under N stressed levels (1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3) compared to the optimal N level (1.44 mM NH_4NO_3) which was set to 1.0 of the relative gene expression graphs.

The *Os03g0860100* and *Os07g0410700* from the AP2-EREBP family showed differences in the expression levels between the susceptible TN1 and resistant IR70 in response to the reduced levels of N (Figure 4.3 A and B). The *Os03g0860100* was

down-regulated (~ 2 fold) in TN1 under 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3 compared to the optimal N level (1.44 mM NH_4NO_3). This TF only showed significant differences ($p< 0.05$) under the lowest N level (0.24 mM NH_4NO_3) compared to the optimal N level. The expression level of this gene under the 1.04 mM NH_4NO_3 remained unchanged as the optimal N level (~ 1 fold) in this rice cultivar. Therefore N deficiency stress resulted in down-regulation of *Os03g0860100* gene in the susceptible TN1. In contrast, IR70 showed up-regulation of *Os03g0860100* in response to reduced levels of N. The expression of *Os03g0860100* under 1.04 mM NH_4NO_3 remained the same (~ 1 fold up-regulation) as the optimal N level. However, the expression of this gene was significantly ($p<0.05$) up-regulated to a 3.8 fold and 5.1 fold respectively under 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3 respectively in IR70. The expression profile of *Os03g0860100* showed that this gene is more responsive to N deficiency stress in the resistant IR70 compared to the susceptible TN1 rice cultivar.

The *Os07g0410700* showed up-regulation in response to N deficiency stress in both TN1 and IR70 rice cultivars. The resistant IR70 showed a higher level of expression across all the N deficiency stress levels compared to the susceptible TN1. As compared to the optimal N level, this gene was up-regulated to a 2.7 fold and 7.6 fold under 1.04 mM NH_4NO_3 and 0.64 mM NH_4NO_3 respectively in IR70. On the other hand, the expression of this gene in TN1 remained unchanged as the optimal N level under 1.04 mM NH_4NO_3 (1.2 fold up-regulation) and increased to a 2.3 fold up-regulation under 0.64 mM NH_4NO_3 respectively. The *Os07g0410700* recorded the highest level of expression under the lowest N level (31.1 fold up-regulation) in IR70 rice which was 11x higher compared to the expression in TN1 (2.8 fold up-regulation). Interestingly, TN1 and IR70 showed significant up-regulation ($p<0.05$) under 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3 compared to the respective optimal N levels. The differences in the expression levels of *Os07g0410700* in both the rice cultivars revealed that this gene is more responsive to N deficiency stress in the resistant IR70 compared to the susceptible TN1 rice cultivar.

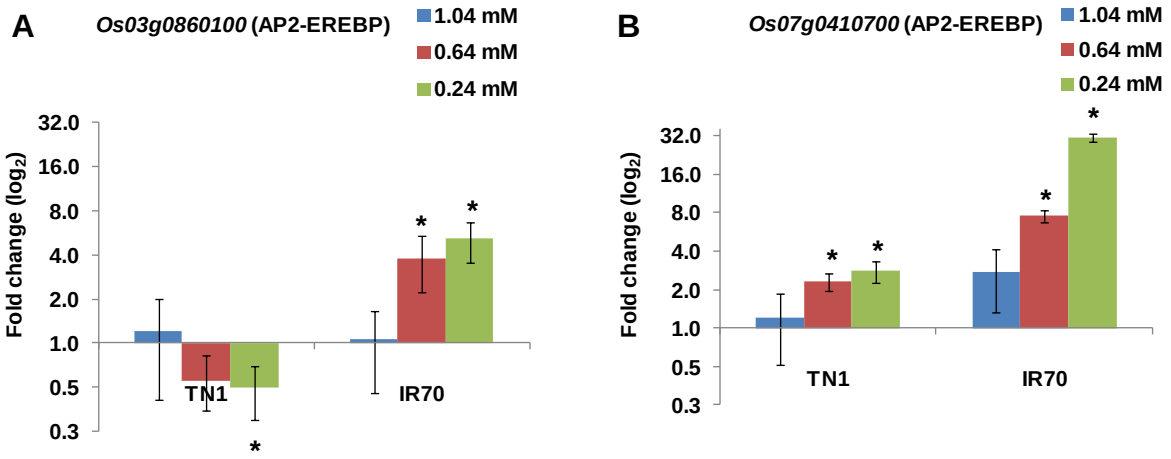


Figure 4.3 qPCR analysis of (A) *Os03g0860100* and (B) *Os07g0410700* in response to reduced levels of N (1.04 mM HN_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3) in TN1 and IR70. Fold change (log_2 scale) indicates the expression of each gene as compared to the respective optimal N levels (1.44mM NH_4NO_3) which were set at 1.0. Significant differences between optimal N and N stress samples were shown by a single asterisk *, $p<0.05$ according to one way ANOVA followed by Tukey Post-Hoc test, n=3. The error bars of each data set represent the mean ± SD of three biological replicates.

Os08g0157600 from the MYB-related family showed a similar pattern of gene expression in the susceptible TN1 and resistant IR70 under different N levels (Figure 4.4 A). Both the rice cultivars demonstrated a significant ($p<0.05$) increase in fold change under 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3 compared to the respective optimal N levels (1.44 mM NH_4NO_3). The expression of this TF in TN1 was recorded to a 2.2 fold up-regulation under 1.04 mM NH_4NO_3 and increased to a 2.7 fold up-regulation followed by 5.1 fold up-regulation under 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3 respectively. A similar level of gene expression (~ 3 fold up-regulation) was observed in the resistant IR70 under the 1.04 mM NH_4NO_3 and 0.64 mM NH_4NO_3. The highest level of gene expression in this rice cultivar was recorded at a 6.0 fold up-regulation under the lowest N level (0.24 mM NH_4NO_3). The expression profile indicates that this gene may be involved in response to N deficiency stress in both the susceptible TN1 and resistant IR70.

The *Os06g0728700* also from the MYB-related family was significantly ($p<0.05$) up-regulated in the susceptible TN1 and resistant IR70 across all N deficiency stress levels compared to the respective optimal N level (Figure 4.4 B) and interestingly both rice cultivars exhibited a similar level of gene expression. The expression profiles of TN1 and IR70 showed that this gene was up-regulated to a 3.2 fold and 3.5 fold respectively under the 1.04 mM NH_4NO_3. The *Os06g0728700* also

demonstrated an up-regulation of a ~ 4 fold under the 0.64 mM NH_4NO_3 in TN1 and IR70. The highest level of expression was recorded at a 5.5 fold up-regulation and a 6.2 fold up-regulation under 0.24 mM NH_4NO_3 in TN1 and IR70 respectively. The expression of this TF gene showed a similar pattern of expression as *Os08g0157600* in response to N deficiency stress in both the susceptible TN1 and resistant IR70 rice cultivars.

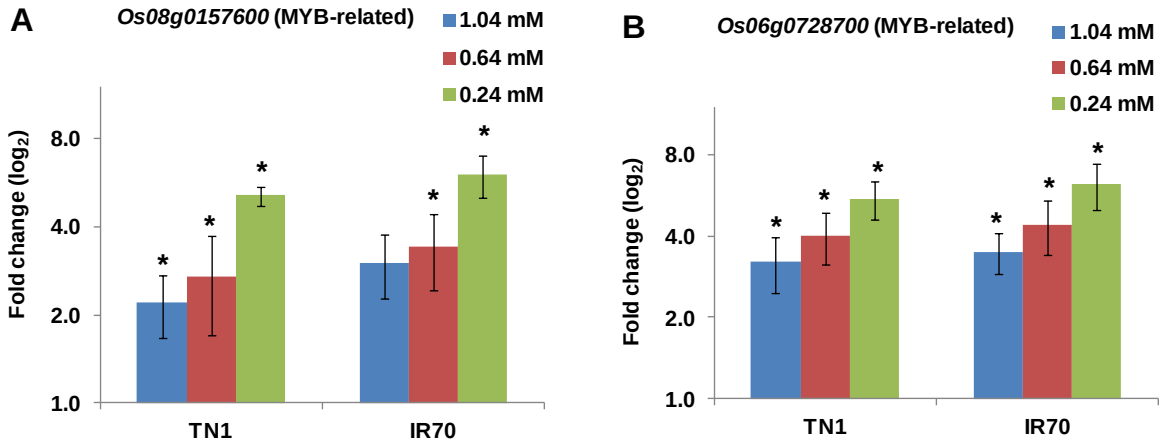


Figure 4.4 qPCR analysis of (A) *Os08g0157600* and (B) *Os06g0728700* (B) in response to reduced levels of N (1.04 mM HN_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3 in TN1 and IR70. Fold change (log_2 scale) indicates the expression of each gene as compared to the respective optimal N levels (1.44mM NH_4NO_3) which were set at 1.0. Significant differences between optimal N and N stress samples were shown by a single asterisk *, $p<0.05$ according to one way ANOVA followed by Tukey Post-Hoc test, n=3. The error bars of each data set represent the mean ± SD of three biological replicates.

Os01g0108400 gene which belongs to the bHLH family (Figure 4.5 A) was significantly ($p<0.05$) down-regulated across all N stress levels with a ~ 4 fold down-regulation under 1.04 mM HN_4NO_3 and a ~ 6 fold under the 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3 respectively in the susceptible TN1 compared to the optimal N level (1.44 mM NH_4NO_3). In contrast, this gene was significantly ($p<0.05$) up-regulated in the resistant IR70 under the reduced levels of N compared to the optimal N level. The *Os01g0108400* demonstrated an up-regulation in IR70 from a 19.8 fold to 23.0 fold and 26.4 fold under 1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3 respectively. Among all the TFs analyzed in this study, *Os01g0108400* gene recorded the highest level of expression in IR70 under 1.04 mM NH_4NO_3 and 0.64 mM NH_4NO_3. Hence this TF gene showed to be more responsive to N deficiency stress in the resistant IR70 rice cultivar.

The expression of *Os02g0214500* gene from the NAC family in the susceptible TN1 remain unchanged (~ 1 fold) under the 1.04 mM NH_4NO_3 and 0.64 mM NH_4NO_3 and increased to a 2.4 fold up-regulation compared to the optimal N level (1.44 mM NH_4NO_3) (Figure 4.5B). Therefore this gene only showed a response to N deficiency under the lowest N level (0.24 mM NH_4NO_3) in TN1. On the other hand, the expression level of *Os02g0214500* in the resistant IR70 increased along with the increased levels of N stress. The expression level of this gene was recorded at a 2.5 fold up-regulation, 8.0 fold up-regulation and 8.3 fold up-regulation respectively under 1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3 compared to the optimal N level. *Os02g0214500* demonstrated the highest expression level under the lowest N level in IR70 which was 3.5x higher compared to the expression in the susceptible TN1. Hence the expression profile revealed that this TF gene is more responsive to N deficiency stress in the resistant IR70 compared to the susceptible TN1.

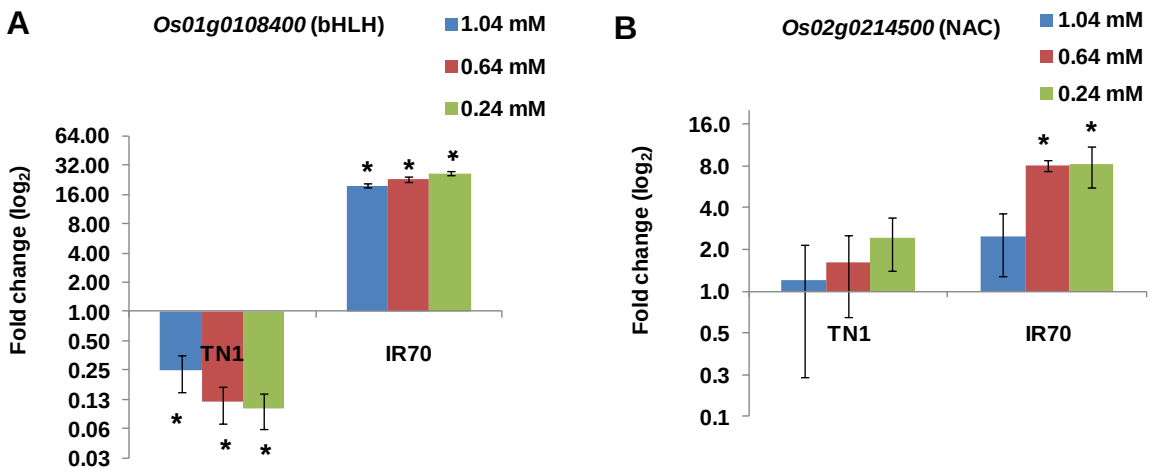


Figure 4.5 qPCR analysis of (A) *Os01g018400* and (B) *Os02g0214500* in response to reduced levels of N (1.04 mM HN_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3 in TN1 and IR70. Fold change (log_2 scale) indicates the expression of each gene as compared to the respective optimal N levels (1.44mM NH_4NO_3) which were set at 1.0. Significant differences between optimal N and N stress samples were shown by a single asterisk *, $p<0.05$ according to one way ANOVA followed by Tukey Post-Hoc test, n=3. The error bars of each data set represent the mean ± SD of three biological replicates.

Os06g0298200 gene from the C2C2-CO-like family also showed a similar pattern of gene expression as the *Os02g0214500* (Figure 4.6 A) in the susceptible TN1. In the susceptible TN1, the expression of this gene remained unchanged (~ 1 fold) under 1.04 mM NH_4NO_3 and 0.64 mM NH_4NO_3 as to the optimal N level (1.44 mM NH_4NO_3) whereas the resistant IR70 showed a significant ($p<0.05$) up-regulation of a 2.8 fold and 5.6 fold under these N levels compared to the optimal N level. The highest level

of gene expression was recorded under the lowest N level (0.24 mM NH_4NO_3) at a 2.6 fold up-regulation in TN1 and 6.1 fold up-regulation in IR70 compared to the respective optimal N levels. The IR70 was significantly ($p<0.05$) up-regulated to a 2.3x higher expression level compared to TN1 under this N level. The differences in the gene expression profile showed that *Os06g0298200* is more responsive to N deficiency stress in the resistant IR70 compared to the susceptible TN1.

The *Os09g0240200* gene which is also from the C2C2-CO-like family demonstrated up-regulation in both the susceptible TN1 and resistant IR70 (Figure 4.6 B). This gene was expressed to a higher level under the reduced N levels in IR70 compared to TN1. *Os09g0240200* was significantly ($p<0.05$) up-regulated to a 5.0 fold, 18.4 fold and 22.1 fold in IR70 under 1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3 respectively compared to the optimal N level (1.44 mM NH_4NO_3). The expression of *Os09g0240200* in TN1 demonstrated that this TF was up-regulated to a 2.6 fold (1.04 mM NH_4NO_3) which then gradually increased to a 3.6 fold (0.64 mM NH_4NO_3) and 6.2 fold (0.24 mM NH_4NO_3) respectively. The expression profile also demonstrated that this gene was significantly up-regulated to a 3.6x higher magnitude in IR70 compared to TN1 under the lowest N level. The expression profile of *Os09g0240200* revealed that this gene is more responsive to N deficiency stress in the resistant IR70 than the susceptible TN1.

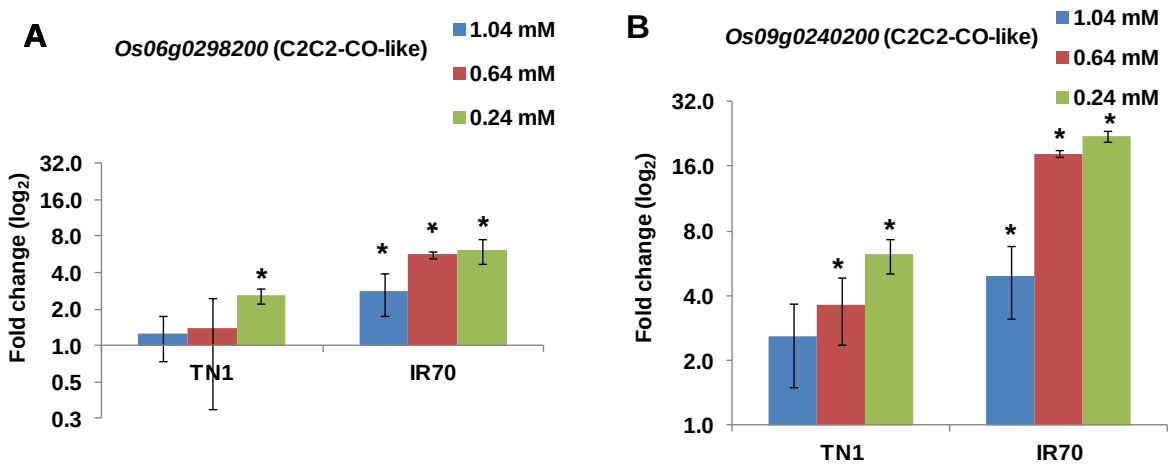


Figure 4.6 qPCR analysis of (A) *Os06g0298200* and (B) *Os09g0240200* in response to reduced levels of N (1.04 mM HN_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3 in TN1 and IR70. Fold change (log_2 scale) indicates the expression of each gene as compared to the respective optimal N levels (1.44mM NH_4NO_3) which were set at 1.0. Significant differences between optimal N and N stress samples were shown by a single asterisk *, $p<0.05$ according to one way ANOVA followed by Tukey Post-Hoc test, n=3. The error bars of each data set represent the mean ± SD of three biological replicates.

The *Os09g0439200* from the Tify family showed a different pattern of expression profile compared to the other 11 TFs analysed in this study. This gene was not differentially expressed in the susceptible TN1 under the reduced N levels (Figure 4.7 A). This might be due to a very low expression level which was not within the detection limit of the qPCR reaction. A semi-quantitative PCR reaction showed that this gene could not be amplified from the susceptible TN1 but a 140 bp amplicon was amplified in the resistant IR70 (data not shown). In contrast, the expression level of this gene in IR70 increased significantly ($p<0.05$) under the reduced N levels. The maximum level of expression was recorded under the lowest N level (0.24 mM NH_4NO_3) with a 6.5 fold up-regulation followed by a 5.5 fold up-regulation and 3.5 fold up-regulation under 0.64 mM NH_4NO_3 and 1.04 mM NH_4NO_3 respectively. Hence, the *Os09g0439200* gene was only responsive to N deficiency stress in the resistant IR70.

The O*s03g0180800* TF gene which is also from the Tify family showed a significant ($p<0.05$) up-regulation in the gene expression profile of both TN1 and IR70 (Figure 4.7 B). O*s03g0180800* in the susceptible TN1 showed an increase of a ~2 fold up-regulation under the 1.04 mM NH_4NO_3 and 0.64 mM NH_4NO_3 respectively compared to the optimal N level (1.44 mM NH_4NO_3). This gene then showed a rapid increase of

a 7.0 fold up-regulation under 0.24 mM NH_4NO_3 compared to the optimal N level in this rice cultivar. On the other hand, the resistant IR70 demonstrated a higher level of gene expression under the reduced N levels compared to the susceptible TN1. This TF gene was up-regulated to a 3.3 fold and 13.6 fold under 1.04 mM NH_4NO_3 and 0.64 mM NH_4NO_3 respectively in IR70. O*s03g0180800* also recorded a 14.8 fold up-regulation in this rice cultivar under the lowest N level (0.24 mM NH_4NO_3) compared to the optimal N level. The expression profile of *Os03g0180800* showed that this gene was expressed at a higher level in the resistant IR70 compared to the susceptible TN1 in response to N deficiency stress.

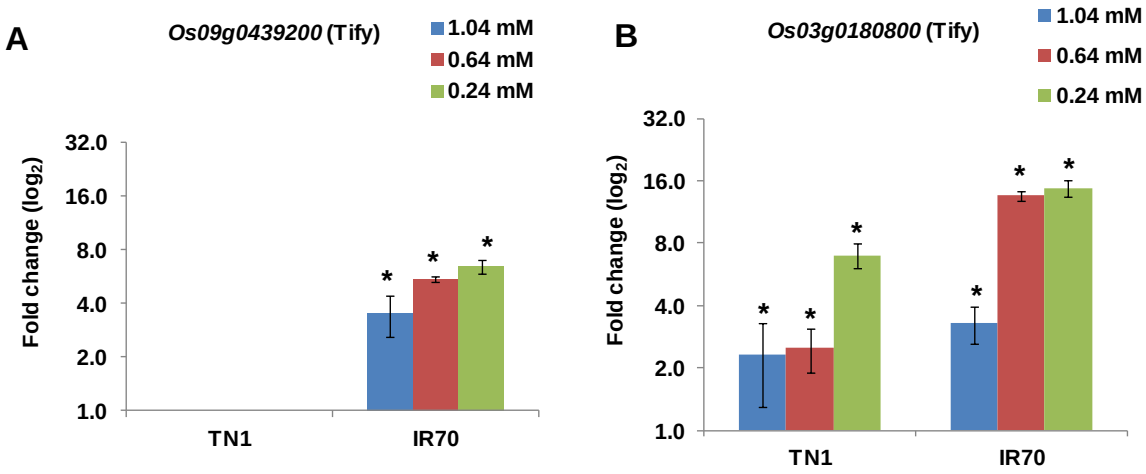


Figure 4.7 qPCR analysis of (A) *Os09g0439200* and (B) *Os03g0180800* in response to reduced levels of N (1.04 mM HN_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3) in TN1 and IR70. Fold change (log_2 scale) indicates the expression of each gene as compared to the respective optimal N levels (1.44mM NH_4NO_3) which were set at 1.0. Significant differences between optimal N and N stress samples were shown by a single asterisk *, $p<0.05$ according to one way ANOVA followed by Tukey Post-Hoc test, n=3. The error bars of each data set represent the mean ± SD of three biological replicates.

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Os03g0437200 which belongs to the C2H2 family showed up-regulation in both the rice cultivars, TN1 and IR70 in response to the reduced N levels (Figure 4.8 A). The expression level in the susceptible TN1 was recorded at a 2.1 fold up-regulation under 1.04 mM and increased significantly ($p<0.05$) to a 3.6 fold up-regulation and 8.0 fold up-regulation under 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3 respectively compared to the optimal N level (1.44 mM NH_4NO_3). The IR70 was significantly ($p<0.05$) up-regulated to a 3.7 fold, 4.6 fold and 9.4 fold under 1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3 respectively. The expression profile of *Os03g0437200* recorded the highest level of expression in the resistant IR70 under

the reduced levels of N showing that this TF is more responsive to N deficiency stress in the resistant IR70 compared to the susceptible TN1.

The *Os01g0971800* TF gene from the G2-like family was up-regulated to a 2.0 fold and 6.6 fold under 1.04 mM NH_4NO_3 in the susceptible TN1 and resistant IR70 respectively compared to the respective optimal N levels (1.44 mM NH_4NO_3) (Figure 4.8 B). The expression level then increased to 8.4 fold up-regulation in IR70 compared to 7.8 fold up-regulation in TN1 under 0.64 mM NH_4NO_3. This gene exhibited a 21.0 fold up-regulation in IR70 whilst 17.2 fold up-regulation in TN1 under the lowest N level (0.24 mM NH_4NO_3). Although both the rice cultivars showed an increased in the expression level under the reduced levels of N, this gene showed to be more responsive to N deficiency stress in the resistant IR70 compared to the susceptible TN1. In comparison to the other 11 TFs tested in this study, the *Os01g0971800* revealed the highest level of expression in the susceptible TN1.

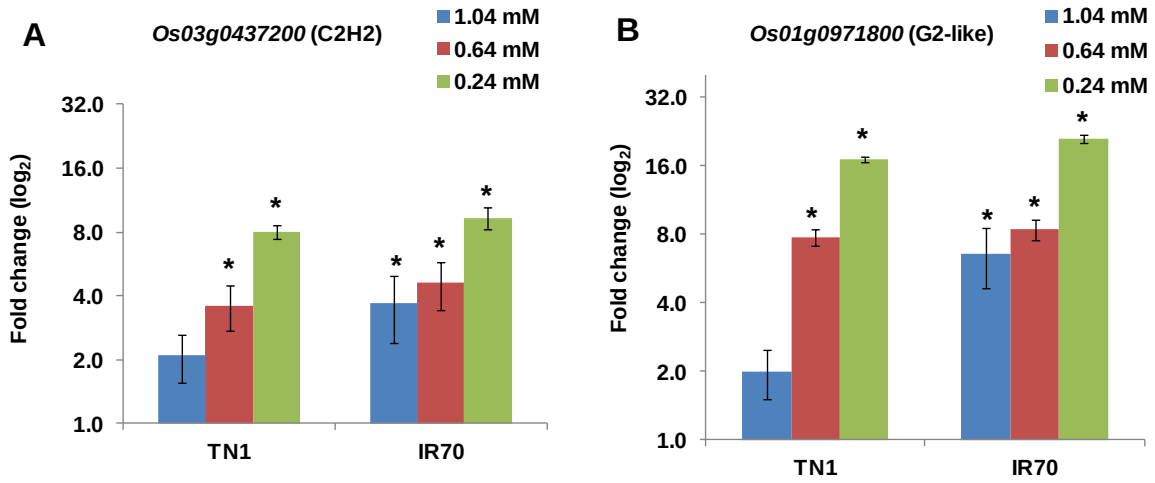

Figure 4.8 qPCR analysis of (A) *Os03g0437200* and (B) *Os01g0971800* in response to reduced levels of N (1.04 mM HN_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3 in TN1 and IR70. Fold change (log_2 scale) indicates the expression of each gene as compared to the respective optimal N levels (1.44mM NH_4NO_3) which were set at 1.0. Significant differences between optimal N and N stress samples were shown by a single asterisk *, $p<0.05$ according to one way ANOVA followed by Tukey Post-Hoc test, n=3. The error bars of each data set represents the mean ± SD of three biological replicates.

Overall the qPCR analysis revealed the differences in expression of the 12 TFs between BPH-resistance IR70 and BPH-susceptible TN1 rice cultivars grown under decreasing nitrate availability. Interestingly, the 12 TFs were up-regulated to a higher level of expression in the resistant IR70 in response to the reduced N levels compared to the susceptible TN1. Hence, findings from this study have identified TF

genes (which were reported to be involved in BPH resistance,) that potentially may play an important role to enhance tolerance to N deficiency stress.

4.5 Discussion

4.5.1 *qPCR efficiency*

The quality of RNA and its integrity is one of the most important aspects of qPCR analysis. A good quality of RNA allows synthesis of efficient cDNA products which leads to an accurate qPCR expression profile. Therefore several precautions and validations including primer optimization and assay specificity were carried out to ensure reliable and consistent results.

The C_T value obtained during qPCR amplification is based on the initial amount of template. Hence it is important to standardize the amount of cDNA across the entire qPCR analysis. An optimal qPCR assay is essential to produce accurate and reproducible quantification results of the samples analyzed. In order to evaluate whether the qPCR assay is optimized, the linear regression equation along with the coefficient of determination (R^2) was calculated. In a perfect qPCR analysis, doubling of each amplification cycle and the spacing of the fluorescence curves will be determined by the equation 2^n = dilution factor, where n is the number of cycles between curves at the fluorescence threshold (BIO-RAD, 2006). In this study, a 2-fold serial dilution with a total of 7 concentrations of cDNA showed that most C_T values were separated by 1 cycle. The R^2 value shows how strongly the regression line fits the data. Significant differences in observed C_T values between replicates will lower the R^2 value whereby the recommended R^2 value is; $R^2 > 0.980$ for the qPCR reactions (BIO-RAD, 2006). The R^2 value obtained for most primers and endogenous gene, *Actin* used in this study were more than 0.980 (Table 4.6) and produced reliable efficiency results. Furthermore, the melt curve analysis also demonstrated a single peak throughout the analysis which showed the specificity of the amplification.

The accuracy and validity of the real-time data also depend on the endogenous gene used for the data normalization. Proper validation and stability testing are important to avoid incorrect conclusions (George *et al.*, 2017). Primer optimization and assay specificity demonstrated that the *Actin* gene used in this study was a stable endogenous gene with a low variation within the biological replicates under all N deficiency stress conditions which gave accurate data normalization. The C_T value of

Actin for all samples under the reduced levels of N was not significantly different and therefore showed that this gene was stably expressed across these different level of N stresses. Therefore, no other endogenous genes were used throughout the qPCR analysis of this study. Moreover, *Actin* genes have been widely used as an endogenous gene in plant gene expression (Gu *et al.*, 2011) because they are involved in basic and essential processes in the cell. Previous studies showed that *Actin* was found to be stable for expression for many different abiotic stress studies in rice (Du *et al*., 2013 and Ramamoorthy *et al.*, 2008). The *Actin* gene was also used as an endogenous gene in gene expression analysis to identify TFs in BPH resistance (Wang *et al.*, 2012 and Jannoey *et al.*, 2017).

4.5.2 *Gene expression in response to N deficiency stress*

This study used a nutrient-free soil to investigate the transcriptional changes of genes that were affected by different levels of N deficiency stress in the susceptible TN1 and resistant IR70. A similar study conducted by Kant *et al*. (2010) revealed that several genes differentially expressed under mild or severe chronic N stress were identified using plants grown under N limiting condition either hydroponically or using nutrient-free soil.

During stress, genes that are induced by the particular stress or various stresses will protect cells from these stresses by producing important metabolic proteins (functional proteins) and also regulate genes (regulatory proteins) for signal transduction. These regulatory proteins are transcription factors (TFs) that up-regulate the expression of many secondary responsive genes which results in abiotic stress tolerance (Nakashima *et al.*, 2007). Plants exposed to N deficiency will shut down activities involving energy and nutrient consuming such as photosynthesis and TCA cycle to survive, as an adaptive mechanism (Lian *et al.*, 2006).

The qPCR analysis showed that most TFs investigated showed a higher level of gene expression under the reduced levels of N in both TN1 and IR70. Rice cultivars grown under the reduced levels of N showed a reduction in physiological responses. It has been established that plant growth is suppressed by N deficiency stress and that the expression of many genes changes with N availability (Watanabe *et al.*, 2010).

The gene expression results of all the 12 TFs analysed in this study in response to reduced N levels in TN1 and IR70 is summarised in the table below.

Table 4.6 Gene expression of TFs in response to reduced levels of N in TN1 and IR70 rice cultivars.

Transcription factors	**N stress levels / Gene expression**					
	1.04 mM NH_4NO_3		0.64 mM NH_4NO_3		0.24 mM NH_4NO_3	
	TN1	**IR70**	**TN1**	**IR70**	**TN1**	**IR70**
Os03g0860100	U	U	D	U	D	U
Os07g0410700	U	U	U	U	U	U
Os08g0157600	U	U	U	U	U	U
Os06g0728700	U	U	U	U	U	U
Os01g0971800	U	U	U	U	U	U
Os09g0439200	ND	U	ND	U	ND	U
Os03g0180800	U	U	U	U	U	U
Os06g0298200	U	U	U	U	U	U
Os09g0240200	U	U	U	U	U	U
Os02g0214500	U	U	U	U	U	U
Os01g0108400	D	U	D	U	D	U
Os03g0437200	U	U	U	U	U	U

U = up-regulated D = down-regulated ND = Not Detected

The outcome of this study has provided an overview of the expression profiles of TF genes which were involved in BPH resistance in the susceptible TN1 and resistant IR70 under reduced levels of N. TFs from different families showed differential expression patterns between both the TN1 and IR70. All 12 TFs analyzed in the present study were up-regulated to a greater magnitude in the resistant IR70 in response to the reduced levels of N compared to the susceptible TN1. In TN1, 2 TFs were down-regulated and the others were all up-regulated in response to the reduced levels of N. The transcriptional results from this present study was similar to the findings by (Bi *et al.*, 2007) who reported greater transcriptional changes under severe N deficiency stress compared to the mild N stress in Arabidopsis.

Previous studies revealed that the molecular response to N stress are complex in rice and has been proved by repression of many stress response genes and TFs. Low N deficiency stress was subjected to repression of photosynthesis and energy metabolism genes in rice seedlings (Vinod and Heuer, 2012). Studies also demonstrated that DREB1/CBF, DREB2, AREB/ABF, MYB/MYC, bHLH, ZFPs, WRKY, and NAC are important stress-responsive genes that play important roles in

abiotic stresses by activating or deactivating the expression of these genes in rice (Gujjar *et al*., 2014).

Two TFs investigated in this study, *Os03g0860100* and *Os07g0410700* from the AP2-EREBP family were differentially expressed under the reduced levels of N. The *Os03g0860100* and *Os07g0410700* were up-regulated in the resistant IR70 in a dose-dependent manner. Up-regulations of TF genes under N deficiency indicate that these genes may play important roles in protecting the plants against low N stress (Lian *et al.*, 2006). The expression of *Os03g0860100* in TN1 and IR70 remained similar as the optimal N levels under 1.04mM NH_4NO_3 showed that expression of this gene was not affected under the medium N level. The down-regulation of *Os03g0860100* in TN1 showed that this gene may not be involved in response to N deficiency stress in this rice cultivar. Interestingly in IR70, the *Os07g0410700* was highly expressed (31.1 fold up-regulation) at the lowest N level compared to TN1 (2.8 fold up-regulation), showing that this gene may be more responsive to N stress in the resistant IR70 compared to the susceptible TN1.

Several studies revealed that the AP2/ EREBP family is involved in plant developmental processes and stress tolerance against various abiotic stresses in plants. Erpen *et al.* (2017) reported that AP2/ethylene (ERP) family TF play an important role in the regulation of the abiotic stress response. Takehisa *et al.* (2013) reported that *Os03g0860100* was up-regulated at 24 hours after K-deficiency treatments in the rice roots. Another study revealed that *Os03g0860100* was down-regulated in the roots at 1 hour of N and phosphorus (P) starvation stress in the Hejiang 19 rice cultivar (Cai *et al*., 2013). *Os03g0860100* which was reported as *OsEBP1* was up-regulated in response to the iron excess in the Nipponbare rice variety (*O. sativa* ssp. *japonica*) (Finatto *et al.*, 2015). This gene was also up-regulated in many other abiotic stresses such as drought stress in rice (Oh *et al.*, 2009). Findings from these studies showed that *Os03g0860100* was involved in response to different type of abiotic stress in particularly to nutrient deficiency.

The *Os07g0410700* has a generic name of AP2/EREBP #074 (EREP1) and belongs to the subgroup IIIb of the AP2/ERF gene. The *OsAP2/EREBP-074* gene in wheat and cotton was shown to be involved in pathogen resistance and abiotic stress tolerance in these transgenic plants (Rashid *et al.*, 2012). Chandran *et al.* (2016) reported that *Os07g0410700* was up-regulated in the Dongjin rice variety after

exposure to a ¼ strength of Yoshida nutrient medium (0.09 mM NH_4NO_3) for 3 days which was similar to the findings of this study. Moreover, the gene ontology analysis (GO) of the PlantPAN 2.0 (http://plantpan2.itps.ncku.edu.tw/) showed that for both the TFs were involved in the cellular nitrogen compound metabolic process (GO: 003461). This study suggested that *Os03g0860100* and *Os07g0410700* may be involved in the regulation of gene expression in N deficiency stress in the resistant IR70 rice cultivar. Overexpression of these TFs is likely to control the expression of other TF or genes required by the plant to maintain homeostasis under these low nitrogen level. Although these final genes and or the biochemical pathways they construct are not known at this stage.

Two other important genes *Os08g0157600* and *Os06g0728700* from the MYB-related family were up-regulated in both the susceptible TN1 and resistant IR70 when subjected to N deficiency stress. Both TFs showed a similar level of gene expression in response to the reduced levels of N in TN1 and IR70. The previous study showed that members of the MYB family have highly diversified biological functions (Wang *et al.*, 2012). Moreover, Li *et al*. (2014) revealed that MYB proteins are involved in the regulation of several stress-related genes which are involved in response to abiotic stresses. Recently, Erpen *et al.* (2017) reported that MYB proteins play an important role in the ABA-dependent pathway and induced tolerance in abiotic stresses such as drought and salinity in plant species. Nitrogen-responsive MYB were identified in several plant species including rice which suggests that MYB genes have a role in nitrogen utilization in rice (Chandran *et al.*, 2016). Interestingly, the overexpression of *OsMYB48-1* in rice demonstrated enhanced tolerance to drought and salinity stress (L. *et al.*, 2017).

One of the studies revealed that *Os08g0157600* and *Os06g0728700* were strongly down-regulated under potassium (K) deficiency and up-regulated upon resupply of this nutrient in IR64 (Shankar *et al.*, 2013). The *Os06g0728700* showed adaptation to salt and drought stress in wheat which indicated to be a potential candidate gene for genetic manipulation to improve salt and drought tolerance in this plant (Rahaie *et al.*, 2010). The findings above showed that *Os08g0157600* and *Os06g0728700* were involved in response to nutrient deficiency and also other abiotic stresses. To date, no previous research had been conducted on this gene in relation to N deficiency stress. Similar expression profiles of these TF genes under different stresses indicate their central role in a generic stress response of rice and demonstrate a degree of

cross-talk in the response to different stress factors. Thus this present study concluded that *Os08g0157600* and *Os06g0728700* were responsive to N deficiency stress in both the susceptible TN1 and resistant IR70.

Another TF investigated in this study is *Os01g0108400*. This TF from the bHLH family was significantly down-regulated in the susceptible TN1 in response to the reduced levels of N. The gene expression profile showed that this TF may be a negative regulator of N deficiency stress in the TN1. According to Lian *et al.* (2006), genes involved in photosynthesis and energy metabolisme were rapidly down-regulated under low N stress in rice plants. In contrast, this gene displayed a drastic increase in expression level in IR70 in response to N deficiency stress. This result showed that *Os01g0108400* is involved in response to N deficiency stress in the resistant IR70.

Overall the basic-helix-loop-helix (bHLH) family is reported to be involved in several abiotic stresses responses such as salt and drought tolerance. Some of these genes from this family are also shown to be responsive to nutrient tolerance such as iron (Fe) uptake and phosphate (Pi) starvation (Wang *et al.*, 2017). bHLH is also a key regulatory component in the transcriptional regulatory network which controls the growth and development signalling pathways and abiotic stress responsive genes (Ji *et al.*, 2015). One of the findings showed that the *Os01g0108400* (LOC_Os01g018400) was down-regulated in the *SNAC2*-overexpressed rice Zhonghua 11 (*O. sativa* L. spp. *japonica*) in response to cold stress (Hu *et al.*, 2008). The present study suggested that overexpression of *Os01g0108400* may potentially enhance tolerance to N deficiency in rice cultivars.

The *Os02g0214500* is a TF which belong to the NAC family. Previous studies demonstrated that NAC TFs are involved in stress tolerance in plants (Khong *et al.*, 2008). The present study demonstrated that *Os02g0214500* was only up-regulated (~ 2 fold) the susceptible TN1 in response to N deficiency stress under 0.24 mM NH_4NO_3. Hence, this gene is only responsive to N deficiency under lowest N level in TN1. In contrast, *Os02g0214500* showed a marked increase in expression levels in response to the reduced N levels in IR70. This expression profile clearly shows that *Os02g0214500* is more responsive to N deficiency stress in IR70. The *Os02g0214500* is reported to be involved in water-deficit stress and seed development in rice (Ray *et al.*, 2011). Presently this gene is not reported to be

involved in response to N deficiency stress. Therefore, the response in IR70 indicates that this gene may be a useful marker to screen for N deficiency and other abiotic stress resistance in new rice cultivars.

The *Os06g0298200* from the C2C2-CO-like also demonstrated a similar pattern of expression as the *Os02g0214500* in the susceptible TN1. The expression level of this TF in this rice cultivar remained unchanged under 1.04 mM NH_4NO_3 and 0.64 mM NH_4NO_3 compared to the optimal N level (1.44 NH_4NO_3) and was only responsive to N stress (~ 2 fold up-regulation) under the lowest N level (0.24 mM NH_4NO_3). This expression profile showed that the *Os06g0298200* is only responsive under lowest N availability in TN1. However, this gene was consistently up-regulated in the resistant IR70 in a dose-dependent manner. The resistant IR70 which was up-regulated 3x more than the susceptible TN1 under the lowest N level showed that this gene is more responsive to N deficiency stress in IR70 compared to TN1.

Os06g0298200 also known as the *OsBB19* was involved in diurnal expression in rice (Huang *et al.*, 2012) and is a negative regulator of long day photoperiodism/flowering (GO:0048579) (http://plantpan2.itps.ncku.edu.tw/). To date, there was no other finding to show that this TF was involved in other abiotic stress in particular nutrient deficiency. However, this study shows that *Os06g0298200* may be involved in N deficiency stress in the resistant IR70. In addition, *Os06g0298200* is also reported to be involved in several biological processes (GO: 0008150) and molecular functions (GO: 0003674) which indicates that this TF is potentially involved in various stress-response pathways. Therefore, *Os06g0298200* is potentially a good candidate for improved growth under low-nitrogen conditions, an agronomically important trait to improve NUE.

Os09g0240200 which is also from the C2C2-CO like family was up-regulated in both the susceptible TN1 and resistant IR70 in a dose-dependent manner under N deficiency stress but to a greater magnitude in the IR70. Up-regulation of *Os09g0240200* in TN1 and IR70 showed that this gene is involved in early responses to N deficiency stress. The previous study showed that this gene was proven to be involved in response to drought stress in rice. This gene showed up-regulation in response to drought tolerance in the HNZ rice variety which is sensitive to drought stress (Zhang *et al.*, 2016). Shankar *et al.* (2013) showed that this gene was down-regulated in response to K deficiency and up-regulated upon resupply of this nutrient

in IR64 (*O. sativa* ssp. *indica*) rice cultivar. The present study demonstrated that *Os09g0240200* is a low N stress- inducible gene and is potentially useful to understand the mechanism of rice in response to N deficiency and improvement of NUE in the rice crop.

Among all the 12 TFs tested, the *Os09g0439200* from the Tify family demonstrated gene expression only in the resistant IR70 and was not differentially expressed in the susceptible TN1 under the reduced levels of N. *Os09g0439200* which is known as the *OsJAZ8* were found to be involved in high carbon and low N up-regulation in Nipponbare (*O. sativa ssp. japonica*) rice variety (Huang *et al.*, 2016) . Therefore, the present study showed that this gene was only responsive to N deficiency stress in IR70. The PlantPAN 2.0 (http://plantpan2.itps.ncku.edu.tw/) gene ontology analysis showed that *Os09g0439200* is involved in plant hormone signal transduction (map04075). One of the studies reported that TFs involved in signal transduction were both up and down-regulated under low N condition (Lian *et al.*, 2006). However, Cai *et al*. (2013) reported that plant response and signal transduction pathways for N deficiency are poorly understood. The present study suggested further investigation is required understand the function of this gene in response to N stress in rice cultivars.

Os03g0180800 which also belongs to the Tify family showed a differential expression profile in the susceptible TN1 and resistant IR70 rice cultivars. *Os03g0180800* was up-regulated in both the rice cultivars in response to the reduced levels of N in a dose-dependent manner. The expression profile which showed similar level of expression in the TN1 under 1.04 mM NH_4NO_3 and 0.64 mM NH_4NO_3 (~ 2 fold up-regulation) exhibited a rapid increase in gene expression under 0.24 mM NH_4NO_3, hence indicated that this gene is very responsive to N deficiency stress under lowest N level. Therefore, *Os03g0180800* is more responsive to N deficiency stress in the resistant IR70 compared to the susceptible TN1.

Previous studies reported that *Os03g0180800* was involved in salt stress tolerance in Minghui63 (*O. sativa* L. ssp. *indica*) rice variety (Wu *et al.*, 2015). Interestingly most of the OsJAZ including *OsJAZ9* (Os03g0180800) were up-regulated in rice under N deficiency (Singh *et al.*, 2015) which is consistent with the finding of this study. Dhakarey *et al*. (2016) reported that the overexpression of *OsJAZ9* also showed an increased salt tolerance while its suppression reduced salt tolerance which indicated

the function of JA signalling in abiotic stress tolerance. Another study showed that this gene which was known as *OSJAZ23* was up-regulation in response to cold stress in different rice cultivar tested (Chawade *et al.*, 2013). Hence this study revealed that *Os03g0180800* is potentially more responsive to N deficiency stress in the resistant IR70 rice cultivar compared to the susceptible TN1. The present study suggested that overexpression of this gene will potentially increase tolerance to N deficiency in rice cultivars.

The *Os03g0437200* from the C2H2 family demonstrated an increase in gene expression in the susceptible TN1 and resistant IR70 in a dose-dependent manner but to a greater magnitude in the resistant IR70. Previous studies reported that C2H2 zinc finger proteins are involved in several stress responses. To date, a total of 26 rice C2H2 zinc finger protein were identified to be up-regulated in response to cold, drought or salt stress in *indica* rice varieties (Huang *et al.*, 2009). One of the studies revealed that *Os03g0437200* was up-regulated under juglone stress in rice (Chi *et al.*, 2011). This gene was also reported to be extensively up-regulated in the phytoalexin biosynthesis in rice (Fujino and Matsuda, 2010). Most importantly it was reported that *Os03g0437200* was induced in the sheaths of rice seedlings under N deficiency condition (Huang *et al.*, 2016) which is similar to the findings of this study. The previous study showed that genes involved in protein degradation were up-regulated under severe N stress (Bi *et al.*, 2007). The present study suggested that *Os03g0437200* is potentially involved in response to N deficiency and may play important role in improving NUE in rice production.

Os01g0971800 from the G2-like was up-regulated in a dose-dependent manner in the susceptible TN1 and resistant IR70 in response to N deficiency stress. *Os01g0971800* demonstrated a high level of expression in both rice cultivars in response to N deficiency stress but to a greater magnitude in IR70. Interestingly, among all the 12 TFs tested in this study, *Os01g0971800* recorded the highest level of expression in TN1 under the lowest N level (0.24 mM NH_4NO_3). According to the PlantPAN 2.0 (http://plantpan2.itps.ncku.edu.tw/) gene ontology analysis, this gene was also involved in the cellular nitrogen compound metabolic process (GO: 0034641) and regulation of transcription (GO: 0034641). Moreover, Lian *et al.* (2006) reported that rapid changes in gene expression of the regulatory elements, such as genes involved in signal transduction and transcription regulation represent the primary response of the regulatory machinery to the low N stress in rice. Therefore,

expression profile of the present study demonstrated that *Os01g0971800* showed an early response to N deficiency and is a low N stress inducible gene which showed to be responsive to this stress in both TN1 and IR70.

4.6 Conclusions

Abiotic stress such as nutrient deficiency causes significant constraints to the growth and yield of rice cultivars. In order to cope with abiotic stresses, plants will demonstrate drastic changes in biochemical and physiological aspects within the plants. In this study, all TFs which were involved in BPH resistance were analyzed in response to N deficiency stress in the susceptible TN1 and resistant IR70 rice cultivars. Changes in gene expression are the important key points for understanding the responses to N deficiency stress. Overall, the susceptible TN1 and resistant IR70 rice cultivars responded differentially at the physiological and molecular levels when subjected to N deficiency stress. As the stress increased then the magnitude of the measured responses to physiological and molecular, also increased.

As expected, these TFs were more responsive to N deficiency stress in the resistant IR70 compared to the susceptible TN1. All the TFs in IR70 were up-regulated in a dose-dependent manner. Up-regulation at higher expression level suggested that these genes potentially played an important role in response to N deficiency stress. Severe N deficiency stress (0.24 mM NH_4NO_3) exhibited greater transcriptional changes compared to the mild N deficiency levels (1.04 mM NH_4NO_3 and 0.64 mM NH_4NO_3).

In TN1, *Os03g0860100* and *Os01g0108400* were down-regulated in response to reduced levels of N. This could be the strategy used by this rice cultivar to survive and make them more vulnerable to the stress condition. This study also revealed that *Os09g0439200* was not induced under N deficiency stress in TN1 but was expressed at a greater level in IR70. Interestingly, TFs from the MYB family *Os08g0157600* and *Os06g0728700* exhibited a similar level of expression in response to N deficiency stress in TN1 and IR70. This finding suggested that both the rice cultivars are potentially involved in response to N deficiency stress.

Findings from this study also demonstrated that five TFs (*Os01g0971800*, *Os01g0108400*, *Os09g0240200*, *Os03g0180800* and *Os07g0410700*) were highly expressed in response to N deficiency stress in the resistant IR70. *Os07g0410700*

and *Os01g0108400* recorded the highest expression level at the lowest N level in IR70 concluded that these genes may be responsive to N deficiency stress in this rice cultivar. Based on the expression levels, *Os01g0971800* is one of the TFs tested that was very responsive to N deficiency stress in the susceptible TN1.

As hypothezied in this study, the TF genes revealed higher level of gene expression in the resistant IR70 compared to the susceptible TN1 in response to the reduced N levels. The expression or repression of all the 12 TF genes in response to N deficiency stress will provide information for future breeding strategies towards N stress tolerance rice crops. The present study showed potential occurrences of crosstalk between abiotic (N deficiency) and biotic (BPH resistance; Wang *et al.*, (2012)) which can lead to enhanced resistance to both the stresses.

Further studies to investigate the response of these TF genes in the combination of N and BPH infestation will be discussed in Chapter 5 of this study. However, there were a few factors that should be taken into consideration in carrying out this study. This study on N deficiency stress in TN1 and IR70 rice cultivars was carried out over a short term. There might be possibilities that molecular responses to N deficiency stress during short and long-term exposure could differ and most importantly the results of these TFs might not reflect the actual tolerance in the field which involves factors such as biomass and the yield of the crop. Long-term response of the rice cultivars under different N deficiency stress may have to be further investigated. Limited knowledge of N deficiency stress is still a major gap in understanding the N stress tolerance in rice. Therefore, comprehensive profiling of N deficiency stress-associated genes of the rice crop will be a key factor in molecular breeding for tolerance to N stress.

Chapter 5: Identification of transcription factors involved in the response of TN1 and IR70 to both BPH infestation (biotic stress) and different levels of nitrogen (abiotic stress)

Abstract

Plants have evolved to develop astonishing survival strategies to adapt to variations in environmental conditions include rapid onset of abiotic and biotic stresses. These extreme conditions have caused constraints on the growth and development of plants. Identification of transcription factors (TFs) involved in the combination of both abiotic and biotic stresses is an important strategy to study the gene expression and molecular mechanism in a plant. The present study was carried out to identify TF genes which were involved in the combination of the reduced levels of nitrogen (N) (abiotic stress) and brown planthopper (BPH) infestation (biotic stress) in the TN1 (susceptible to BPH) and IR70 (resistant to BPH). Twelve TF genes from different TF families which were previously reported to be potentially related to BPH-resistance showed differential expression patterns in response to the dual stress. Seven TFs were down-regulated in IR70 compared to two TFs in TN1 in response to the dual stress. In contrast, there were more up-regulated TFs in TN1 than IR70. Most TFs showed an increase in expression level in response to the reduced levels of N (1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3) compared to the optimal N level (1.44 mM NH_4NO_3) at different time points of BPH infestation. The resistant IR70 also exhibited a higher level of expression compared to TN1 in response to the reduced levels of N and BPH infestation. This study revealed that two TF genes, *Os06g0728700* from the MYB-related family and *Os09g0240200* from the C2C2-CO-like was down-regulated in both the TN1 and IR70 but to a greater magnitude in the IR70. Overall, this study demonstrated that *Os08g0157600*, *Os02g0214500*, *Os09g0439200* and *Os03g0437200* are potentially involved in response to BPH infestation and reduced levels of N in the resistant IR70.

Keywords: Transcriptional factors, brown planthopper, nitrogen, gene expression

5.1 Introduction

Previous studies revealed that plant anti-herbivore resistance is directly associated with the physiology of plants and therefore any factors affecting a plant's physiology could potentially alter its resistance to insect pests. Application of synthetic fertilizers is reported to increase the nutrients in the plants and enhanced crop growth. At the same time, application of these fertilizers has altered the production of defence-related secondary compounds that can impact the susceptibility of a given crop to insect herbivore (Wu *et al.*, 2017).

N is proven to be one of the essential macronutrients which have contributed towards the growth and development of most herbivorous insect species and the level of N in the host-plant is the most important factor affecting the performance of these insects. Previous studies have shown that application of high levels of N fertilizer in crop plants has influenced plant-insect interactions, increased growth, survival, reproductive rate and the population of insect herbivores. In contrast, deficiency of N potentially alters plant metabolism and triggers insect resistance in the plants. The increasing population of major insect pest of rice including brown planthoppers are associated with a long-term excessive application of N fertilizers in most of the rice growing areas in Asia (Wu *et al.*, 2017). The relationship between application of N fertilizers and BPH outbreaks is widely established in the literature.

Crop plants under their natural environment are exposed to abiotic and biotic stresses either individually or in a combination of both stresses and have evolved many defence mechanisms in order to adapt and survive (Kissoudis *et al.*, 2016). One of the studies suggested that plants have the ability to cope with simultaneous biotic and abiotic stresses through different defence responses which cannot be understood by directly extrapolating the results from individual stress studies where each stress is applied. Plants tolerating two or more independently occurring stresses need not necessarily tolerate these stresses when they occur simultaneously. Many studies on gene expression data from independent biotic and abiotic stress experiments have identified stress-responsive genes under the combination of both stresses. Despite the need for understanding simultaneous biotic and abiotic tolerance of plants, not many studies were carried out in this direction (Ramegowda and Senthil-Kumar, 2015). Kissoudis *et al.* (2016) also reported that studies conducted to understand the interaction between the combination of abiotic and biotic stresses are limited.

Therefore understanding the molecular responses to the combination of biotic and abiotic stresses is an important tool for identification of new targets and development of novel genes to improve crop yield and enhance plant resistance to insect pest (Kissoudis *et al.*, 2016). qPCR is one of the technologies which has been well developed and several studies have revealed the function of many TF genes under individual stress or combination of abiotic and biotic stresses by gene expression profiles. The relationship between gene expression responses to different stresses demonstrates a fundamental basis for understanding the genetics and functional foundation of multiple-stress tolerance (Swindell, 2006).

5.1.1 *Impact of a high level of N on BPH population in rice crop*

Extensive studies have been carried out to enhance an effective pest management strategy to reduce rice crop yield loss. Host plant tolerance to BPH is an important aspect of BPH management in rice growing areas. BPH has been reported to show rapid adaptation to adverse environmental factors which has caused changes in their virulent levels, biotypes, resistance to insecticides, the emergence of adult macropterous and occurrence of long-distance immigration in the BPH population (Lu *et al.*, 2005). These authors also reported that BPH survives better with an increased rate of fecundity in host plants with a high level of N. According to a choice test experiment, BPH showed a preference to N-rich host plants compared to the host plants grown in low N conditions to feed and oviposit. BPH grown in a rice field with high level of N has the potential to find new habitats over a long distance in a new rice field and newly transplanted field. This is because female adults feed on host plants with high N content have a higher tolerance to starvation.

The findings also showed that the female BPH were heavier, laid more eggs and the egg hatchability increased significantly with the increased level of N. Therefore BPH potentially has higher ecological fitness over a long time in the rice growing areas which are overloaded with N (Lu and Heong, 2009). Moreover, the hopper also had higher feeding rates and honeydew excretion in host plant with high N content. This is because of the high content of amino acids such as aspartic acid and glutamic acids which stimulates the feeding rate of the hopper. Hence, increased colonization resulted in rapid increase of BPH population and contributes to potential BPH outbreak (Lu *et al.*, 2005).

Therefore, excessive application of N will not only increase the BPH population but also cause a severe environmental impact which has been discussed in 1.4.3 of Chapter 1. Thus, developing rice cultivars which do not depend on a high level of N is very important in order to overcome the problems as discussed. However, lacking knowledge in the regulation of plants in response to low N stress has been a setback to identify genes involved in both stresses (Lian *et al.*, 2006).

Wang *et al.* (2012) identified 37 induced and 26 constitutive transcription factors (TFs) related to BPH resistance in two rice cultivars TN1 and Rathu Heenati (RHT). RHT is a resistant rice carrying *Bph3* which has similar resistance to the IR70 (Peñalver Cruz *et al.*, 2011). TFs from different families showed a differential in gene expression upon BPH infestation in both the rice cultivars. Some of these genes were probably related to BPH-induced resistance because their expression profiles changed, positively and negatively, in response to BPH infestation. The induced TF genes showed interesting patterns at two time points (8 h or 24 h) after BPH infestation and more TN1 was induced at these time points compared to the RHT.

TF genes that were related to constitutive resistance were up-regulated or down-regulated in RHT compared to the respective expressions in TN1 at the same time points of BPH infestation. These genes were considered as being specific to resistant rice variety, RHT. These authors revealed that the levels of gene expression play an important role in plant defence reaction. The study also showed that the resistant RHT suffered less damage than the susceptible TN1 at the molecular level. This present study carried out gene expression analysis with twelve TFs used by Wang *et al.* (2012) to identify TF genes which were potentially responsive to both simultaneous abiotic (reduced levels of N) and biotic (BPH infestation) stresses.

5.2 Objectives of study

Hypothesis:

The transcription factors (TFs) shown in Table 4.1 were more responsive to the combination of the reduced levels of N and BPH infestation in the resistant IR70 compared to the susceptible TN1.

The specific objectives of this study are to:

(i) Investigate the responses of susceptible TN1 and resistant IR70 rice cultivar to the combination of reduced levels of N deficiency stress and BPH infestation at different time points simultaneously using qPCR technology.

(ii) Identify TFs which are responsive to combination of both abiotic (N deficiency) and biotic (BPH infestation) stress in the susceptible TN1 and resistant IR70 rice cultivars.

5.3 Material and methods

5.3.1 *Plant materials and growth condition*

The TN1 (susceptible to BPH) and IR70 (resistant to BPH) rice cultivars were germinated and grown at the same leaf stage and conditions as described in 3.3.1 and 3.3.3 of Chapter 3 .

5.3.2 *Insects*

The BPH population was obtained from Syngenta Switzerland and reared on TN1 rice cultivar in the insectary at 28°C with a photoperiod of 16/8 h day/night. The BPH population was continuously maintained under the same temperature and light regime as described above. This present study used fourth to fifth BPH instars for BPH infestation experiments.

5.3.3 *BPH infestation and sample collection*

In this experiment, TN1 and IR70 rice cultivars grown in silica sand under different N levels (1.44 mM NH_4NO_3, 1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3) were covered with a clear perforated bag and infested with 10 nymphs of BPH per plant. The experiment was conducted with six replicates for each plant. BPH infestation on the rice cultivars began at the same time point and stopped at different time points of BPH infestation (4 h, 8 h, 12 h, 24 h and 36 h). Each set of TN1 and IR70 experiment was provided with six replicates of non-infested plants for each N level. The experiment for TN1 and IR70 were conducted separately due to space constraint at the exact same conditions as described above. Each plant was supplied with sufficient nutrient solutions throughout the experiment. The leaves of each

sample from the six replicates were harvested at different time points and immediately immersed in liquid nitrogen. The samples were then stored in -80°C for further analysis.

5.3.4 *RNA extraction*

RNA extraction was conducted as described in 2.3.5 of Chapter 2.

5.3.5 *cDNA synthesis*

cDNA synthesis was conducted as described in 4.3.4 of Chapter 4.

5.3.6 *Quantitative PCR Analysis (qPCR)*

qPCR analysis was carried out using *Actin* as the endogenous gene and twelve primers as described in 2.3.9 of Chapter 2 and 4.3.5 of Chapter 4.

5.3.7 *Validation of reference gene in dual stress*

In order to determine the stability of *Actin* in response to combination of N deficiency stress and BPH infestation, the average C_T value of the respective infested and non-infested samples under all the different N levels. The significance of C_T value obtained from the infested and non-infested TN1 and IR70 samples across all of the different N levels were analyzed using ANOVA and further evaluated using Tukey Post-Hoc test ($p<0.05$).

5.3.8 *Data analysis*

The relative expression of the target genes was calculated according to the method of $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001) using the equation as follows:

$\Delta\Delta C_T = (C_{T,\,Target} - C_{T,\,Actin})_{Time\ x} - (C_{T,\,Target} - C_{T,\,Actin})_{Time\ 0}$, where time *x* represents the time points of 4, 8, 12, 24 and 36 h for BPH infestation and time 0 represents the non-infested control samples at different levels of N.

5.4 Results

5.4.1 *Validation of reference gene in dual stress*

The stability of the reference gene was assessed under optimal N level (1.44 mM NH_4NO_3) and reduced N levels (1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3) at 36 h of BPH infestation and were compared to the respective TN1 and IR70 grown under different N levels (non-infested plants). The stability and suitability of this gene analyzed using two-way ANOVA showed that there were no significant variations in the average C_T value between each N level in the non-infested and the respective infested plants upon BPH infestation. Therefore, this confirmed the suitability of *Actin* as an endogenous control to enable normalization against total amount of cDNA across all conditions tested in both TN1 and IR70 rice cultivar. Specificity and amplification efficiency of the *Actin* gene used in this study was verified as described in 4.3.7 of Chapter 4.

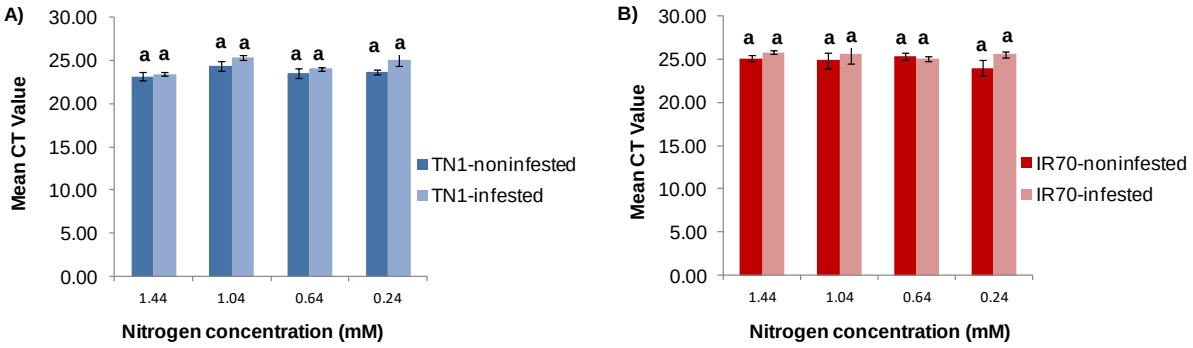

Figure 5.1 Mean C_T value of the endogenous gene, *Actin* across different N levels in non-infested and infested A) TN1 and B) IR70 rice cultivars. Data refers to mean ±SD (n=3) and $p<0.05$ according to two-way ANOVA followed by Tukey Post-Hoc Test for each rice variety. Bars with the same letter are not significantly different.

5.4.2 *Gene expression analysis in response to the combination of the reduced level of Nitrogen and BPH infestation*

A qPCR analysis was carried out using twelve TFs (potentially involved in BPH resistance) to investigate the molecular response of susceptible TN1 and resistant IR70 in response to the combination of reduced N levels and BPH infestation. The gene expression profiles of the TFs under simultaneous stresses (N input and BPH infestation) in TN1 and IR70 were displayed as four graphs (Figure 5.2 to Figure 5.13) representing expression profiles upon BPH infestation at different time points

(4 h, 8 h, 12 h, 24 h and 36 h) under optimal N level (1.44 mM NH_4NO_3) and the reduced N levels (1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3). The gene expressions of all TF genes under the different N levels were compared to the respective non-infested N levels.

The *Os03g0860100* gene from the AP2-EREBP family (Figure 5.2) showed up-regulation upon BPH infestation across all time points in the optimal N level (1.44 mM NH_4NO_3), 1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3 in the susceptible TN1 and resistant IR70. This gene remained ~ 1 fold up-regulated in TN1 under 1.44 mM NH_4NO_3 upon BPH infestation at 4 h and 8 h and was up-regulated to a ~ 2 fold at 12 h, 24 h and 36 h of BPH infestation. The *Os03g0860100* was up-regulated to a ~ 2 fold (4 h, 8 h and 12 h), 3.0 fold (24 h) and increased to 3.9 fold (36 h) in IR70 upon BPH infestation under this N level. The expression profile of *Os03g0860100* in IR70 exhibited that this gene was up-regulated to a 3.3 fold, 3.6 fold, 12.1 fold, 3.4 fold and 4.3 fold respectively at 4 h, 8 h, 12 h, 24 h and 36 h of BPH infestation under 1.04 mM NH_4NO_3 whilst the susceptible TN1 showed similar level expression as in 1.44 mM NH_4NO_3 at 4 h, 8 h and 12 h of BPH infestation. TN1 showed a higher level of expression at 24 h (3.6 fold up-regulation) and 36 h (4.0 fold up-regulation) upon BPH infestation under 1.04 mM NH_4NO_3. This gene also showed a ~ 2 fold up-regulation at 4 h and 8 h and increased to a 3.1 fold (12 h) then decreased to a 3.6 fold (24 h) and 4.7 fold (36 h) of BPH infestation in IR70 under 0.64 mM NH_4NO_3.

In addition, the expression level of *Os03g0860100* in IR70 was increased to a higher level upon BPH infestation at all time points under the lowest N level (0.24 mM NH_4NO_3) to a 5.3 fold (4 h), 4.8 fold (8 h), 8.2 fold (12 h), 3.9 fold (24 h) and 5.5 fold (36 h). The susceptible TN1 showed higher levels of expression at most time points (4 h, 8 h and 36 h) of BPH infestation under 0.24 mM NH_4NO_3. The expression of this gene in TN1 under the lowest N level was up-regulated to a 4.1 fold (4 h), 3.4 fold (8 h), 3.1 fold (12 and 24 h) and 4.5 fold (36 h) of BPH infestation. *Os03g0860100* exhibited a higher level of expression under the lowest N level at most time points of BPH infestation compared to the expression level under the other N stress levels and the optimal N level in both TN1 and IR70.

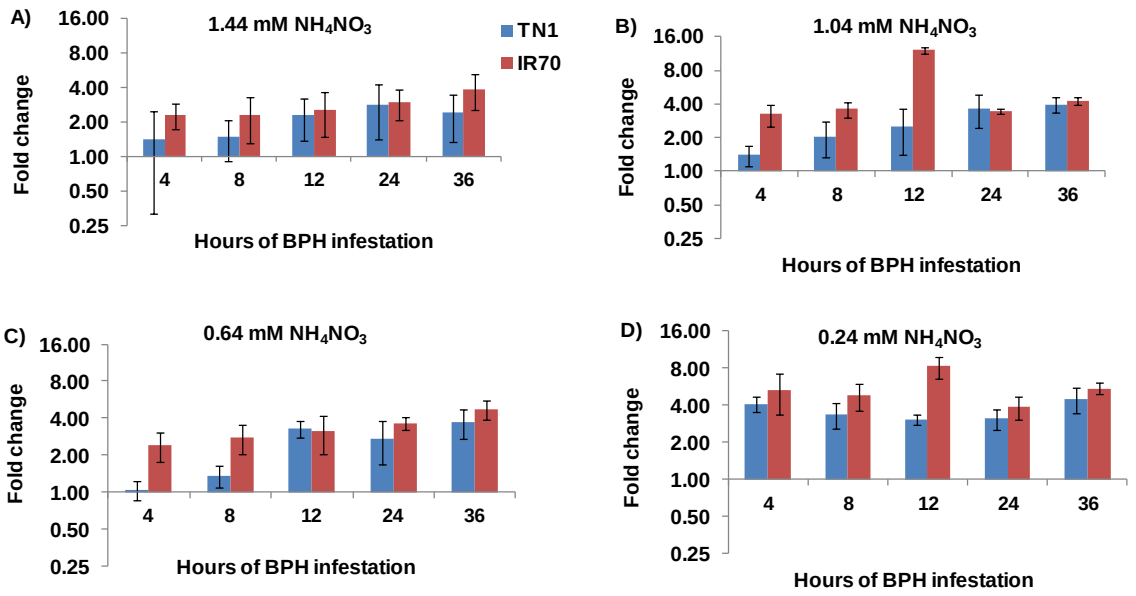

Figure 5.2 Gene expression profiles of *Os03g0860100* in response to different levels of N A) 1.44 mM NH_4NO_3, B) 1.04 mM NH_4NO_3, C) 0.64 mM NH_4NO_3 and D) 0.24 mM NH_4NO_3 and BPH infestation at 4 h, 8 h, 12 h, 24 h, and 36 h in TN1 and IR70. Gene expression levels were normalized to their respective non-infested N condition which was set as 1.0. Data are shown as the log2 value of the fold change in response to the combined stress. Error bars represent ± SD for three biological replicates per time point.

Interestingly the expression of *Os07g0410700* in both TN1 and IR70 increased upon BPH feeding in response to the reduced levels of N but to a greater magnitude in the resistant IR70 (Figure 5.3). The expression of this gene in TN1 remained ~ 1 fold up-regulation at 4 h of BPH infestation under 1.44 mM NH_4NO_3 and 1.04 mM NH_4NO_3. The expression level of *Os03g0860100* in TN1 was then recorded at a 2.1 fold up-regulation, 3.9 fold up-regulation, 2.1 fold up-regulation and 2.6 fold up-regulation respectively at 8 h, 12 h, 24 h and 36 h upon BPH infestation under the 1.44 mM NH_4NO_3. In the resistant IR70, this gene was up-regulated to a ~ 3 fold at most of the time points (4 h, 8 h and 24 h), 4.5 fold (12 h) and to a 6.4 fold (36 h) of BPH infestation under 1.44 mM NH_4NO_3. The susceptible TN1 showed changes in gene expression after 8 h of BPH infestation under the 1.04 mM NH_4NO_3. The highest level of expression under this N level was observed at 12 h and 36 h (5.7 fold up-regulation) of BPH infestation. The expression of *Os07g0410700* increased to a ~ 3 fold up-regulation (4 h and 8 h), 5.2 fold up-regulation (12 h), 7.3 fold (24 h) and 14.4 fold up-regulation (36 h) under 1.04 mM NH_4NO_3 in the resistant IR70.

Os07g0410700 exhibited a high level of expression in IR70 at early response to BPH infestation (4 hours) to a 7.8 fold up-regulation and 15.6 fold up-regulation under 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3 respectively. *Os07g0410700* showed a similar level of expression of a ~ 5 fold up-regulation at 8 h, 12 h and 24 h and the expression level increased to a 8.4 fold up-regulation at 36 h of BPH infestation in IR70 under 0.64 mM NH_4NO_3. This gene also exhibited a ~ 3 fold up-regulation (4 h and 12 h), 2.4 fold up-regulation (24 h) and a ~ 4 fold up-regulation (8 h and 36 h) of BPH infestation in TN1 under this N level.

An increase in gene expression was observed under the lowest N level (0.24 mM NH_4NO_3) with an up-regulation of 11.2 fold, 10.0 fold, 9.5 fold and 12.3 fold at 8 h, 12 h, 24 h and 36 h respectively in the resistant IR70. The expression of *Os07g0410700* in TN1 remained ~ 2 fold up-regulation (4 h and 8 h), ~ 3 fold up-regulation (12 h and 24 h) and increased to a 4.8 fold up-regulation (36 h) under the lowest N level. Overall, the expression profiles of *Os07g0410700* showed a higher level of expression at most time points of BPH infestation under the reduced levels of N in the resistant iR70 compared to the susceptible TN1.

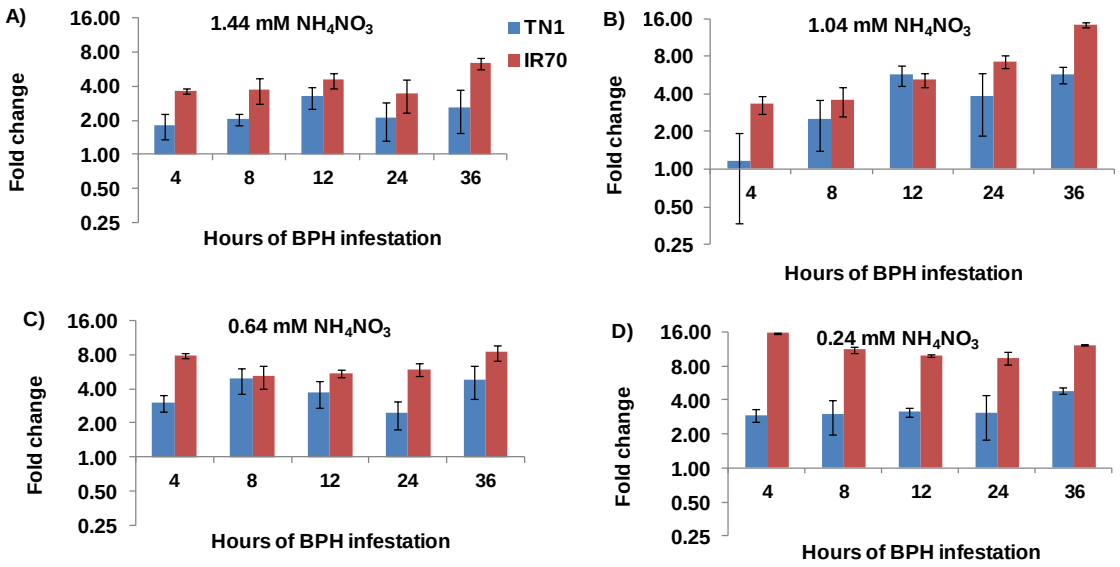

Figure 5.3 Gene expression profiles of *Os07g0410700* in response to different levels of N A) 1.44 mM NH_4NO_3, B) 1.04 mM NH_4NO_3, C) 0.64 mM NH_4NO_3 and D) 0.24 mM NH_4NO_3 and BPH infestation at 4 h, 8 h, 12 h, 24 h and 36 h in TN1 and IR70. Gene expression levels were normalized to their respective non-infested N condition which was set as 1.0. Data are shown as the log2 value of the fold change in response to the combined stress. Error bars represent ± SD for three biological replicates per time point.

The expression profile of *Os08g0157600* revealed that this gene was down-regulated at most of the time points of BPH infestation in IR70 and at fewer time points in the TN1 (Figure 5.4). In IR70, this gene demonstrated a greater magnitude of down-regulation after 8 h of BPH infestation under 1.44 mM NH_4NO_3. In this rice cultivar, *Os08g0157600* was down-regulated to a ~ 5 fold at 8 h and 12 h and to a greater magnitude of ~ 6 fold at 36 h of BPH infestation at the optimal N level. This gene then was down-regulated to a ~ 3 fold (4 h), ~ 6 fold (8 h) and ~ 9 (12 h) in IR70 under 1.04 mM NH_4NO_3. The expression level at 36 hours of BPH infestation under this level was similar as recorded under 1.44 mM NH_4NO_3. A similar pattern of down-regulation with a greater magnitude of expression was observed under the lower N levels (0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3) in the resistant IR70. The expression profile showed that IR70 recorded a 2 fold down-regulation (4 h), ~ 6 fold down-regulation (8 h) and ~ 11 fold down-regulation (12 h) and ~ 9 fold down-regulation (36 h) under 0.64 mM NH_4NO_3. *Os08g0157600* was strongly down-regulated in IR70 at the lowest N level (0.24 mM NH_4NO_3) to a ~ 5 fold (4 h), ~ 9 fold (8 h) and ~ 11 fold (12 h and 36 h) of BPH infestation.

However, in the susceptible TN1, this gene was expressed to less than 2 fold under most N levels at 4 h and 8 h of BPH infestation with an exception of a ~ 2 fold up-regulation at 4 h under 0.64 mM NH_4NO_3. This gene was consistently down-regulated at 12 h and 36 h of BPH infestation under all N levels in the TN1. This gene was down-regulated in TN1 to a greater magnitude of a ~ 6 fold at 12 h under 0.24 mM NH_4NO_3 and to ~ 5 fold under 1.04 mM NH_4NO_3 at 36 h of BPH infestation. An interesting pattern of gene expression was observed at 24 h of BPH infestation whereby this gene was up-regulated in both TN1 and IR70 but to a greater magnitude in the IR70 across all different N levels.

The expression profile of *Os08g0157600* exhibited a 7.4 fold up-regulation in IR70 compared to 1.5 fold up-regulation in the susceptible TN1 under the optimal N level (1.44 mM NH_4NO_3) upon BPH infestation at 24 h. This TF gene then was up-regulated to a 11.1 fold and 10.0 fold under 1.04 mM NH_4NO_3 and 0.64 mM NH_4NO_3 respectively which was 4.9x and 3.8x higher then in TN1 at this time point. The IR70 recorded a 9.2 fold up-regulation compared to 2.3 fold up-regulation in TN1 at 24 h of BPH infestation under the lowest N level. The expression profile of *Os08g0157600* showed that this gene was more responsive to the dual stress in IR70 and was constantly down-regulation to a greater magnitude compared to the susceptible TN1.

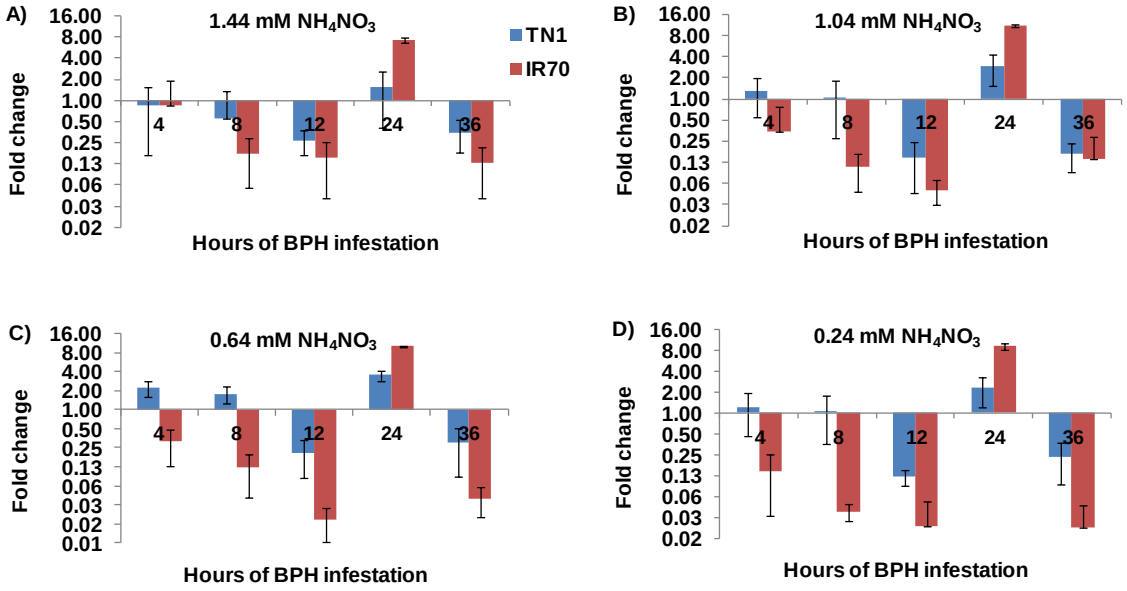

Figure 5.4 Gene expression profiles of *Os08g0157600* in response to different levels of N A) 1.44 mM NH_4NO_3, B) 1.04 mM NH_4NO_3, C) 0.64 mM NH_4NO_3 and D) 0.24 mM NH_4NO_3 and BPH infestation at 4 h, 8 h, 12 h, 24 h and 36 h in TN1 and IR70. Gene expression levels were normalized to their respective non-infested N condition which was set as 1.0. Data are shown as the $\log_2$ value of the fold change in response to the combined stress. Error bars represent ± SD for three biological replicates per time point.

qPCR analysis of *Os06g0728700* (Figure 5.5) showed that the expression of this gene under the optimal N level (1.44 mM NH_4NO_3) in the susceptible TN1 was down-regulated to less than 2 fold at 4 h and 8 h and to a greater extent of a ~ 3 fold down-regulation at 12 h and 36 h of BPH infestation. The resistant IR70 showed a marked down-regulation in gene expression upon BPH infestation to a ~ 4 fold (4 h and 36 h), 2 fold (8 h) and to a ~ 9 fold (12 h) under 1.44 mM NH_4NO_3. The expression of this gene in TN1 and IR70 remained similar ~ 3 fold down-regulation at 4 h of BPH infestation under 1.04 mM NH_4NO_3. This gene was down-regulated to a ~ 4 fold at 8 h and ~ 5 fold at 12 h and 36 h of BPH infestation under 1.04 mM NH_4NO_3 in the resistant IR70. The *Os06g0728700* in TN1 *was* down-regulated to a similar level of expression at 4 h, 8 h and 12 h of BPH infestation and to a greater magnitude (~ 4 fold down-regulation) at 36 h of BPH infestation under this N level.

The *Os06g0728700* was down-regulated to a ~ 7 fold (12 h) and ~ 5 fold (24 h) under 0.64 mM NH_4NO_3 in IR70 compared to a ~ 5 fold and ~ 4 fold respectively in TN1 at both the time points. Both TN1 and IR70 exhibited a similar level of expression of a ~ 2 fold down-regulation and ~ 3 fold down-regulation respectively at an early stage of BPH infestation (4 h and 8 h) under 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3. The

expression profile indicated that this gene was strongly down-regulated to a ~ 8 fold at 12 h and 36 h of BPH infestation in the resistant IR70 and to a lesser magnitude (~ 4 fold down-regulation) at these time points in the susceptible TN1 under 0.24 mM NH_4NO_3.

The *Os06g0728700* also demonstrated a similar pattern of expression as *Os08g0157600* at 24 h of BPH infestation whereby this gene was up-regulated at all N levels upon BPH infestation in both TN1 and IR70. This TF was up-regulated to a ~2 fold under 1.44 mM NH_4NO_3, 1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3 at 24 h of BPH infestation in TN1. However, this gene showed a higher level of expression in IR70 under these N levels compared to TN1 at 24 h of BPH infestation. The expression profile demonstrated that this gene was up-regulated to a 6.1 fold, 9.2 fold, 5.5 fold and 6.5 fold in IR70 under 1.44 mM NH_4NO_3, 1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3 respectively. Overall, this gene was down-regulated to a greater magnitude in the resistant IR70 than the susceptible TN1 at 4 h, 8 h, 12 h and 36 h of BPH infestation.

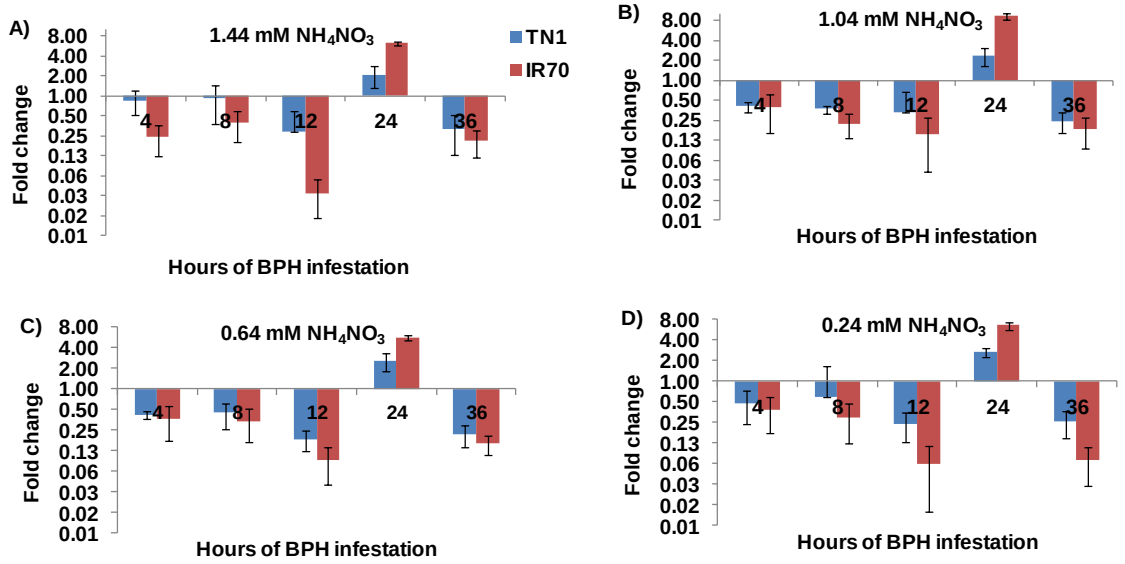

Figure 5.5 Gene expression profiles of *Os06g0728700* in response to different levels of N A) 1.44 mM NH_4NO_3, B) 1.04 mM NH_4NO_3, C) 0.64 mM NH_4NO_3 and D) 0.24 mM NH_4NO_3 and BPH infestation at 4 h, 8 h, 12 h, 24 h and 36 h in TN1 and IR70. Gene expression levels were normalized to their respective non-infested N condition which was set as 1.0. Data are shown as the log2 value of the fold change in response to the combined stress. Error bars represent ± SD for three biological replicates per time point.

The gene expression of *Os01g0108400* (Figure 5.6) in the resistant IR70 was up-regulated to a ~ 3 fold (4 h, 8 h and 12 h), 5.3 fold (24 h) and 6.4 fold (36 h) under the optimal N level (1.44 mM NH_4NO_3) upon BPH infestation. However, the expression of this gene in TN1 under optimal N level remained unchanged (~1 fold up-regulation) at all time points of BPH infestation and was up-regulated to a ~ 2 fold at all time points of BPH infestation under 1.04 mM NH_4NO_3. In IR70, *Os01g0108400* was up-regulated to a 5.7 fold (4 h), 6.6 fold (8 h), 11.2 fold (12 h) and 9.1 fold (24 h and 36 h) upon BPH infestation under 1.04 mM NH_4NO_3. This gene exhibited a similar level of expression as 1.04 mM NH_4NO_3 under 0.64 mM NH_4NO_3 in TN1 which recorded a ~ 3.0 fold (4 h, 8 h, 12 h and 24 h) and ~ 2 fold (36 h).

In contrast, the expression profile of *Os01g0108400* in IR70 demonstrated that this gene was up-regulated to a ~ 4 fold (4 h and 8 h), 8.7 fold (12 h), 6.5 fold (24 h) and 8.1 fold (36 h) upon BPH infestation under 0.64 mM NH_4NO_3. The highest level of gene expression in *Os01g0108400* was recorded at a 13.8 fold up-regulation (12 h) and 12.2 fold up-regulation (36 h) under the lowest N level (0.24 mM NH_4NO_3) in the resistant IR70. However, the susceptible TN1 demonstrated a similar level of gene expression (~ 3 fold up-regulation) under the lowest N at different time points of BPH infestation. The expression profile of the *Os01g0108400* indicated that this gene showed an early response to BPH infestation in the resistant IR70 and to a lesser extent in the susceptible TN1 under the reduced N levels. Interestingly, the IR70 demonstrated the highest level of expression under these N levels at 12 h of BPH infestation. Moreover, this gene also exhibited a higher level of expression in IR70 compared to the TN1 under all N levels at all time points of BPH infestation.

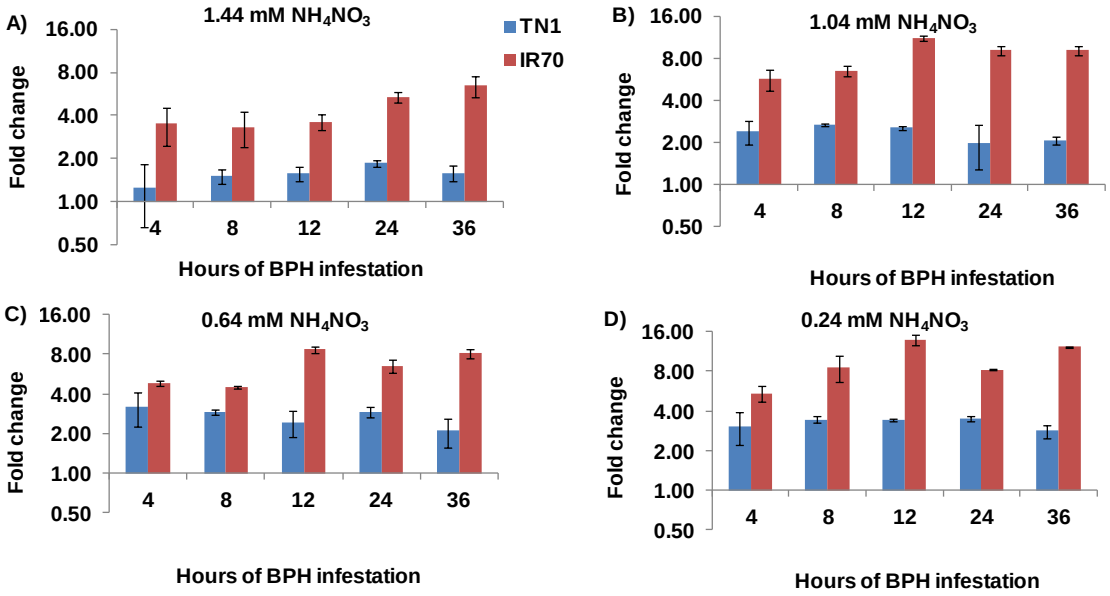

Figure 5.6 Gene expression profiles of *Os01g0108400* in response to different levels of N A) 1.44 mM NH_4NO_3, B) 1.04 mM NH_4NO_3, C) 0.64 mM NH_4NO_3 and D) 0.24 mM NH_4NO_3 and BPH infestation at 4 h, 8 h, 12 h, 24 h and 36 h in TN1 and IR70. Gene expression levels were normalized to their respective non-infested N condition which was set as 1.0. Data are shown as the log2 value of the fold change in response to the combined stress. Error bars represent ± SD for three biological replicates per time point.

The *Os02g0214500* showed an up-regulation at the early stage BPH infestation under the optimal N level (4 h and 8 h) and 1.04 mM NH_4NO_3 (4 h) in the resistant IR70 (Figure 5.7). However, the expression of *Os02g0214500* was down-regulated at the later stage of BPH infestation (12 h, 24 h and 36 h) in this rice cultivar under this optimal N level and was constantly down-regulated at most time points under 1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3. *Os02g0214500* was down-regulated to a ~2 fold (12 h and 36 h) and ~ 4 fold (24 h) in IR70 under 1.44 mM NH_4NO_3. The expression profile showed that this gene was up-regulated to a higher level of expression (3.1 fold) in TN1 compared to IR70 (2.6 fold) under 1.04 mM NH_4NO_3 at 4 h of BPH infestation.

This gene was strongly down-regulated at 24 h and 36 h of BPH infestation under the reduced levels of N in the resistant IR70. The IR70 showed down-regulation of ~ 3 fold at 8 h, 12 h and 24 h and ~ 6 fold at 36 h of BPH infestation under 1.04 mM NH_4NO_3. The *Os02g0214500* gene was down-regulated to a ~ 5 fold (8 h and 36 h), ~ 4 fold (12 h) and ~ 7 fold (24 h) respectively under 0.64 mM NH_4NO_3 in this rice cultivar. In addition, this gene was down-regulated to a ~ 3 fold (4 h and 8 h) and ~ 6 fold (12 h, 24 h and 36 h) of BPH infestation under lowest N level (0.24 mM NH_4NO_3) in IR70.

In contrast to the expression of *Os02g0214500* in IR70, this gene was up-regulated in TN1 across all different N levels at different time points of BPH infestation. The expression level of *Os02g0214500* in TN1 decreased from 4.5 fold up-regulation at 4 h to ~ 3 fold up-regulation at 8 h and 12 h and to ~ 2 fold up-regulation at 24 h and 36 h of BPH infestation under 1.44 mM NH_4NO_3. However, this gene was up-regulated to a higher level at 2.6 fold (4 h), 4.9 fold (8 h), 3.9 fold (12 h), 5.7 fold (24 h) and 4.8 fold (36 h) respectively under 1.04 mM NH_4NO_3 in this rice cultivar. A similar pattern of expression in TN1 was also observed under 0.64 mM NH_4NO_3 with the highest level of expression at 36 h of BPH infestation (4.8 fold up-regulation). The susceptible TN1 recorded the highest level of up-regulation at most times of BPH infestation under lowest N level (0.24 mM NH_4NO_3) which was recorded at a 4.3 fold (4 h), 5.7 (8 h), 4.5 fold (12 h), 3.6 fold (24 h) and 5.8 fold (36 h) upon BPH infestation.

The expression level of this gene in TN1 increased at some time points of BPH infestation under the reduced levels of N compared to the optimal N level. However, this rice cultivar did not exhibit drastic changes in the expression levels in response to the reduced levels of N and BPH infestation. Therefore, it was interesting to observe that *Os02g0214500* was down-regulated in IR70 whilst up-regulated in TN1 in this dual stress.

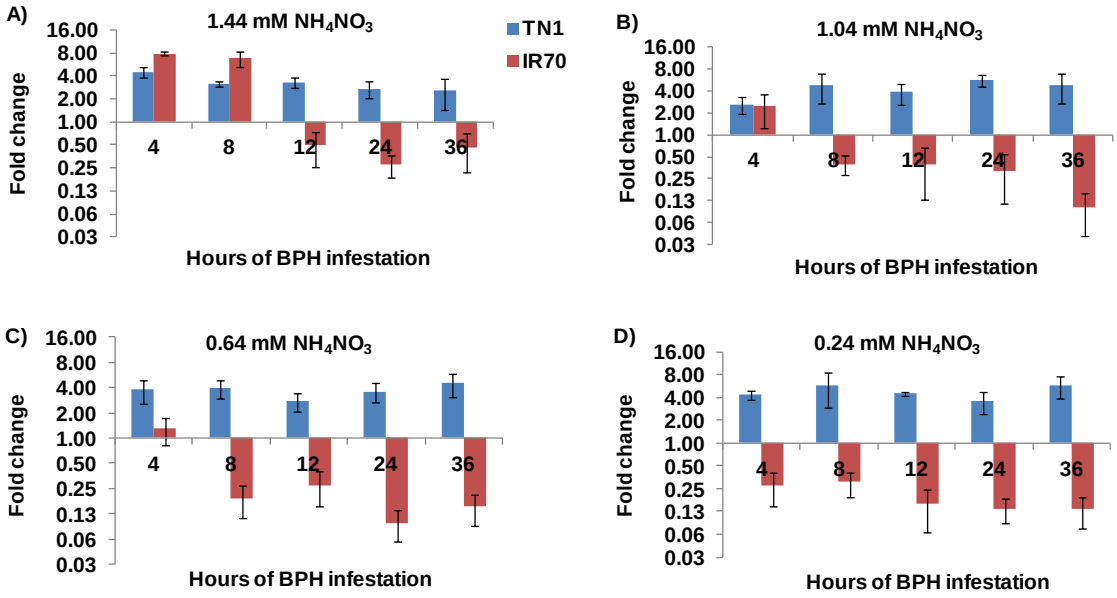

Figure 5.7 Gene expression profiles of *Os02g0214500* in response to different levels of N A) 1.44 mM NH_4NO_3, B) 1.04 mM NH_4NO_3, C) 0.64 mM NH_4NO_3 and D) 0.24 mM NH_4NO_3 and BPH infestation at 4 h, 8 h, 12 h, 24 h, and 36 h in TN1 and IR70. Gene expression levels were normalized to their respective non-infested N condition which was set as 1.0. Data are shown as the $\log_2$ value of the fold change in response to the combined stress. Error bars represent ± SD for three biological replicates per time point.

The *Os06g0298200* gene which belongs to the C2C2-CO like family (Figure 5.8) was up-regulated in both TN1 and IR70 under different N levels at most time points of BPH infestation. This gene was constantly down-regulated at two time points, 12 h and 24 h of BPH infestation in TN1 under 1.44 mM NH_4NO_3, 1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3. The gene expression profile showed that this gene was strongly down-regulated to a ~ 4 fold and a ~ 3 fold under 1.44 mM NH_4NO_3 at 12 h and 24 h of BPH infestation in the susceptible TN1. However, the expression of this gene remained at a ~ 3 fold down-regulation at 12 h of BPH infestation under the reduced levels of N in this rice cultivar. At 24 hours of BPH infestation, *Os06g0298200* was down-regulated to less than 2 fold under 1.04 mM NH_4NO_3 and 0.24 mM NH_4NO_3 respectively and to a ~ 2 fold down-regulation under 0.64 mM NH_4NO_3 in the susceptible TN1.

The expression profile of *Os06g0298200 showed* that this gene was up-regulated to a 3.8 fold (4 h), 9.3 fold (8 h), 10.7 fold (12 h), 7.5 fold (24 h) and 15.3 fold (36 h) in IR70 under the optimal N level (1.44 mM NH_4NO_3) upon BPH infestation. This gene was also expressed to a similar level of expression (~ 2 fold up-regulation) at 4 hours of BPH infestation in TN1 and IR70 under 1.04 mM NH_4NO_3. A drastic increase in expression was observed in IR70 under 1.04 mM NH_4NO_3 whereby this gene was up-regulated 2.4x (16.6 fold) and 2.7x (39.0 fold) higher compared to TN1 at 8 hours and 36 hours of BPH infestation. This expression pattern was repeatedly observed under 0.64 mM NH_4NO_3 whereby IR70 demonstrated a higher level of expression at 8 h (22.9 fold) and 36 h (44.4 fold) of BPH infestation compared to 9.5 fold (8 h) and 11.1 (36 h) in TN1.

The resistant IR70 showed a decreased in expression level at some points of BPH infestation under 0.24 mM NH_4NO_3 compared to 1.04 mM NH_4NO_3 and 0.64 mM NH_4NO_3. This expression of *Os06g0298200* in IR70 showed that this gene was up-regulated to a 3.2 fold (4 h), 12.8 (8 h) and a marked increase to 31.6 fold (12 h) upon BPH infestation under lowest N level. However, the expression then decreased to a 16.9 fold (24 h) and 23.7 fold (36 h) upon BPH infestation. Overall the expression profile of *Os06g029200* showed that this gene is more responsive to the combined reduced levels of N and BPH infestation in IR70. The expression profile also showed that this gene was down-regulation in TN1 particularly at 12 h and 24 h which was an interesting pattern of gene expression and was only observed in *Os06g0298200*.

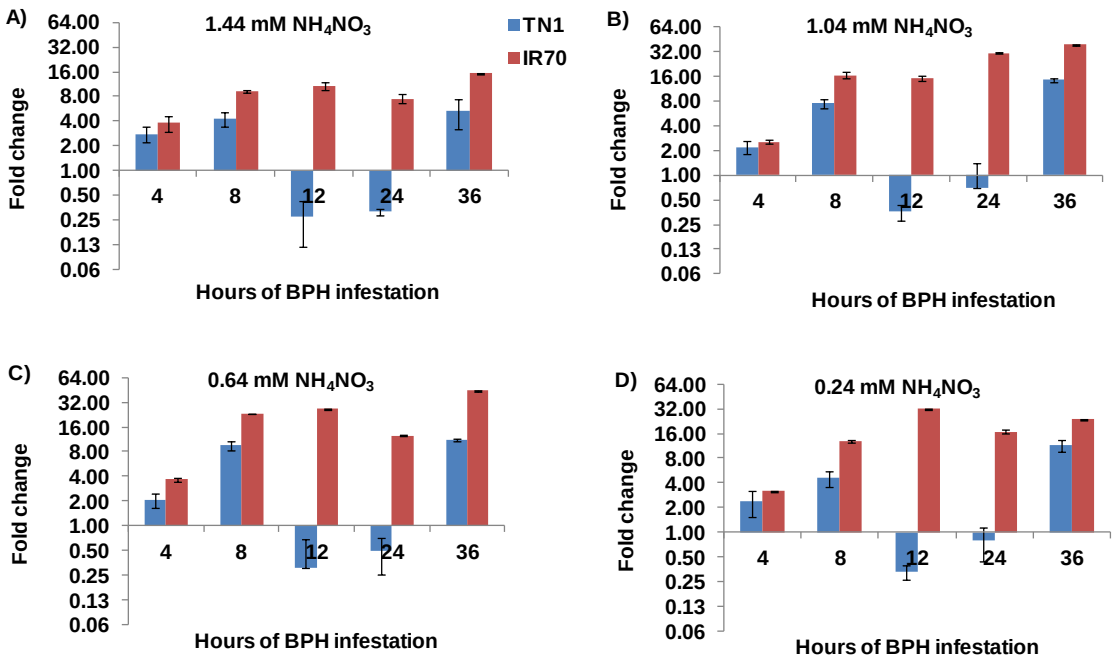

Figure 5.8 Gene expression profiles of *Os06g0298200* in response to different levels of N A) 1.44 mM NH_4NO_3, B) 1.04 mM NH_4NO_3, C) 0.64 mM NH_4NO_3 and D) 0.24 mM NH_4NO_3 and BPH infestation at 4 h, 8 h, 12 h, 24 h and 36 h in TN1 and IR70. Gene expression levels were normalized to their respective non-infested N condition which was set as 1.0. Data are shown as the log2 value of the fold change in response to the combined stress. Error bars represent ± SD for three biological replicates per time point.

The *Os09g0240200* was up-regulated to a 7.3 fold in the susceptible TN1 and to a lesser magnitude (4.2 fold) in the resistant IR70 at the early stage of BPH infestation (4 hours) under the optimal N level (Figure 5.9). However, a drastic decrease in the expression level of both the rice cultivars was observed after 8 hours of BPH infestation at 1.44 mM NH_4NO_3 and 1.04 mM NH_4NO_3. Expression of this gene in TN1 remained at ~ 1.1 fold up-regulation at 8 h and was down-regulated to less than ~ 2 fold down-regulation at 12 h, 24 h and 36 h of BPH infestation under 1.44 mM NH_4NO_3. In contrast, this gene was down-regulated to a ~ 2 fold (8 h), ~ 3 fold (12 h and 24 h) and ~ 4 fold (36 h) upon BPH infestation under the optimal N level in IR70.

A similar pattern of gene expression as the optimal N level was also observed under 1.04 mM NH_4NO_3 in both TN1 and IR70. This gene was down-regulated to a ~ 3 fold at 8 h, 12 h and 24 h and to ~ 4 fold at 36 h of BPH infestation in IR70 under this N level. However, this gene was expressed to a greater magnitude in TN1 under 0.64 mM NH_4NO_3 after 4 h of BPH infestation. This gene was down-regulated to a ~ 7 fold at 8 h, 12 h and 24 h and to ~ 6 fold at 36 h of BPH infestation in this rice cultivar. The expression profile of *Os09g0240200* showed that this gene was down-regulated

to a greater level under 0.64 mM NH_4NO_3 in IR70 compared to the expression under the optimal N level and 1.04 mM NH_4NO_3 upon BPH infestation. This gene was down-regulated to a ~ 4 fold (4 h), ~ 8 fold (8 h), ~ 10 fold (12 h) and ~ 7 fold (24 h and 36 h) in IR70 under this N level.

Os09g0240200 exhibited down-regulation in both IR70 and TN1 under the lowest N level (0.24 mM NH_4NO_3) at all time points of BPH infestation but to a greater magnitude in the resistant IR70. This gene was down-regulated from a ~ 4 fold at 4 h to ~ 7 fold (8 h and 12 h), ~ 8 fold (24 h) and ~ 9 fold at 36 h of BPH infestation in IR70. The TN1 was down-regulated to a ~ 7 fold at 8 h and ~ 6 fold at 12 h, 24 h and 36 h of BPH infestation. The expression of *Os09g0240200* showed that this gene was constantly down-regulated in IR70 upon BPH infestation and was expressed to a greater magnitude under 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3. The susceptible TN1 was also down-regulated upon BPH infestation under these N deficiency levels but to a lesser magnitude than the resistant IR70. This TF showed correlating gene expression profiles whereby both the rice cultivars showed to be responsive to the combined stress especially under 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3.

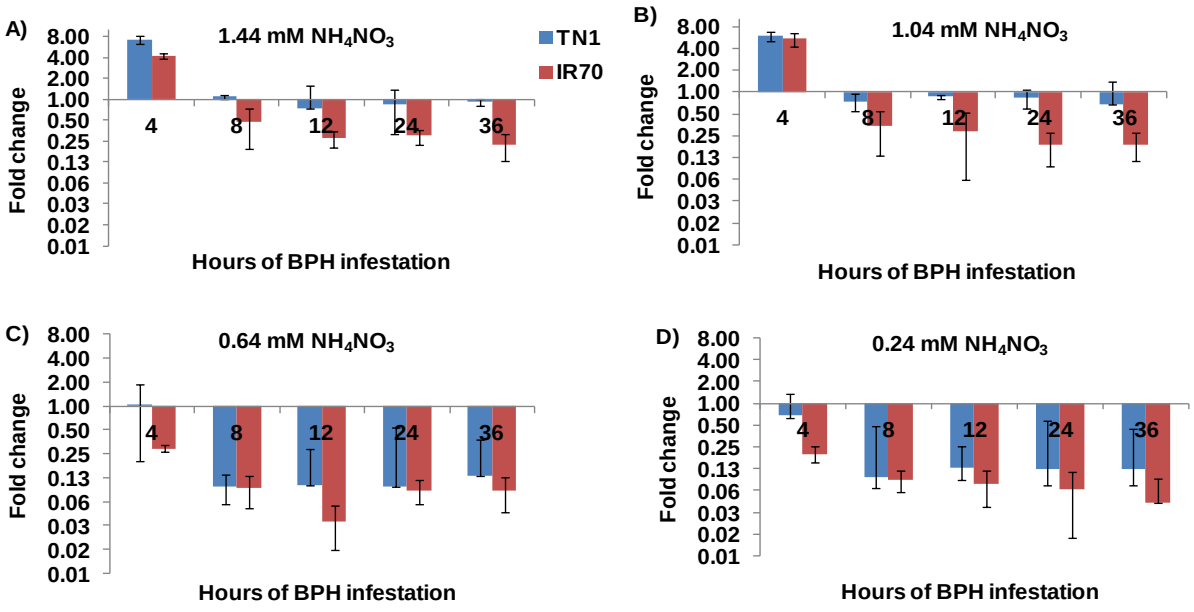

Figure 5.9 Gene expression profiles of *Os09g0240200* in response to different levels of N A) 1.44 mM NH_4NO_3, B) 1.04 mM NH_4NO_3, C) 0.64 mM NH_4NO_3 and D) 0.24 mM NH_4NO_3 and BPH infestation at 4 h, 8 h, 12 h, 24 h and 36 h in TN1 and IR70. Gene expression levels were normalized to their respective non-infested N condition which was set as 1.0. Data are shown as the log2 value of the fold change in response to the combined stress. Error bars represent ± SD for three biological replicates per time point.

The *Os09g0439200* (Figure 5.10) was not differentially expressed in the susceptible TN1 under all different N levels upon BPH infestation. In IR70, this gene was down-regulated under all N levels at different time points of BPH infestation. The expression of this gene in IR70 showed down-regulation of a ~ 2 fold at most time points (4 h, 12 h, 24 h and 36 h) and ~ 3 fold (8 h) of BPH infestation at the optimal N level (1.44 mM NH_4NO_3). The *Os09g0439200* was down-regulated to a ~ 3 fold (4 h), ~ 6 fold (8 h) and ~ 5 fold (12 h) and decreased to a ~ 4 fold (24 h and 36 h) in IR70 under 1.04 mM NH_4NO_3.

A similar pattern of down-regulation was also observed under 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3. The expression level of this gene at 4 h of BPH infestation remains at the similar level as 1.04 mM NH_4NO_3 (~ 3 fold down-regulation) under both these levels. This gene was down-regulated to a ~ 4 fold (8 h, 12 h and 24 h) and ~ 6 fold (36 h) under 0.64 mM NH_4NO_3. The expression of *Os09g0439200* in IR70 demonstrated a greater magnitude of down-regulation to a ~ 7 fold down-regulation at 8 h and 36 ho of BPH infestation under the lowest N level (0.24 mM NH_4NO_3). The expression level at 12 h and 24 h of BPH infestation were recorded to a ~ 5 fold down-regulation and ~ 6 fold down-regulation respectively under this N level. Therefore, expression profiles of *Os09g0439200* showed that this gene is responsive to the reduced levels of N and BPH infestation in the resistant IR70.

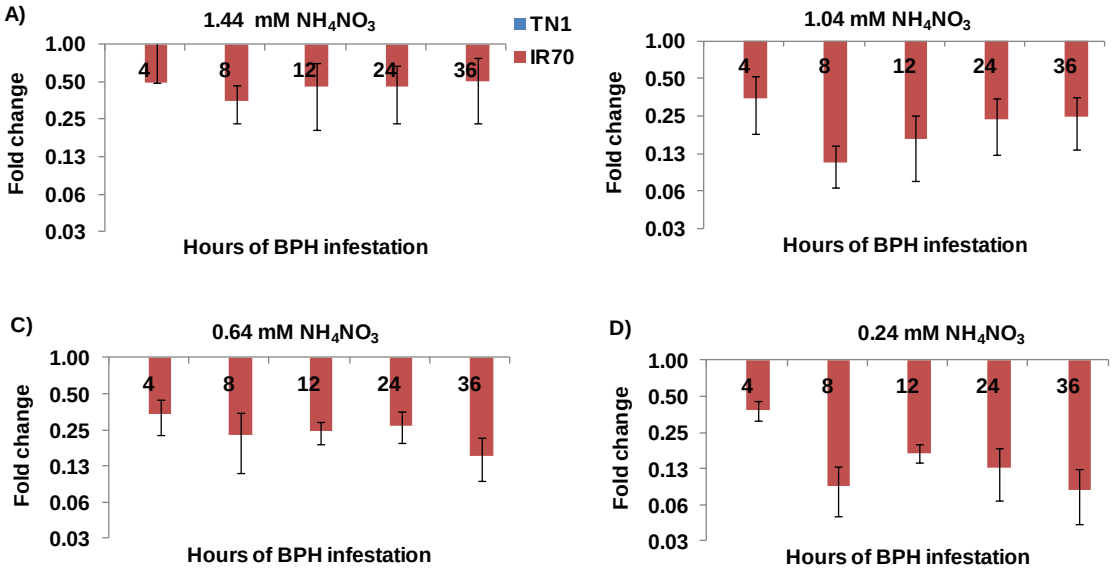

Figure 5.10 Gene expression profiles of *Os09g0439200* in response to different levels of N A) 1.44 mM NH_4NO_3, B) 1.04 mM NH_4NO_3, C) 0.64 mM NH_4NO_3 and D) 0.24 mM NH_4NO_3 and BPH infestation at 4 h, 8 h, 12 h, 24 h and 36 h in TN1 and IR70. Gene expression levels were normalized to their respective non-infested N condition which was set as 1.0. Data are shown as the log2 value of the fold change in response to the combined stress. Error bars represent ± SD for three biological replicates per time point.

The expression profile of *Os03g0180800* showed that this gene was up-regulated to a 2.5 fold (4 h) and 3.1 fold (8 h) in TN1 compared to a 1.3 fold (4 h) and 2.9 fold (8 h) respectively in IR70 under 1.44 mM NH_4NO_3 (Figure 5.11). However, this gene was down-regulated to ~ 3 fold in IR70 at 12 h of BPH infestation. At 24 h and 36 h of BPH infestation, *Os03g0180800* showed a drastic up-regulation of a 12.3 fold and 15.5 fold in IR70 compared to a 5.0 fold and 3.7 fold in TN1 under this N level. In IR70, this gene exhibited more down-regulation at 4 h, 8 h and 12 h of BPH infestation under the reduced levels of N compared to the optimal N level. The expression profile showed that this gene was expressed to less than 2 fold down-regulation in IR70 at 4 h of BPH infestation under the reduced levels of N.

Os03g0180800 was down-regulated to a ~ 2 fold at 8 h and ~ 3 fold at 12 h of BPH infestation in IR70 under 1.04 mM NH_4NO_3. Prolonged BPH infestation at 24 h and 36 h showed that this gene was highly expressed in the IR70 at both the time points with 11.3 fold up-regulation and 9.5 fold up-regulation respectively. The susceptible TN1 also demonstrated the highest level of expression at a 8.2 fold up-regulation (24 h) and 7.4 fold up-regulation (36 h) under the 1.04 mM NH_4NO_3. The TN1 exhibited similar level of expression (~ 3 fold up-regulation) at 4 h and 8 h and the expression

level increased to a 5.0 fold up-regulation at 12 h of BPH infestation under this N level.

In IR70, the *Os03g0180800* was down-regulated to a ~ 3 fold at 8 h and ~ 4 fold at 12 h of BPH infestation under 0.64 mM NH_4NO_3 whilst showed an increased in the expression level (~ 4 fold up-regulation) in TN1 at these time points. In addition, TN1 exhibited a similar level of expression (~ 5 fold up-regulation) at 24 h and 36 h of BPH infestation compared to a 6.3 fold up-regulation and 5.0 fold up-regulation in the resistant IR70 at these time points respectively under 0.64 mM NH_4NO_3. The expression of this gene in the resistant IR70 was down-regulated to a greater magnitude at 8 h (~ 6 fold down-regulation) and 12 h (~ 7 fold down-regulation) of BPH infestation under the lowest N level (0.24 mM NH_4NO_3). Prolonged BPH infestation (24 h and 36 h) showed that this gene was expressed to a similar level of expression (~ 2.0 fold up-regulation) in TN1 whilst showed an increase of ~ 5 fold up-regulation in IR70 under this N level. The *Os03g0180800* also showed a decrease in expression level under 0.24 mM NH_4NO_3 compared to the expression inder 0.64 mM NH_4NO_3 in TN1 whereby this gene was expressed to a ~ 2 fold up-regulation across different time point of BPH infestation under this lowest N level.

Os03g0180800 was up-regulated to its highest level in the IR70 under the optimal N level (1.44 mM NH_4NO_3) showed a decrease in expression level under the lowest N availability at some time points (24 h and 36 h). The expression of this gene in IR70 showed drastic down-regulation only under the lowest level of N at 8 hours and 12 hours of BPH infestation. Decreased expression levels were also observed in the susceptible TN1 under the lowest level of N compared to the other level of N in response to the combined stress.

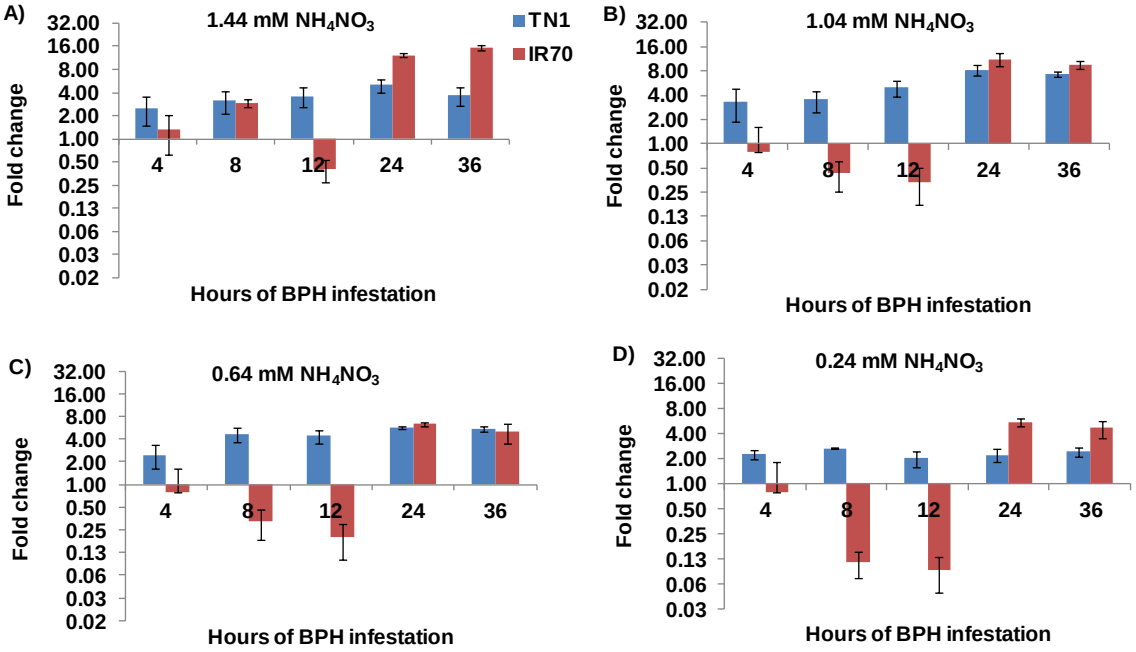

Figure 5.11 Gene expression profiles of *Os03g0180800* in response to different levels of N A) 1.44 mM NH_4NO_3, B) 1.04 mM NH_4NO_3, C) 0.64 mM NH_4NO_3 and D) 0.24 mM NH_4NO_3 and BPH infestation at 4 h, 8 h, 12 h, 24 h and 36 h in TN1 and IR70. Gene expression levels were normalized to their respective non-infested N condition which was set as 1.0. Data are shown as the log2 value of the fold change in response to the combined stress. Error bars represent ± SD for three biological replicates per time point.

The expression profile of *Os03g0437200* showed that this gene was up-regulated to a 6.8 fold in IR70 compared to a 2.0 fold in TN1 at the early stage of BPH infestation (4 h) (Figure 5.12) under 1.44 mM NH_4NO_3. The expression level of *Os03g0437200* in IR70 showed a decrease after 8 h of BPH infestation under this N level. The susceptible TN1, however, exhibited a higher level of expression at a 3.9 fold up-regulation (24 h) and 4.9 fold up-regulation (36 h) compared to a ~ 2 fold up-regulation in the resistant IR70 at both the time points. Interestingly this gene was down-regulated under all N levels at 12 h of BPH infestation in both the TN1 and IR70. The expression profile indicated that this gene was down-regulated to ~ 6 fold (1.44 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3) and ~ 5 fold (1.04 mM NH_4NO_3) in IR70 and to a lesser extent in TN1 at 12 h of BPH infestation.

In addition, *Os03g0437200* exhibited more down-regulation in IR70 at most time points of BPH infestation in response to the reduced N levels compared to TN1. The expression profile indicated that this gene was up-regulated to a 2.9 fold, 3.3 fold, 7.3 fold and 7.7 fold at 4 h, 8 h, 24 h and 36 h respectively in TN1 under 1.04 mM NH_4NO_3. Conversely, *Os03g0437200* was down-regulated to a ~ 2 fold (8 h), ~ 3 fold

(24 h) and ~ 5 fold (36 h) in IR70. Interestingly a similar pattern of gene expression as 1.04 mM NH_4NO_3 was also observed under 0.64 mM NH_4NO_3 in TN1 and IR70. At 12 h of BPH infestation, this gene was down-regulated to a ~ 4 fold (1.44 mM NH_4NO_3) and the expression of this gene decreased to a ~ 3 fold down-regulation (1.04 mM NH_4NO_3), ~ 2 fold down-regulation (0.64 mM NH_4NO_3) and < 2 fold down-regulation (0.24 mM NH_4NO_3) in the TN1. The expression profile demonstrated that this gene was down-regulated to a ~ 3 fold at 8 h and ~ 4 fold at 24 h and 36 h of BPH infestation respectively in IR70 under 0.64 mM NH_4NO_3.

The gene expression profile at the lowest N level (0.24 mM NH_4NO_3) demonstrated that this gene was down-regulated in IR70 at all time points of BPH infestation. This gene was down-regulated to a ~ 2 fold (4 h), ~ 5 fold (8 h) and ~ 6 fold (12 h, 24 h and 36 h) in IR70 under this N level. The expression of this gene in TN1 remained ~ 2 fold up-regulation at 4 h and 8 h of BPH infestation and increased to ~ 5 fold up-regulation at 24 h and 36 h of BPH infestation under 0.64 mM NH_4NO_3. However, *Os03g0437200* showed a decrease in expression level under the lowest N level in TN1 at all time points of BPH infestation as compared to the expression under all other N levels. The expression of this gene under the lowest N level in TN1 was recorded at < then 2 fold up-regulation (4 h), ~ 2 fold up-regulation (8 h and 24 h) and ~3 fold up-regulation (36 h).

Overall, *Os03g0437200* was down-regulated at most time points of BPH infestation (8 h, 12 h, 24 h and 36 h) under the reduced levels of N in the IR70. On the other hand, this gene was up-regulated at most time point upon BPH infestation except at 12 hours of BPH infestation in the susceptible TN1. The expression profile of *Os03g0437200* showed that this gene is more responsive to the combined stress in the resistant IR70 compared to the susceptible TN1.

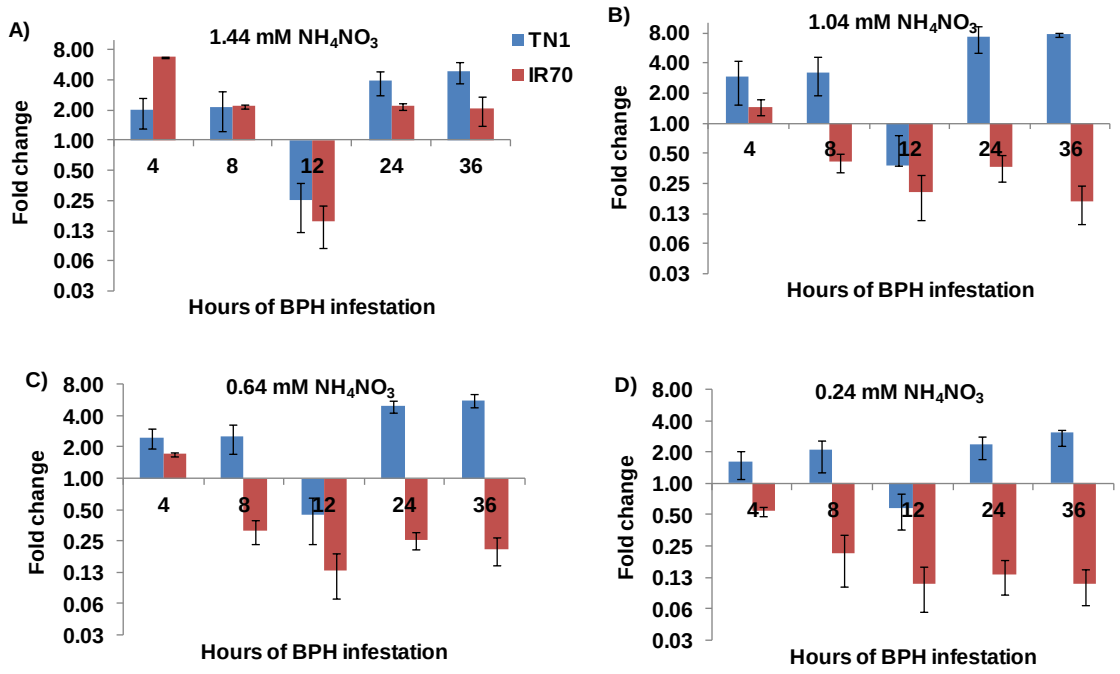

Figure 5.12 Gene expression profiles of *Os03g0437200* in response to different levels of N A) 1.44 mM NH_4NO_3, B) 1.04 mM NH_4NO_3, C) 0.64 mM NH_4NO_3 and D) 0.24 mM NH_4NO_3 and BPH infestation at 4 h, 8 h, 12 h, 24 h and 36 h in TN1 and iR70. Gene expression levels were normalized to their respective non-infested N condition which was set as 1.0. Data are shown as the log2 value of the fold change in response to the combined stress. Error bars represent ± SD for three biological replicates per time point.

Among the other TFs analyzed, the *Os01g0971800* was up-regulated to a higher level in the IR70 compared to the susceptible TN1 in response to the reduced levels of N and BPH infestation. This gene expression profile indicated that *Os01g0971800* was up-regulated to a 5.3 fold, 7.9 fold, 9.2 fold, 9.8 fold and 11.3 fold respectively in IR70 at all time points of BPH infestation under the 1.44 mM NH_4NO_3. The expression of *Os01g0971800* in TN1 also increased upon BPH infestation to a ~ 2 fold up-regulation at 4 h, 8 h and 12 h, 3.0 fold up-regulation at 24 h and 5.1 fold up-regulation at 36 h under the optimal N level.

Os01g0971800 exhibited a higher level of expression at 4 h, 8 h and 24 h (~ 10.0 fold up-regulation) followed by a marked increase in the expression at 12 h (18.4 fold up-regulation) and 36 h (25.8 fold up-regulation) in the resistant IR70 under 1.04 mM NH_4NO_3. This TF gene also demonstrated a high level of expression in TN1 at 12 h and 36 h but to a lesser magnitude than the resistant IR70. The expression of *Os01g0971800* in TN1 remained ~ 2 fold up-regulation (4 h, 8 h and 24 h) and increased to a 6.2 fold (12 h) and 4.6 fold (36 h) upon BPH infestation. This gene was constantly up-regulated to a greater magnitude in IR70 under 0.64 mM NH_4NO_3

upon BPH infestation. The gene expression profile indicated that this gene was up-regulated to a 12.7 fold (4 h), 14.1 fold (8 h), 14.5 fold (12 h), 16.3 fold (24 h) and 20.3 fold (36 h) in IR70 compared to 2.0 (4 h), 2.3 (8 h), 2.5 (12 h), 1.5 fold (24 h) and 1.9 fold (36 h) in TN1.

Under the lowest N level (0.24 mM NH_4NO_3), *Os01g0971800* was expressed 7x higher in IR70 upon BPH infestation at 4 h (16.6 fold up-regulation) and 8 h (16.0 fold up-regulation) compared to TN1. However, the expression level was reduced to a 8.8 fold up-regulation (12 h), 6.9 fold up-regulation (24 h) and increased to 12.5 fold up-regulation at 36 h of BPH infestation in IR70. The expression level of *Os01g0971800* in TN1 remained ~ 2 fold up-regulation at most time points of BPH infestation under this lowest N level. This gene exhibited a similar level of gene expression in the optimal N level and the reduced levels of N in TN1 at most time points of BPH infestation. The expression profile indicated that *Os01g0971800 showed* a lower level of expression under the reduced levels of N (0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3) in TN1. Therefore, the expression profile of *Os01g0971800* clearly showed that this gene was more responsive to the combination of the reduced N levels and BPH infestation in the resistant IR70 compared to the susceptible TN1.

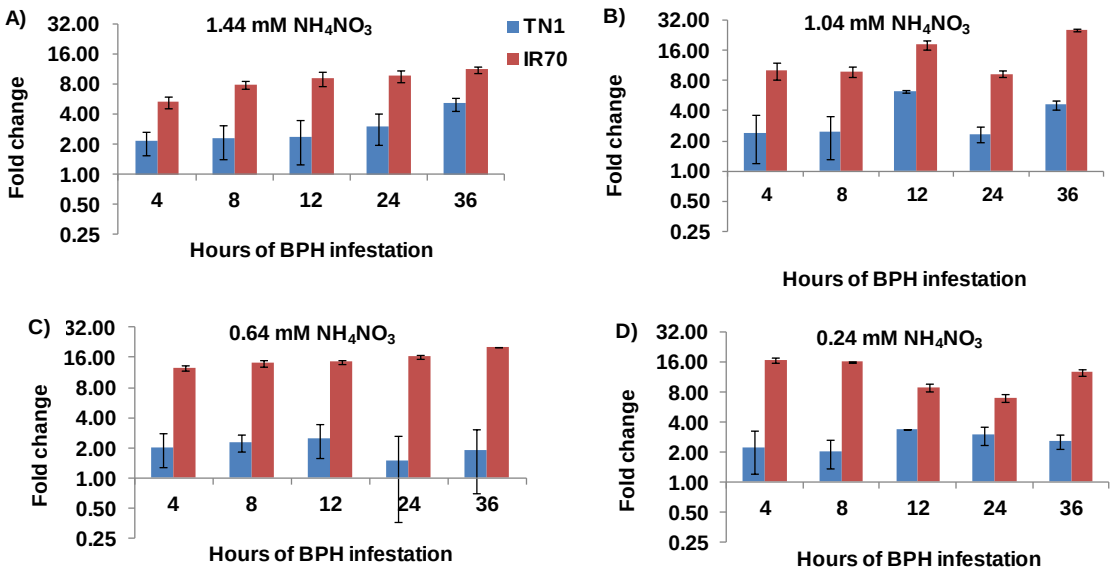

Figure 5.13 Gene expression profiles of *Os01g0971800* in response to different levels of N A) 1.44 mM NH_4NO_3, B) 1.04 mM NH_4NO_3, C) 0.64 mM NH_4NO_3 and D) 0.24 mM NH_4NO_3 and BPH infestation at 4 h, 8 h, 12 h, 24 h and 36 h in TN1 and IR70. Gene expression levels were normalized to their respective non-infested N condition which was set as 1.0. Data are shown as the $\log_2$ value of the fold change in response to the combined stress. Error bars represent ± SD for three biological replicates per time point.

5.5 Discussion

5.5.1 *Validation of endogenous gene, Actin in response to the dual stress*

Despite being an efficient technique, qPCR still has some technical shortfalls that can affect analysis such as the integrity of RNA samples, qPCR efficiency and the amount of cDNA used in the reaction. All of these technical issues could be compensated by normalizing the expression of the gene of interest against a stable expressed endogenous control. The stable endogenous gene demonstrates steady and constitutive expression at different growth stage and environmental conditions (Kundu *et al*., 2013). In the present study, qPCR was conducted to evaluate the stability of endogenous gene, *Actin* in response to the combined N deficiency stress and BPH infestation. Results of the analysis demonstrated that there were no significant differences in the mean of C_T value between the non-infested and infested rice cultivars at different levels of N. Therefore, *Actin* is considered to be a stable and reliable endogenous gene for accurate interpretation of the data which generated consistent results in response to the combined stress.

As described in 4.5.1 of Chapter 4, this present study did not carry out a comparison study with other endogenous genes as *Actin* has been used as an endogenous gene in a gene expression analysis to identify TFs in BPH resistance which was used by Wang *et al.* (2012) and other study involving rice and BPH (Liu *et al.*, 2017). In addition, this gene also showed low variations in response to N deficiency stress in TN1 and IR70 rice cultivars as described in the 4.4.3 of Chapter 4.

5.5.2 *Gene expression profiles of TFs in response to combined stress of N and BPH infestation*

Previous studies have identified TFs of rice responsive to individual stress, N deficiency (abiotic stress) (Cai *et al.*, 2012) or BPH infestation (biotic stress) (Wang *et al.*, 2012). However, our current work firstly identified the TFs which were responsive to simultaneous N deficiency stress and BPH infestation in susceptible TN1 and resistant IR70. The TF genes used in this present study were previously shown to be potentially involved in induced and constitutive resistance to BPH in TN1 and RHT rice cultivars.TF genes that were induced by BPH were divided into two categories based on their fold change (FC) value upon BPH infestation (Wang *et al.*, 2012). These authors identified *Os03g0860100*, *Os09g0240200*, *Os03g0437200*,

Os02g0214500, *Os03g0180800*, *Os07g0410700*, *Os06g0298200*, *Os01g0971800*, *Os08g0157600* and *Os06g0728700* as TF genes related to BPH-induced resistance (FC>10). The other two TF genes *Os01g0108400* and *Os09g0439200* were genes that showed constitutive differential expression (FC>10).

The present study investigated these TFs based on their expression patterns in response to the reduced N levels (abiotic stress) and the combined stress of reduced N levels and BPH infestation in TN1 and IR70. The differences in expression in the combined stress were compared to the relative expression level of the non-infested plants (set as 1.0) under the respective N levels in order to measure the changes before and after BPH infestation. In the susceptible TN1, there were nine TF genes which were up-regulated, two TF genes down-regulated and one TF which was not differentially expressed in response to the reduced levels of N and BPH infestation. In contrast, there were more down-regulated (seven TFs) TF genes than the up-regulated (five TFs) TF genes in the resistant IR70 under this combined stress.

Two TFs which showed a similar pattern of expression under the N deficiency stress (abiotic stress) and combined stress (N deficiency and BPH infestation) in both the susceptible TN1 and resistant IR70 was the *Os03g0860100* (AP2-EREBP family) and the *Os01g0108400* (bHLH family). Both TFs were down-regulated in the TN1 and up-regulated in IR70 in response to the reduced N levels (abiotic stress). However, these genes were up-regulated in both TN1 and IR70 but to a greater magnitude under the combined stress. The gene expression results of the TFs analysed in response to the combined stress in TN1 and IR70 rice cultivar is tabulated in Table 4.7.

Table 5.1 Gene expression of TFs in response to combination of reduced levels of N and BPH infestation in TN1 and IR70 rice cultivars.

TF/hpi	N stress NH_4NO_3	4		8		12		24		36	
		TN1	IR70	TN1	IR70	TN1	IR70	TN1	IR70	TN1	IR70
Os03g0860100	1.44	U	U	U	U	U	U	U	U	U	U
	1.04	U	U	U	U	U	U	U	U	U	U
	0.64	U	U	U	U	U	U	U	U	U	U
	0.24	U	U	U	U	U	U	U	U	U	U
Os07g0410700	1.44	U	U	U	U	U	U	U	U	U	U
	1.04	U	U	U	U	U	U	U	U	U	U
	0.64	U	U	U	U	U	U	U	U	U	U
	0.24	U	U	U	U	U	U	U	U	U	U
Os08g0157600	1.44	D	D	D	D	D	D	U	U	D	D
	1.04	U	D	U	D	D	D	U	U	D	D
	0.64	U	D	U	D	D	D	U	U	D	D
	0.24	U	D	U	D	D	D	U	U	D	D
Os06g0728700	1.44	D	D	U	D	D	D	U	U	D	D
	1.04	D	D	D	D	D	D	U	U	D	D
	0.64	D	D	D	D	D	D	U	U	D	D
	0.24	D	D	D	D	D	D	U	U	D	D
Os01g018400	1.44	U	U	U	U	U	U	U	U	U	U
	1.04	U	U	U	U	U	U	U	U	U	U
	0.64	U	U	U	U	U	U	U	U	U	U
	0.24	U	U	U	U	U	U	U	U	U	U
Os02g0214500	1.44	U	U	U	U	U	D	U	D	U	D
	1.04	U	U	U	D	U	D	U	D	U	D
	0.64	U	U	U	D	U	D	U	D	U	D
	0.24	U	D	U	D	U	D	U	D	U	D
Os06g0298200	1.44	U	U	U	U	D	U	D	U	U	U
	1.04	U	U	U	U	D	U	D	U	U	U
	0.64	U	U	U	U	D	U	D	U	U	U
	0.24	U	U	U	U	D	U	D	U	U	U
Os09g0240200	1.44	U	U	U	D	D	D	D	D	D	D
	1.04	U	U	D	D	D	D	D	D	D	D
	0.64	D	D	D	D	D	D	D	D	D	D
	0.24	D	D	D	D	D	D	D	D	D	D
Os09g0439200	1.44	ND	D	ND	D	ND	D	ND	D	ND	D
	1.04	ND	D	ND	D	ND	D	ND	D	ND	D
	0.64	ND	D	ND	D	ND	D	ND	D	ND	D
	0.24	ND	D	ND	D	ND	D	ND	D	ND	D
Os03g0180800	1.44	U	U	U	U	U	D	U	U	U	U
	1.04	U	D	U	D	U	D	U	U	U	U
	0.64	U	D	U	D	U	D	U	U	U	U
	0.24	U	D	U	D	U	D	U	U	U	U
Os03g0437200	1.44	U	U	U	U	D	D	U	U	U	U
	1.04	U	U	U	D	D	D	U	D	U	D
	0.64	U	U	U	D	D	D	U	D	U	D
	0.24	U	D	U	D	D	D	U	D	U	D
Os03g0860100	1.44	U	U	U	U	U	U	U	U	U	U
	1.04	U	U	U	U	U	U	U	U	U	U
	0.64	U	U	U	U	U	U	U	U	U	U
	0.24	U	U	U	U	U	U	U	U	U	U

U = up-regulated D = down-regulated ND = Not Detected

Overall, there was little difference in the magnitude of response observed in the expression level of *Os03g0860100* in response to the combined stress in both TN1 and IR70 at most time points. The expression level of this TF gene in TN1 and IR70 remained ~ 2 fold at most time points of BPH infestation under the optimal N level. Moreover, this gene also demonstrated similar level of expression (FC<5) under 1.04 mM NH_4NO_3 and 0.64 mM NH_4NO_3 in both the rice cultivars at most time points of BPH infestation. The *Os03g0860100* recorded the highest level of expression under the lowest level of N (0.24 mM NH_4NO_3) at most time points of BPH infestation compared to the other N levels in TN1 and IR70.

The expression profile showed that *Os03g0860100* may only be responsive to the combined stress under very low N level in IR70 and TN1. According to Wang *et al.* (2012), this gene was up-regulated in the TN1 but was barely affected in the resistant RHT (FC< 5) upon BPH infestation (biotic stress) which concluded that this gene might not be involved in BPH resistance. The previous studies indicated that the AP2/ERF family TFs are generally involved in several abiotic stress responses but the biotic stress responses depend on compatible or incompatible interactions between pathogen and plants which leads to susceptibility or resistance in the plants (Jisha *et al.*, 2015). The simultaneous occurrence of biotic and abiotic stresses in plants under natural environment and different regulatory roles of AP2/ERF raised the importance to understand the regulatory roles of these AP2/ERF proteins in plants. Although the AP2/ERF proteins involved in abiotic and biotic stress tolerance have been characterized, the molecular mechanisms involved in these cross-talk remains unclear (Mishra *et al.*, 2015). The results obtained from the present study suggested that the *Os03g0860100* may not be a suitable candidate TF gene in response to the combination of reduced levels of N and BPH infestation.

The present study showed that the *Os01g0108400* was strongly up-regulated in response to the reduced N levels and BPH infestation in the resistant IR70. The expression level of IR70 under 1.44 mM NH_4NO_3 remained similar at 4 h, 8 h and 12 h of BPH infestation showed an increase in expression at the later stage of BPH infestation (24 h and 36 h). Interestingly, the expression of this gene under the reduced levels of N was higher than the expression under the optimal N level in IR70

and the highest level of expression under the reduced levels of N were observed at 12 h of BPH infestation. In contrast, the expression profile of this gene in TN1 exhibited less than 2 fold up-regulation under 1.44 mM NH_4NO_3 and remained ~ 2 fold up-regulation under 1.04 mM NH_4NO_3 and 0.64 mM NH_4NO_3 and increased to a ~ 3 fold under the lowest level of N at most time points. The expression profile clearly indicated that there were large quantifiable differences in the expression between the susceptible TN1 and resistant IR70 upon BPH infestation at all time points under the different levels of N.

Wang *et al.* (2012) showed that *Os01g0108400* was up-regulated more in the resistant RHT at some point compared to the susceptible TN1 (2 h, 4 h, 12 h, 24 h and 36 h) of BPH infestation which was similar to the findings of the present study (Figure 5.6A). These authors also reported that this gene was strongly up-regulated in RHT compared to TN1 before BPH infestation and showed slight changes in gene expression upon BPH attack in the RHT. Therefore, this gene was constitutively expressed at a high level during the normal life cycle and may not likely to be involved in the BPH resistance responses. However, the results from the present study showed that this gene showed an increase in the expression level under the optimal N level and to a higher level in response to the reduced N levels and BPH infestation in the resistant IR70.

The expression profile of *Os01g0108400* demonstrated that IR70 is not only responsive to reduced levels of N stress but was also strongly induced in response to the combination of N deficiency stress and BPH infestation. Therefore, crosstalk between these stresses may induce enhance resistance to rice genotypes. The gene expression profile of *Os01g0108400* showed that this gene may not be involved in response to the reduced levels of N and also in response to the combined stress in TN1. The previous study revealed that the bHLH TFs were involved in plant defence responses. *OsbHLH65* in rice showed responses to *Magnaporthe grisea* and defence related-related genes hormones such as methyl jasmonate (MeJA) and salicylic acid (SA) (Shin *et al.*, 2014). Generally, the bHLH TFs regulates cellular processes such as responses to abiotic and biotic stress, hormone signalling and biosynthesis of specialised metabolites (Pireyre and Burow, 2015). Therefore, this present study suggested that *Os01g0108400* from the bHLH is potentially involved in response to simultaneous N deficiency stress and BPH infestation in the resistant IR70 which provides important information for breeding insect-resistant rice varieties.

The *Os07g0410700* (AP2-EREBP) and *Os01g0971800* (G2-like) which was up-regulated in response to N deficiency stress (abiotic stress) were also highly up-regulated in response to the combined N deficiency stress and BPH infestation in the susceptible TN1 and resistant IR70. The *Os07g0410700* exhibited an increase in expression level in IR70 upon BPH infestation at different time points under the 1.44 mM NH_4NO_3. Overall the IR70 demonstrated an increase in gene expression at most time points of BPH infestation under 1.04 mM NH_4NO_3 and 0.64 mM NH_4NO_3 and the highest level of expression was recorded under 0.24 mM NH_4NO_3 at all time points of BPH infestation. In addition, this TF gene was also expressed to a higher magnitude in IR70 compared to TN1 under all N levels at most time points of BPH infestation.

In TN1, this gene was not expressed to a higher level in response to the reduced levels of N and BPH infestation as compared to IR70. TN1 exhibited similar level at most time points of BPH infestation under all N levels with an exception of ~ 5 fold up-regulation at 12 h and 36 h of BPH infestation under 1.04 mM NH_4NO_3. According to Wang *et al.* (2012), *Os07g0410700* was expressed to a higher expression in TN1 compared to the resistant RHT at most of the time point of BPH infestation. The findings of these authors were different from the results obtained from the present study (Figure 4.3 A). Overall, the expression profile of *Os07g0410700* showed that this TF is more responsive to N deficiency stress and the dual stress in the resistant IR70.

The AP2/ERF TFs have multiple binding abilities which is a very useful target providing resistance and multiple stress responses (Mishra *et al.*, 2015). One of the reports showed that overexpression of *OsEREBP1* from the AP2/ERF family confers resistance to Xoo infection, drought and submergence tolerance in transgenic rice. *OsEREBP1* also activates the jasmonate and abscisic acid signalling pathways to enhanced survival under abiotic and biotic stress conditions and is involved in multiple stress tolerance (Jisha *et al.*, 2015). Another study showed that Ethylene responsive TAERF1 from wheat (*Triticum aestivum*) also activates stress response genes such as the pathogen and cold response genes which lead to an improved pathogen and abiotic stress response in transgenic plants (Mishra *et al.*, 2015). In addition, Vega *et al.* (2015) also reported that TFs belongs to the ERF family are involved in stress responses and plays important role in SA/JA crosstalk. The present study suggested that *Os07g0410700* may play an important role in response to simultaneous N deficiency and BPH infestation. Moreover, the PlantPan2.0 gene

information of *Os07g0410700* showed that this gene is involved in transcription (GO:0006351), regulation of transcription (GO:0006355) and biological process (GO:0008150) (http://plantpan2.itps.ncku.edu.tw/) which is potentially related to defence responses such as JA.

The *Os01g0971800* from the G2-like TF family also demonstrated a similar pattern of expression as the *Os07g041700*. This gene exhibited a higher level of expression under 1.44 mM NH_4NO_3 and continued to be highly expressed under the reduced N levels upon BPH infestation. The *Os01g0971800* was up-regulated to a greater magnitude in IR70 at all levels of N compared to the susceptible TN1 at all time points of BPH infestation. The susceptible TN1 showed a marked increase in response to BPH infestation at two time points, 8 h (9.7 fold up-regulation) and 12 h (6.2 fold up-regulation) under 1.04 mM NH_4NO_3. However, the expression level of this gene in TN1 under 0.64 mM NH_4NO_3, and 0.24 mM NH_4NO_3 decreased and was similar to the expression profile of 1.44 mM NH_4NO_3 optimal N level upon BPH infestation. TN1 which demonstrated high expression in response to N deficiency stress (abiotic stress), did not show large differences in the expression level in response to the combined stress. Therefore, this gene may not be involved in response to the dual stress in the susceptible TN1.

The present study suggested that resistant IR70 may potentially play an important role in the combined reduced N levels and BPH infestation. The PlantPAN 2.0 gene ontology information showed that the *Os01g0971800* is involved in transcription (GO:0006351) and regulation of transcription (GO:0006355) (http://plantpan2.itps.ncku.edu.tw/) which indicates that this gene is potentially involved in regulation of several stress responses. Wang *et al.* (2012) reported that both the *Os07g0410700* and *Os01g0971800* TF genes were strongly up-regulated at 8 hours after BPH infestation in both TN1 and RHT but showed a similar level of gene expression as the non-infested plants. It was concluded that these genes were participating in the early stage of biological responses. However, the results from the present study showed that both TF genes were up-regulated to a higher level at all time points in the resistant IR70 under the optimal N level (1.44 mM NH_4NO_3) upon BPH infestation. According to Wang *et al.* (2012), the up-regulation of TF genes in rice cultivars was triggered by BPH infestation. The products of these genes and metabolites resulting from activated biochemical pathways are potentially involved in repairing the damage in the phloem to prevent the loss of phloem sap. This is one of

the strategies used by plants to defend themselves against the invasion of pathogens and bacteria. The expression profiles of both the TFs showed that these TFs may share similar components of regulatory networks between both abiotic and biotic stress signalling, indicating crosstalk and existence of general stress responses.

The *Os09g240200* from C2C2-CO like family was responsive to N deficiency stress (abiotic stress) in the resistant IR70 but to a lesser magnitude in the susceptible TN1. This gene was down-regulated in response to the combined reduced levels of N and BPH infestation after 8 h of BPH infestation under the optimal N level (1.44 mM NH_4NO_3) and 1.04 mM NH_4NO_3 in the IR70. The expression level of this gene in IR70 remained similar under the optimal N level and 1.04 mM NH_4NO_3 upon BPH infestation at different time points of BPH infestation and exhibited less than 2 fold down-regulation in TN1 under these levels at all time points. The expression profiles of *Os09g240200* showed that this TF gene is not responsive to the combined stress in TN1 under 1.44 mM NH_4NO_3 and 1.04 mM NH_4NO_3. However, a drastic increase in expression level was observed in both TN1 and IR70 under the 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3 upon BPH infestation. Moreover, both TN1 and IR70 showed a similar level of expression at some time points of BPH infestation under both these N levels.

It was interesting to observe that the trend of gene expression changed under 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3 upon BPH infestation in TN1 and IR70. The expression profiles indicated that both TN1 and IR70 were responsive to the combination of both the stresses under 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3. The results of this present study (Figure 5.9 A) were similar to the expression profile obtained by Wang *et al.* (2012) whereby the resistant RHT was down-regulated upon BPH infestation (biotic stress) at all time points but was not expressed at the most time except 12 h and 36 h of BPH infestation in the susceptible TN1. This study showed that the *Os09g024200* TF gene may play an important role not only in response to N stress (abiotic stress) but also in the combined stress of N deficiency and BPH infestation in both susceptible TN1 and resistant IR70 but to a greater extent in the resistant IR70. The present study proposed that this gene may an important role in abiotic and biotic stress tolerance crosstalk and therefore is useful in breeding improved rice cultivars against combinatorial stress.

The expression profile of *Os09g0439200* from the Tify family demonstrated that this gene was down-regulated in response to the reduced levels of N and BPH infestation in the resistant IR70. However, this gene was not differentially expressed in response to the reduced N levels (abiotic stress), was also not expressed in response to the dual stress in TN1. The *Os09g0439200* which was up-regulated in the resistant IR70 in response to the reduced N levels (abiotic stress) showed down-regulating in response to the combined stress in this rice cultivar. This TF was down-regulated to a ~ 2 fold at most time points all point of BPH infestation in IR70 with an exceptional at 8 hours under the optimal N level (1.44 mM NH_4NO_3) upon BPH infestation. However, the expression profile indicated that this gene was down-regulated to a greater magnitude in response to the reduced levels of N compared to the optimal N level at all time points of BPH infestation.

Interestingly, *Os09g0439200* was down-regulated to a greater magnitude in the IR70 at 8 hours of BPH infestation under the 1.44 mM NH_4NO_3, 1.04 mM NH_4NO_3 and 0.24 mM NH_4NO_3. It was also observed that the expression level at 4 h of BPH infestation remained between ~ 2 fold to ~ 3 fold down-regulation under all N levels. Wang *et al.* (2012) reported that this gene was strongly up-regulated in RHT compared to the TN1 before BPH infestation and showed changes in gene expression upon BPH infestation (biotic stress). These authors also suggested that this gene was probably involved in BPH resistance and may be involved in response to the dual stress as demonstrated in the present study.

The PlantPAN 2.0 gene ontology information (http://plantpan2.itps.ncku.edu.tw) showed that this gene was involved in plant hormone signal transduction (map04075) and plant-pathogen interaction (map04626). The *Os09g0439200* which is known as *OsJAZ8* is reported to be involved in jasmonate-induced resistance to bacterial blight caused by *Xanthomonas oryzae pv oryzae* in rice (Yamada *et al.*, 2012). In addition, Zhang *et al.* (2015) reported that *OsJAZ8/OsTIFY10c* plays a role as a repressor of JA signalling and the JA-induced volatile compound which are involved in plant defence systems. The present study showed that the *Os09g0439200* is potentially involved in response to the reduced level of N and also in response to the combined N deficiency stress and BPH infestation in the resistant IR70.

The *Os02g0214500* from the NAC TF family which was up-regulated in both the TN1 and IR70 but to a lesser extent in the TN1 in response to N deficiency stress (abiotic

stress) showed a different pattern of expression in the combined reduced N levels and BPH infestation. This gene was constantly up-regulatedin TN1 but down-regulated in IR70 upon BPH infestation at different time points across all different levels of N. The expression profile under the optimal N level (1.44 mM NH_4NO_3) showed that there was a decrease in the gene expression level from 4 h to 36 h of BPH infestation in TN1. The expression profile indicated that this TF gene did not exhibit drastic changes in gene expression in response to the dual stress in TN1 and therefore may not be responsive to this stress in the susceptible TN1.

In contrast, this gene which was up-regulated in IR70 upon BPH infestation at 4 h and 8 h of BPH infestation under the optimal N level, was down-regulated at 12 h, 24 h and 36 h of BPH infestation. This may be due to the rice cultivar suppressing the production of certain metabolites to protect them from further damage at the later stage of BPH infestation (12 h, 24 h and 36 h) was also observed in other TFs analyzed in this study. The results from the present study (Figure 4.7 A) was different from the findings of Wang *et al.* (2012) who reported that this gene was strongly up-regulated in the TN1 but was not affected in the resistant RHT (biotic stress).

This gene marked a drastic change in gene expression in IR70 under the 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3. Moreover, this gene was down-regulated at all time points upon BPH infestation in the lowest level of N (0.24 mM NH_4NO_3) in the resistant IR70. The expression profile indicated that the *Os02g214500* was responsive to the combined reduced level of N and BPH infestation especially under the lower N levels (0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3) in the resistant IR70. The gene expression of *Os02g214500* showed that this gene is potentially involved in response to N deficiency stress (abiotic stress) and combined stress in IR70.

The *Os02g214500* is also known as the *OsNAC6* was identified to be involved in abiotic stresses (cold, drought and high salinity) and in biotic stresses (wounding and blast diseases) in rice cultivars (Nakashima *et al.*, 2007). The PlantPAN 2.0 gene information data revealed that this TF gene is involved in regulation of transcription (GO: 0006355) and biological process (GO: 0008150) (http://plantpan2.itps.ncku.edu.tw). The role of NAC was proven to be useful in conferring stress tolerance or disease resistance in many model plants. Many genes from the NAC family were successfully overexpressed in rice (Puranik *et al.*, 2012). The *OsNAC19* gene was identified to be involved in rice defence response to

Magnaporthe grisea infection (Xia *et al.*, 2010a). In addition two NAC TFs, *TaNAC4* (Xia *et al.*, 2010a) and *TaNAC8* (Xia *et al.*, 2010b) were discovered to be involved in stripe rust pathogen infection and salinity stress in wheat. The present study suggested that overexpression or silencing of *Os02g214500* may potentially increase tolerance to the combined reduced N levels and BPH infestation.

The *Os03g0437200* from the C2C2 family demonstrated up-regulation in the susceptible TN1 upon BPH infestation at all N levels with an exception of down-regulation at 12 h of BPH infestation. This gene was down-regulation at 12 h of BPH infestation in both TN1 and IR70 but to a lesser magnitude in the TN1. This gene showed an increase in gene expression in TN1 under the optimal N level (1.44 mM NH_4NO_3) at the later stage of BPH infestation (24 h and 36 h) and was expressed to a higher level at both time points compared to the resistant IR70.

This TF also recorded the lowest level of expression in TN1 under the lowest N level (0.24 mM NH_4NO_3). Therefore *Os03g0437200* which was responsive to the reduced levels of N (abiotic stress) may not be involved in response to the combined reduced levels of N and BPH infestation in TN1. In contrast, this gene was up-regulated to a higher level in IR70 than the TN1 only at 4 hours of BPH infestation and the expression then gradually decreased after 8 hours of BPH infestation under the optimal N level (1.44 mM NH_4NO_3). *Os03g0437200* showed down-regulation at 12 h of BPH infestation under 1.44 mM NH_4NO_3 in IR70 and demonstrated more down-regulation under the reduced levels of N upon BPH infestation. Interestingly this gene exhibited down-regulation in IR70 at all time points of BPH infestation and to a greater magnitude under 0.24 mM NH_4NO_3.

An interesting pattern of expression was observed at 12 h and 36 h of BPH infestation whereby this gene exhibited a greater level of expression under the respective reduced levels of N. The expression profile of this TF showed that this gene may not be responsive to BPH resistance in IR70 under the optimal N level. Wang *et al.* (2012) also reported that the TN1 was expressed to a greater level at most time points (8 h, 12 h, 24 h and 36 h) compared to the resistant RHT (biotic stress). However, the present study showed that the resistant IR70 was more responsive to BPH infestation under the reduced N levels. According to Wang *et al.*, (2012), the resistant RHT was involved in cessation of many metabolic pathways which will prevent the loss of phloem sap. Therefore, the metabolic activity of the

resistant rice cultivar was suppressed and reduced the number of substances supplied to the phloem and prevents BPH feeding which is an important defence mechanism of the plant. This present study suggested that *Os03g0437200* is responsive to N deficiency stress (abiotic stress) and the combined stress (N deficiency and BPH infestation) in the resistant IR70. Responses of this TF gene to abiotic and the combined stress showed that this gene is potentially controlled by different signalling pathways which potentially enhance resistance to this combinatorial stress.

Previous studies have reported that the C2H2-type zinc families are transcriptional regulators in plants which are involved in tolerance to biotic and abiotic stresses and control the expression of many activated genes. These TFs were also reported to participate in plant defence responses. The expression of a novel potato C2H2- type zinc finger protein gene, *StZFP1* showed that this gene was involved in response to *Phytophtora infestans*, salt and dehydration (Kiełbowicz-Matuk, 2012). Another TF, *CaZFP1* from the C2H2 family was identified to enhanced tolerance to drought and infection by *Pseudomonas syringae* pv. tomato when it is expressed in Arabidopsis (Lawrence *et al.*, 2014).

The *Os08g0157600* and *Os06g0728700* TF genes from the MYB family which exhibited a similar level of expression in response to N deficiency stress (abiotic stress) in the TN1 and IR70, however, showed differences in gene expression under the combined stress. The expression profiles of *Os08g0157600* and *Os06g0728700* showed that these genes were down-regulated at most time points of BPH infestation in IR70 under 1.44 mM NH_4NO_3, 1.04 mM NH_4NO_3, 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3. Interestingly, *Os08g0157600* and *Os06g0728700* were down-regulated to a greater magnitude at 12 h under some N levels in IR70. Moreover, the *Os08g0157600* was constantly down-regulated in TN1 at 12 h and 36 h of BPH infestation under all N levels is an interesting pattern of gene expression. In addition, both *Os08g0157600* and *Os06g0728700* genes showed an interesting pattern of up-regulation at 24 h of BPH infestation in TN1 and IR70 but to a higher level of expression in IR70. However, *Os06g0728700* did not show any drastic differences in the expression levels of both rice cultivars at 24 hours of BPH infestation with an exception under 1.04 mM NH_4NO_3 in the resistant IR70.

Wang *et al.* (2012) also showed that the *Os08g0151600* was up-regulated at 24 hours in both the rice cultivars tested but to a greater extent in the resistant RHT. Therefore, this study suggested that *Os08g0157600* is responsive to the combined reduced levels of N and BPH infestation. According to Wang *et al.* (2012) the gene expression profile showed that the RHT was down-regulated to a higher level compared to the susceptible TN1 at 4 h, 8 h, 12 h and 36 h of BPH infestation (biotic stress) which was also observed in the present study under 1.44 mM NH_4NO_3 upon BPH infestation (Figure 5.4A).

The expression profiles showed a greater level of expression in the IR70 under 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3. Although the expression profile of *Os06g0728700* indicates that this gene was down-regulated at most time points in TN1, the expression level in this rice cultivar remained similar at most time point under the reduced N levels upon BPH infestation. Therefore, *Os06g0728700* may be involved in response to the dual stress in TN1and IR70 but to a lesser extent in TN1. Wang *et al.* (2012) reported that *Os06g0728700* was strongly down-regulated only in RHT and likely to be involved in BPH-induced resistance which is similar to the results obtained under 1.44 mM NH_4NO_3 of this present study (Figure 5.5 A). The expression profile of *Os06g0728700* indicated that IR70 is responsive to the reduced level of N deficiency (abiotic stress) and combined stress of N deficiency and BPH infestation.

The previous study showed that transgenic plants overexpressing wheat MYB gene *TaPIMP1* in tobacco enhanced resistance to pathogen *Ralstonia solanacearum* and increased tolerance to drought and salt stresses. The MYB family was also reported to play important roles in photosynthesis and metabolism of the plants (Wang *et al.*, 2012). Down-regulation of the TFs from the MYB family upon BPH infestation was reported by Wang *et al.* (2012) and also in the present study. Members from the MYB TFs are also potentially involved in controlling antagonism between hormone-mediated abiotic stress and pathogen response pathways. Previous studies showed that manipulation of the MYB TFs can confer tolerance to biotic and abiotic stresses in many plant species (Atkinson and Urwin, 2012). The results from the present study showed that *Os08g0157600* and *Os06g0728700* have the potential to induce tolerance towards more than one stress and thefore are good candidates for development of new rice varieties tolerance to the combined N deficiency and BPH infestation. Atkinson and Urwin (2012) reported that common genes that were

induced in both abiotic and biotic stress may play importance role in regulation of crosstalk between pathways.

The expression profile of *Os03g0180800* from the Tify family showed that this gene demonstrated a higher level of expression in TN1 at early stages of BPH infestation (4 h and 8 h) under the optimal N level. Findings by Wang *et al.* (2012) showed that this gene was up-regulated in the TN1 but was not affected in the resistant RHT (biotic stress). However, the findings of this study showed that the IR70 exhibited a drastic increase in expression level at 24 hours and 36 hours of BPH infestation whilst the expression of TN1 remained ~ 3 fold up-regulation at most time points under this optimal N level (Figure 5.11 A).

Os03g0180800 was down-regulated in IR70 at 12 hours of BPH infestation under 1.44 mM NH_4NO_3 and more down-regulation were observed under the reduced levels of N at 4 h, 8 h and 12 h of BPH infestation. Interestingly this gene was down-regulated to a greater magnitude in IR70 at the lowest N level at 8 hours and 12 hours of BPH infestation. *Os03g0180800* which was highly up-regulated at 24 h and 36 h of BPH infestation under the optimal N level, showed a reduction in expression level in response to the reduced levels of N in IR70. Therefore, this gene may be responsive to the combined stress in IR70 under lower level of N deficiency. However, further investigation is required to understand the molecular mechanisme that regulates the response of this TF to the combination of N deficiency and BPH infestation.

On the other hand, the TN1 only showed the highest level of expression at 24 h and 36 h of BPH infestation under 1.04 mM NH_4NO_3. This rice cultivar exhibited a decrease in expression level (~ 2 fold up-regulation) at all time points of BPH infestation under the lowest level N (0.24 mM NH_4NO_3) and the expression level under this N level was lower than the expression recorded under the optimal N level. The expression profile of *Os03g0180800* showed that this TF may not be a good candidate in response to the dual stress in TN1.

The PlantPAN2.0 gene ontology information (http://plantpan2.itps.ncku.edu.tw) showed that *Os03g0180800* is involved in plant hormone signal transduction (map04075) and plant-pathogen interaction (map04626). The *Os03g0180800* which is known as the *OsJAZ9 or OsTIFY11a* acts as a transcriptional regulator in the jasmonate signalling (https://funricegenes.github.io/OsJAZ9~OsTIFY11a/). In rice,

several reports have proven that JA plays an important role in defence against insect (Dhakarey et al., 2016). The different patterns of gene expression of TFs related with hormone responses, suggested the existence of several regulatory pathways which interacts in a complex and highly dynamic manner, linking plant N status, and the plant defence response (Vega *et al.*, 2015).

Unlike the other TFs, the *Os06g0298200* from the C2C2-CO-like family was down-regulated in the susceptible TN1 at 12 h and 24 h of BPH infestation but to a greater magnitude at 12 h of BPH infestation under all N levels. The expression profile showed that this gene was down-regulated to a greater magnitude at 12 h and 24 h under the optimal N level compared to the expression under the reduced N levels upon BPH infestation.

The expression profile of *Os06g0298200* showed a higher level of expression in IR70 compared to the susceptible TN1 under the optimal N level (1.44 mM NH_4NO_3) at all time points of BPH infestation. Interestingly the IR70 showed a drastic increase in the gene expression level under the reduced levels of N after 4 hours of BPH infestation as compared to the optimal N level. Wang *et al.* (2012) reported that this gene was strongly up-regulated after 8 h of BPH infestation in both TN1 and RHT and probably is involved in the early response of biological response. Overall the *Os06g0298200* demonstrated to be responsive to the combined reduced levels of N and BPH infestation in IR70 compared to TN1. Moreover, previous studies reported that the Zn-finger TF families are involved in response to fungal and bacterial pathogens. Many studies showed that the function of TFs from this family depends on the JA and SA signalling pathways which demonstrated BPH-resistance responses (Wang *et al.*, 2012). The present study suggested further characterization of this TF would be useful to confer tolerance to the combined stress.

5.6 Conclusions

This study provided an overview of the TF genes involved in response to the combination of reduced N levels and BPH infestation in two rice cultivars, the susceptible TN1 and resistant IR70. Most of these TF genes showed interesting expression patterns at some time points under this dual stress. Some TFs have interesting patterns of expression at 12 h and others at 24 h of BPH infestation under the reduced levels of N. Overall, this study supported the proposed hypothesis

whereby the gene expression profiles revealed that the expression of TFs in response to the combination stress in IR70 was greater compared to TN1.

Some TFs were down-regulated to a greater level in the resistant IR70 under the reduced N levels at most time points of BPH infestation. Among the TFs which were down-regulated under the reduced N levels at most time points of BPH infestation in the resistant IR70 were the *Os08g0157600*, *Os02g0214500*, *Os09g0439200* and *Os03g0437200*. This study also identified two TF genes *Os06g0728700* and *Os09g0240200* which were down-regulated in both TN1 and IR70 rice cultivars but to a greater magnitude in the resistant IR70. The susceptible TN1 had more up-regulated TF genes than the resistant IR70 in response to the combined stress. Most of the TF genes were down-regulation in the resistant rice cultivar showed that certain metabolic pathways may be turned off to prevent further damage to loss of water and nutrient. This explains the reason why BPH was unable to extract nutrition from the phloem sap of the resistant rice cultivar (Wang *et al.*, 2012).

The present study also demonstrated that some TFs were strongly up-regulated in the IR70 and to a lesser magnitude in the TN1 under the combination of reduced N levels and BPH infestation. These genes were induced to produce metabolites which may be used to help to repair the damage to the phloem sap and defence against the BPH infestation (Wang *et al.*, 2012). TFs such as *Os07g0410700*, *Os01g0108400*, *Os06g0298200* and *Os01g0971800* were up-regulated in response to the dual stress and to a higher level of expression in the resistant IR70 compared to the susceptible TN1.

This study revealed that the TFs in the dual stress responded differentially from the TFs exposed to biotic stress (BPH infestation from the previous study) and N deficiency stress (abiotic stress). Overall identifying plant responses to the combination of abiotic and biotic stresses are very complex and depend on the experimental setting. The developmental stage of the plant, the timing of stress and severity of individual stresses will determine the outcome of the expression levels. The findings of this study enhanced the understanding of responses to the combination of abiotic (N) and biotic (BPH infestation) stress in susceptible TN1 and resistant IR70 rice cultivars which will provide valuable information for breeding insect-resistant rice cultivars. Further characterization of these TF genes would be

useful in choosing the right candidate gene which can be potential targets for conferring tolerance to the combined N stress and BPH infestation.

Chapter 6: General Discussion and Future Perspectives

6.1 The effect of reduced N levels on physiological parameters of rice cultivars

Nitrogen is one of the major abiotic stresses affecting plant physiology and development of rice cultivars. The physiological experiments carried out on the BPH-susceptible TN1 and BPH-resistant IR70 rice cultivars showed that both the rice cultivars were affected by reduced levels of N. Both cultivars were grown for 3 weeks on Yoshida nutrient solutions under 1.04 mM NH_4NO_3 (representing medium level of N), 0.64 mM NH_4NO_3 and 0.24 mM NH_4NO_3 (representing low levels of N). The physiological parameters of these plants were compared with the plants grown under 1.44 mM NH_4NO_3 (representing optimal/control N level). Both the TN1 and IR70 demonstrated significant reductions in growth including the shoot height, number of tillers and leaves, root length, relative water content, leaf area and chlorophyll index in response to reduced levels of N as compared to plant growth on under optimal N levels.

The physiological results clearly indicated that N plays an important role in the growth of rice plants. Although several previous studies have demonstrated that reduced N input affects the physiological growth of rice crops, this study was important in establishing base-line data for subsequent work carried out is this project. The results of the physiological studies showed that plants grown under reduced levels of N are in a stressed condition as compared to the plant's growth under optimal N. Therefore, the plants were grown under the exact conditions and same growth stage to determine the molecular response of TN1 and IR70 to N stress (abiotic stress) and the combination of N stress and BPH infestation presented in Chapter 4 and Chapter 5 of this study respectively.

A thorough understanding of the physiological and molecular responses of rice cultivars to N stress is important for the development of breeding strategies to confer stress tolerance in rice cultivars. Overall, improved NUE and greater understanding of the physiological responses of rice crops to N stress will help to reduce usage of fertilizers and thus reduce water pollution.

6.2 Molecular response of TN1 and IR70 to reduced levels of N

Although physiological mechanisms against abiotic stress in rice are moderately well understood, further investigation is required to establish resistance against abiotic and biotic stresses, and identification of transcription factors (TFs) that contribute towards these stress responses is essential. Hence, knowledge of the molecular response of rice cultivars to abiotic and biotic stress is potentially important in order to develop rice cultivars which can be well grown under sub optimal conditions.

TFs play a vital role in the regulation of physiological and biological processes in plants in response to adverse abiotic and biotic stresses (Rahaie *et al*., 2013). TFs from different families, which are potentially involved in BPH resistance (Wang *et al.*, 2012) were used to identify genes involved in the response to reduced levels of N in the present study. This study demonstrated that all the 12 TFs analyzed by Wang *et al.* (2012) were up-regulated in the resistant IR70 cultivar, whilst 10 TFs were up-regulated and 2 TFs were down-regulated in the susceptible TN1 cultivar in response to the reduced N, compared to their respective controls. In addition, the gene expression profiles indicated that the resistant IR70 exhibited a higher level of expression compared to the susceptible TN1 for all 12 TFs in response to reduced N.

Interestingly, two TFs from the MYB-related family (Os08g0157600 and Os06g0728700) showed a similar level of expression in response to reduced N in both the TN1 and IR70. Moreover, several studies have reported that TFs from the MYB family are involved in nutrient deficiency in many plant species. The gene expression profiles of *Os07g0410700*, *Os09g0240200*, *Os01g0108400* and *Os03g01080800* demonstrated that these genes were up-regulated in both IR70 and TN1 in response to reduced N but to a much greater magnitude in the resistant genotype compared to the susceptible TN1. In contrast, *Os09g0439200* was only differentially expressed in the resistant IR70 in response to this stress.

In conclusion, most of the TF genes which were potentially involved in BPH-resistance were also shown to be responsive to the reduced levels of N (abiotic stress) in the resistant IR70 and to a lesser magnitude in the susceptible TN1. Most of these TF genes were reported to be involved in different abiotic stresses. TF genes responding to low N conditions may be potentially good candidate genes for improvement of NUE in rice production (Ding *et al.*, 2011). Suzuki *et al.* (2014) also reported that in some cases, a specific abiotic stress enhanced the resistance of

plants to biotic stress so demonstrating “cross-talk” between different stress response pathways.

The TFs which were highly expressed under the N stress in the present study would improve crop yield if they were over-expressed in transgenic rice plants. However, the extent of damage caused by the combined stress is important to inform the development of crops with enhanced tolerance to the combination of both the stresses (see Chapter 5 of this study).

6.3 Molecular response of the combination of the reduced level of N and BPH infestation

Whilst many previous studies have focused on TFs which were responsive to a single abiotic or biotic stress in different rice cultivars, very few have focused on simultaneous biotic and abiotic stresses. As described in the previous chapters, although N is an important macronutrient, which plays an important role in the growth of rice plants, excessive use of nitrogenous fertilizers in rice fields has increased the population of BPH. BPH is an important insect pest which causes hopperburn, a condition that leads to complete drying of the rice crop. Hopperburn has been reported to cause significant losses to the crop production across the world (Lu and Heong, 2009).

This present study identified several TFs which potentially play an important role in simultaneous N and BPH infestation stress in the susceptible TN1 and resistant IR70 rice cultivars. Previous studies showed that these genes may be potentially involved in resistant cultivars in response to BPH (Wang *et al.*, 2012). In addition, the present study revealed that some of the TFs which were responsive to N stress alone were also responsive to the combination of both the stresses. TFs from different families showed different expression patterns in response to the combination of reduced N and BPH resistance. Most TFs showed a greater magnitude in gene expression under reduced levels of N compared to optimal N levels in response to BPH infestation in the resistant IR70.

The *Os02g0214500* and *Os03g0437200* TFs from the NAC and C2H2 family, respectively, were down-regulated but only in the resistant IR70 in response to reduced N at different time points of BPH infestation. Interestingly, *Os09g0439200*, from the Tify family, was differentially expressed at all N levels, including the optimal level, (1.44 mM NH_4NO_3) in response to BPH infestation in the resistant IR70. In

contrast, this TF gene was not differentially expressed in susceptible TN1 under either the N stress or the combined stress.

This study also identified that *Os09g0240200* from the C2C2-CO like family and *Os08g0157600* from the MYB-related, were responsive to the dual stress in both IR70 and TN1, but to a greater magnitude in IR70. The gene expression profile also showed that some TFs such as *Os07g0410700* from the AP2-EREBP TF family, *Os01g018400* from the bHLH family and *Os01g0971800* from the G2-like family, were up-regulated to a higher level of expression in response to the combination of the reduced level of N and BPH infestation in the resistant IR70, but not in susceptible TN1.

The present study identified some of the important candidate TF genes for improvement of rice tolerance to the combined stress of reduced N and BPH infestation. Overall the results from Chapter 4 and Chapter 5 demonstrated that five TFs (*Os08g0157600*, *Os02g0214500*, *Os09g0240200*, *Os09g0439200* and *Os03g0437200*) were down-regulated in response to the dual stress (reduced N and BPH infestation) but up-regulated in response to reduced N (abiotic stress) in the resistant IR70. Further analysis and characterization of these genes are important in determining the appropriate candidate TF genes among the single and combined stress response genes which can be potential targets for conferring tolerance in rice cultivars that are sensitive to the combination of both these stresses.

The identification of candidate TF genes can be suitability modulated to confer resistance against the combined stress using tools such as RNA interference (RNAi) allowing the knock-down of these genes. Results from this study suggest that silencing of *Os02g0214500* and *Os03g0437200* TFs, both of which showed differential expression in the resistant IR70 in response to the reduced level of N and BPH infestation compared to the susceptible TN1 may be good candidate genes since they were down-regulated in IR70 but up-regulated in TN1. Down-regulation of these genes in the resistant IR70 cultivar under the reduced levels of N and in the presence of BPH infestation shows that these TFs have repressed many active pathways to prevent further damage and is an efficient method of defence against infestation of the insect pest. Therefore silencing these genes may contribute to the improvement of the breeding of N-tolerant and BPH resistant rice crops.

Overall, the response of plants to combined stress (representing both abiotic and biotic) is more complex and is governed by more severe stresses in the rice field. Combinations of stress are reported to have an entirely different effect on physiological and molecular processes of the plants (Pandey *et al*., 2015). Ultimately understanding the physiological and gene-regulatory network is important to develop or select for stress-tolerant and high yielding rice cultivars.

6.4 Knockdown of β-1,3-glucanase in rice using RNA interference confers resistance to rice brown planthopper

RNA interference (RNAi) is a powerful tool in functional genomics which has been used to develop insect resistant crops such as SmartStax Pro developed by Mosanto (Head *et al.*, 2017). This method is used to down-regulate the expression of a specific gene(s) and has the potential to provide a broad-spectrum resistance against insect pests and pathogens (Younis *et al.*, 2014). Chapter 2 of the present study investigated the knockdown of β-1,3-glucanase (*Gns5*) gene in TN1, a cultivar susceptible to BPH using RNAi to confer resistance to BPH infestation. To the best of our knowledge, the result of this finding is the first to demonstrate that knockdown of *Gns5* confers resistance to BPH in a susceptible rice cultivar.

Gns5 is a pathogenesis-related protein which is classified in the subfamily A of the β-1,3-glucanases family based on its structure and function (Yamaguchi *et al.*, 2017). Du *et al.* (2009) reported that *Gns5* encodes the callose-hydrolyzing enzyme β-1,3-glucanase, which is involved in callose decomposition. These authors demonstrated that BPH feeding induced the expression of *Gns5* causing decomposition of the callose in the susceptible TN1 rice cultivar. Callose (*β*- 1,3-glucan) is a plant polysaccharide that plays an important role in plant growth, development and defence against adverse environmental conditions (Piršelová and Matušíková, 2013). Deposition of callose in the sieve tubes of the plants is an important mechanism of plant defence against the BPH, a major field pest of rice. The previous study by Shoala (2012) showed that *Gns5* was differentially expressed in response to BPH infestation in the resistant IR70 compared to the susceptible TN1. Based on these findings by Shoala (2012) and other previous studies relating to this gene, the *Gns5* was knockdown in the susceptible TN1 rice cultivar in the present study.

The present study verified the role of the β-1,3-glucanase 5 (*Gns5*) gene in BPH susceptibility using antisense RNAi technology with two different constitutive promoters IR462 (pCAMBIA 1300int-Ubi-hpRNAi) and IR463 (pCAMBIA 1300int-

35S-hpRNAi). The present study demonstrated that the RNAi lines, IR463 and IR462 exhibited significantly lower survival rate of BPH compared to their respective empty vector plants and the parental TN1 controls. The study also demonstrated a decrease in the mean number of eggs produced per plant in the RNAi lines compared to the control lines. However, the mean number of eggs produced per adult remained the same in the RNAi lines and the control lines. Therefore, knock-down of *Gns5* did not show an effect on the fitness of this particular trait that is fecundity.

In addition to significantly reduced survival, the suppression of *Gns5* also showed a delay in development of the BPH nymphs to adulthood on both the RNAi lines, whereby only ~ 20-30% of nymphs reached adulthood in the RNAi lines as compared to ~70 -80% on the empty vector lines and the parental, TN1. The study also showed that there were ~ 20% of underdevelopment nymphs on the RNAi lines, with all surviving insects having reached adulthood on the control plants. Microscopic analysis of leaves and stem tissues in the RNAi plants both before and post BPH infestation confirmed that silencing of *Gns5* in the susceptible TN1 plays an important role in callose deposition. These micrographs showed that higher levels of relative callose intensity were present in the sieve tubes of the RNAi plants infested with BPH compared to their respective infested empty vector and parental TN1 controls. However, there were no significant differences recorded in the relative callose intensity in the non-infested RNAi lines and the control plants. Changes in the amounts of callose in the sieve tubes of these plants were only observed upon BPH infestation. Therefore, suppression of *Gns5* prevents callose decomposition, thus keeping the sieve tubes occluded in the RNAi lines.

The EPG analysis recorded the feeding behaviour of BPH on the RNAi lines, parental TN1, BPH-resistant IR70 and empty vector plants. These results showed that the frequency of non-probing and penetration of BPH was significantly longer in the RNAi lines and the resistant IR70, compared to the empty vector and parental control lines. Moreover, the resistant lines recorded the shorter duration of phloem ingestion, which indicated that the BPH feeding was inhibited on the RNAi and resistant IR70 lines. Taken together, the results obtained from all investigations carried out in this study clearly demonstrated that silencing of *Gns5* reduces the feeding, development and survival of BPH in the RNAi lines and therefore confers enhanced resistance to BPH in the transgenic rice lines carrying antisense *Gns5*.

The present study showed that silencing of *Gns5* affects the fitness of several traits such as survival of the BPH nymphs and development of the insect in the RNAi rice lines. These findings will potentially contribute towards the development of transgenic rice cultivars with enhanced levels of BPH resistance and therefore help in reducing the use of a broad spectrum of pesticides in the rice field. One of the future studies recommended is to identify the plant defence responses of these RNAi lines to BPH infestation. It will be interesting to investigate the defence pathways involved in *Gns5*-mediated resistance transgenic rice lines. The expression pattern of plant-defence genes such as *EDS1*, *PAD4*, *PAL* AND *ICS1* (salicylic acid synthesis-related genes), *LOX* and *AOS2* (JA synthesis-related genes), *EIN2* the ethylene signalling pathway receptor and *PR1b* a pathogen related gene will potentially indicate the defence-signalling pathways induced by BPH. Understanding the mechanism of plant defence against this devastating insect pest will be important for breeding rice varieties with high levels of resistance to BPH.

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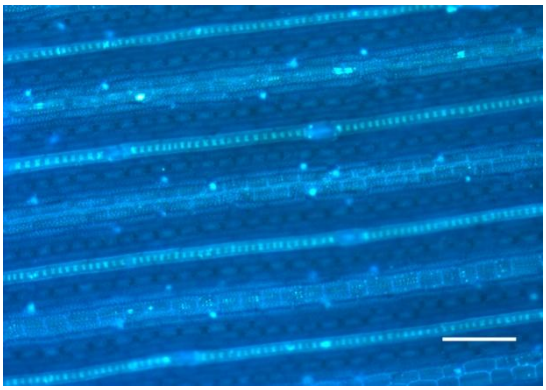
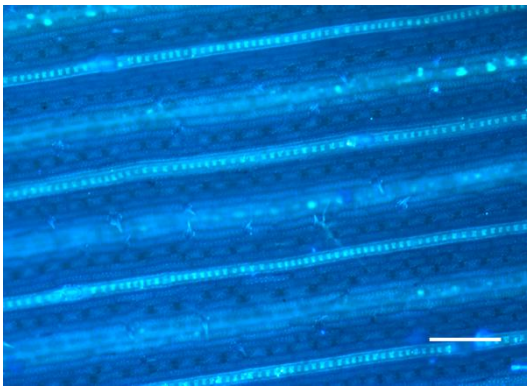
Appendix A

Epifluoresence microscopic images of non-infested and infested leaf samples
Scale bar = 20µm

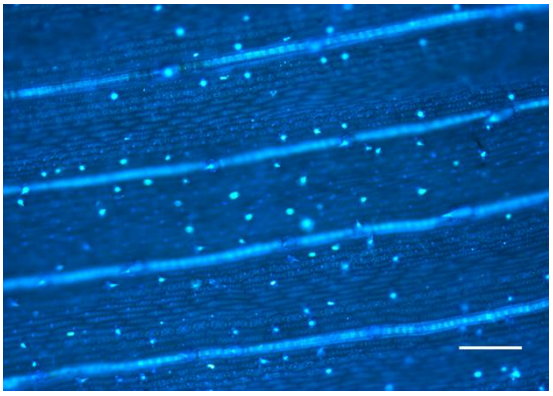
1A) Non-infested TN1 control



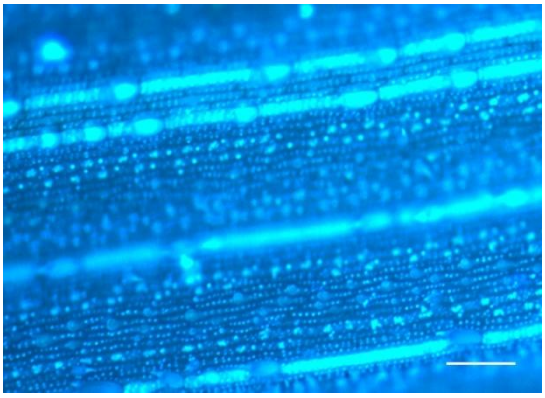
1B) TN1 infested with BPH



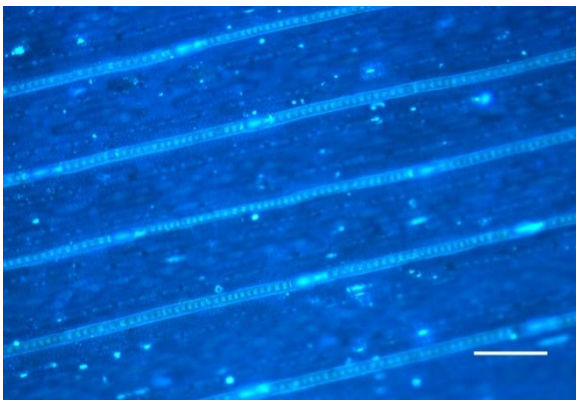
2A) Non-infested IR70 control



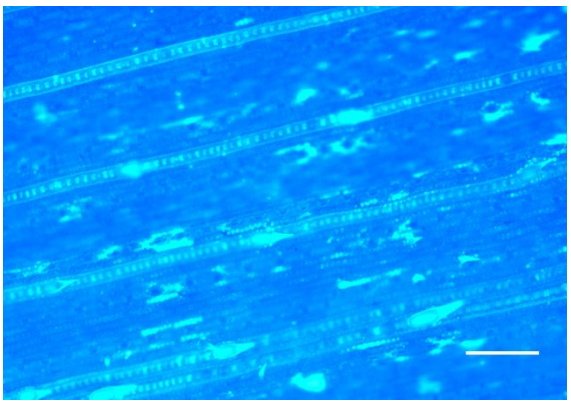
2B) IR70 infested with BPH



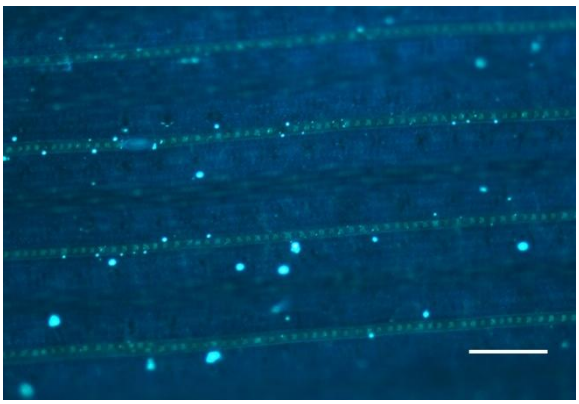
3A) Non-infested 462-T2-18 control



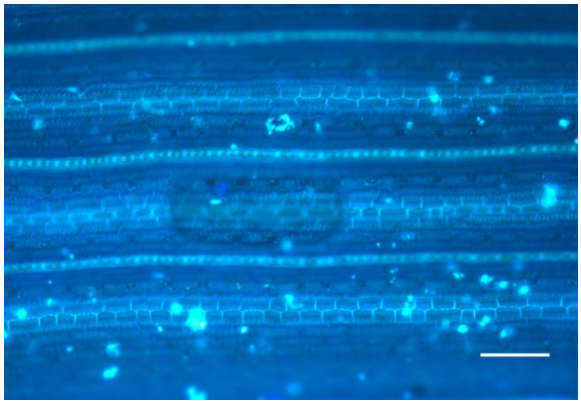
3B) 462-T3-18 infested with BPH



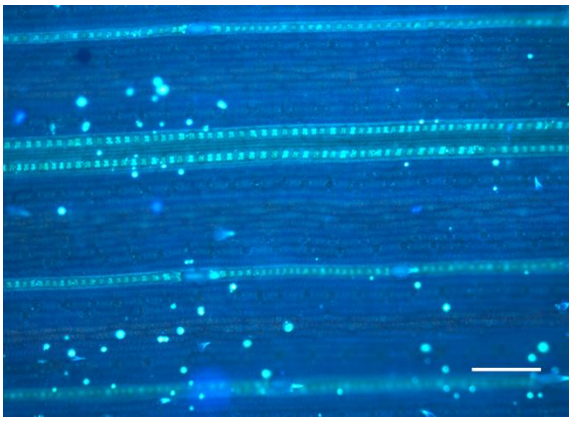
4A) Non-infested 462-T3-16 control



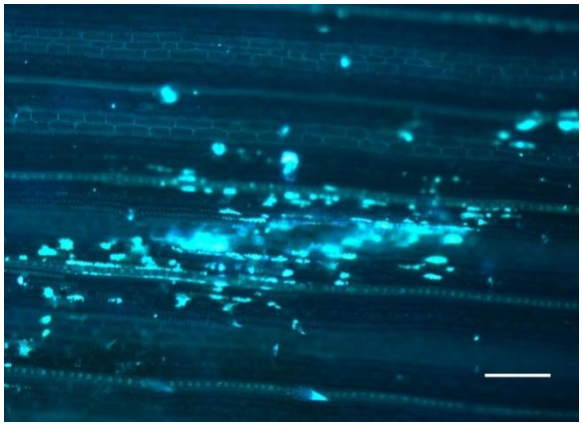
4B) 462-T3-16 infested with BPH



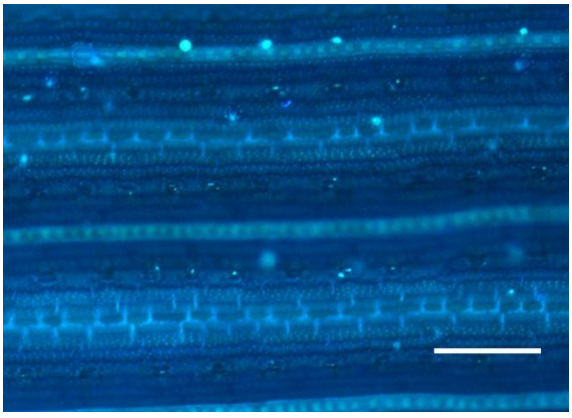
5A) Non-infested 463-T3-26 control



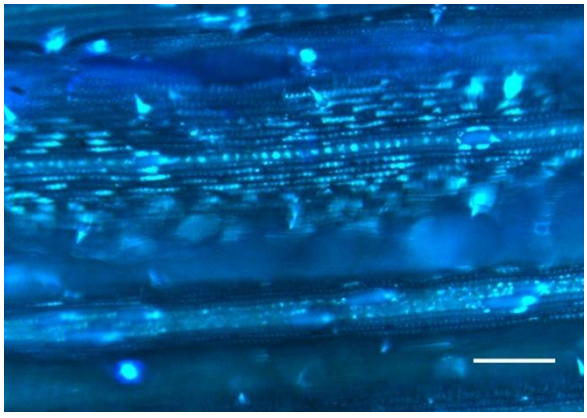
5B) 463-T3-26 infested with BPH



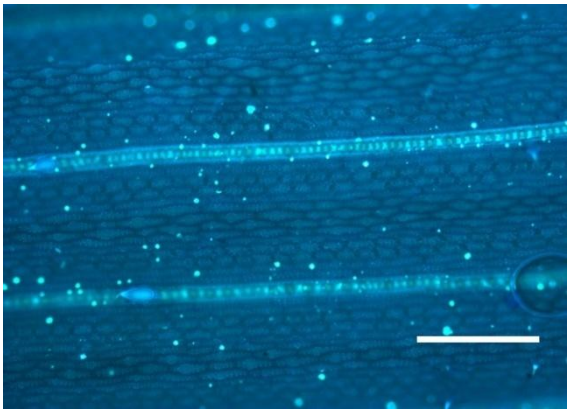
6A) Non-infested 463-T3-41 control



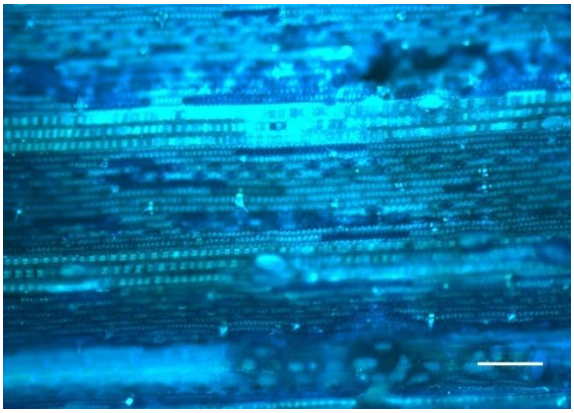
6B) 463-T3-41 infested with BPH



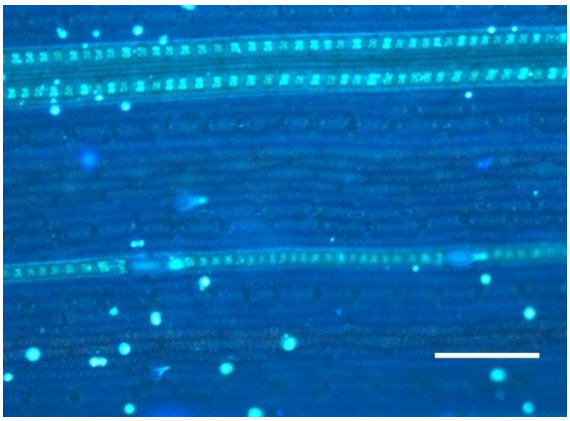
7A) Non-infested 463-T3-8 control



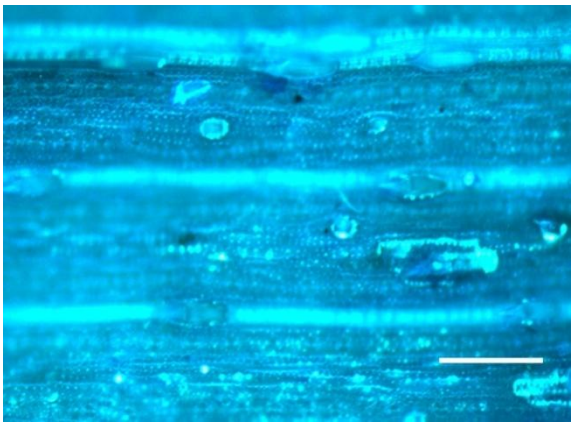
7B) 463-T3-8 infested with BPH



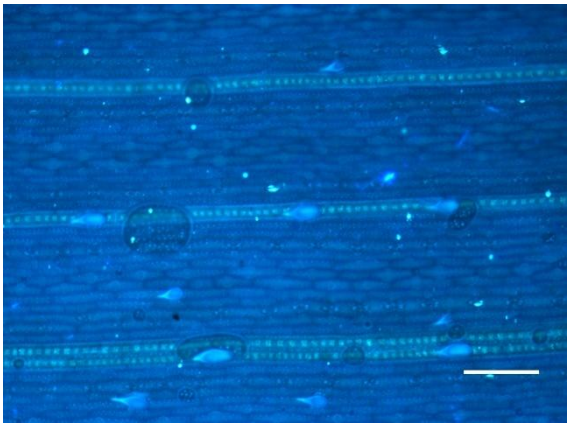
8A) Non-infested 463-T3-22 control



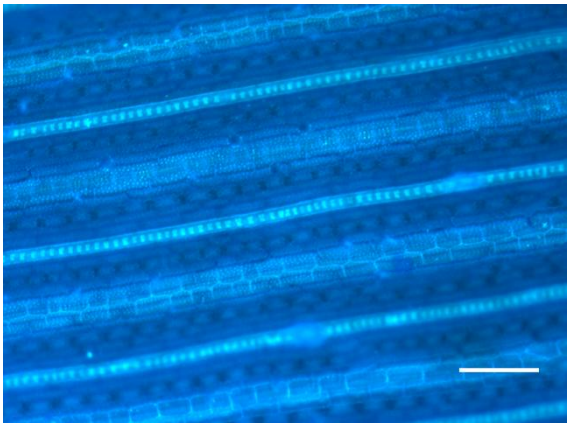
8B) 463-T3-22 infested with BPH



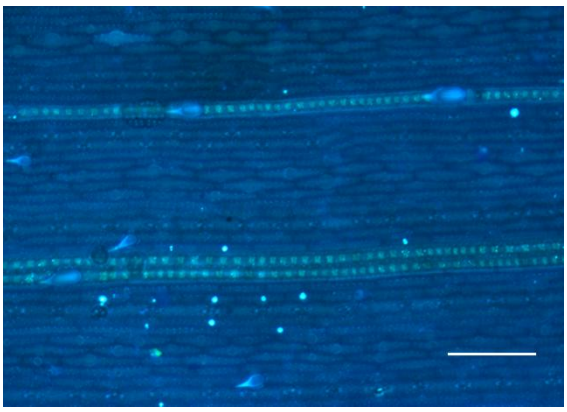
9A) Non-infested 463-4 control



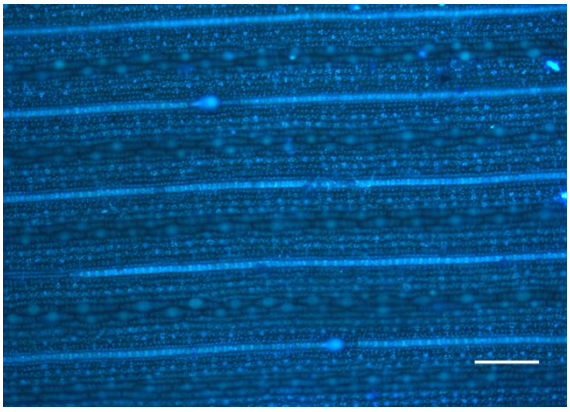
9B) 463-4 infested with BPH



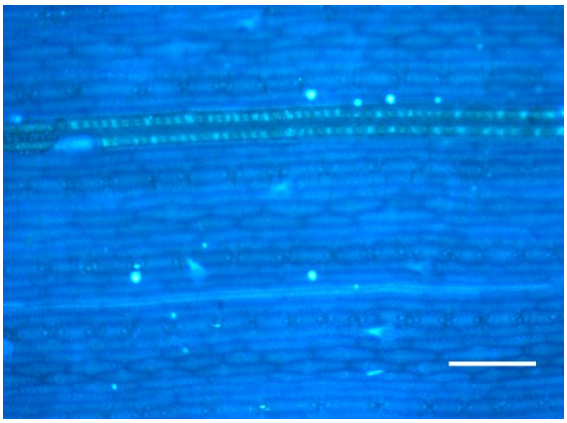
10A) Non-infested 462-20 control



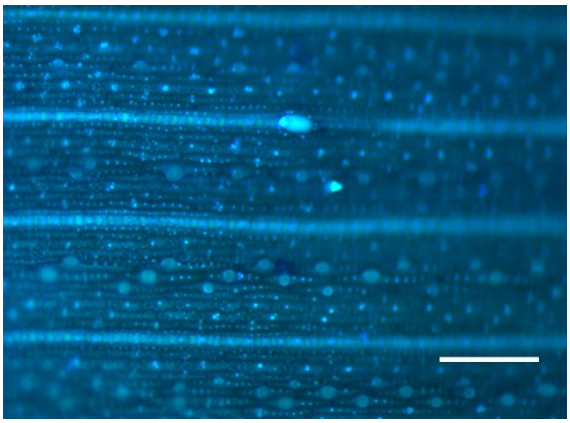
10B) 462-20 infested with BPH



11A) Non-infested 462-33 control

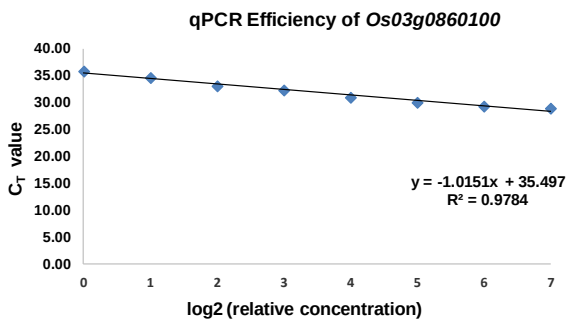


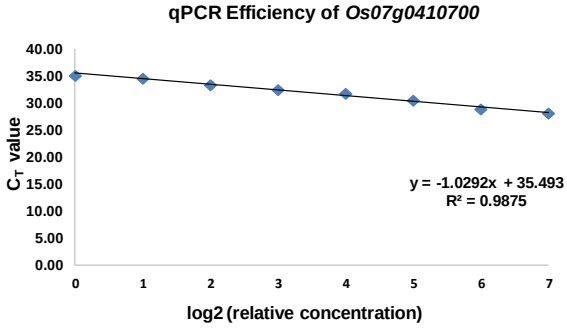
11B) 462-33 infested with BPH

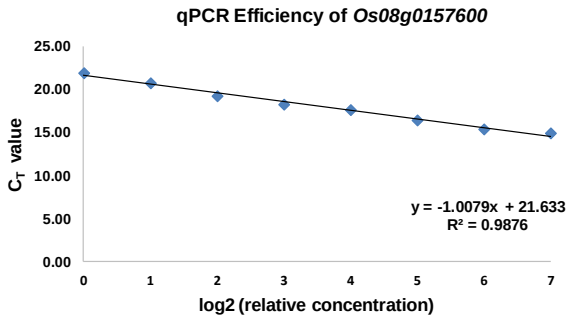


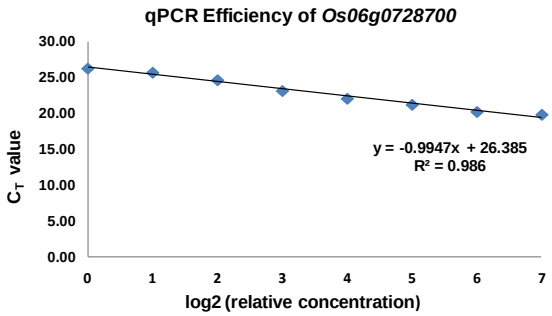
Appendix B

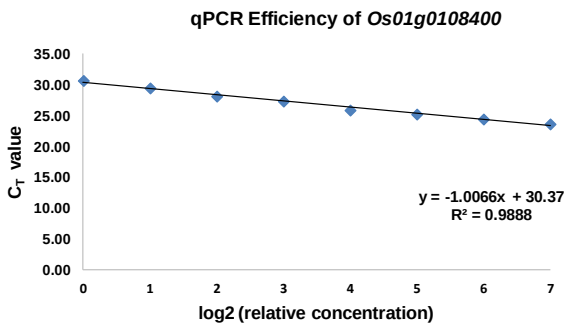
qPCR efficiency of primers used in this present study

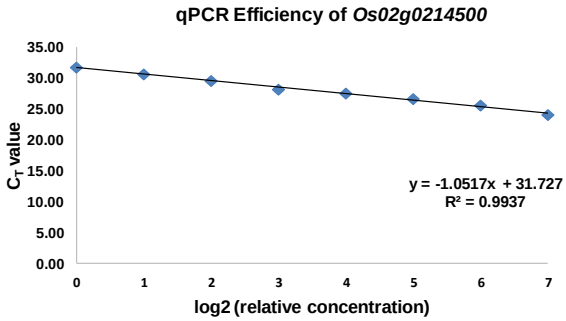


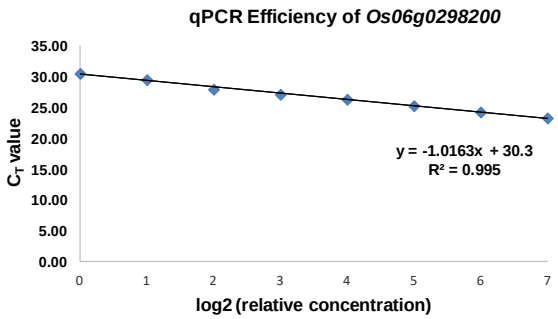


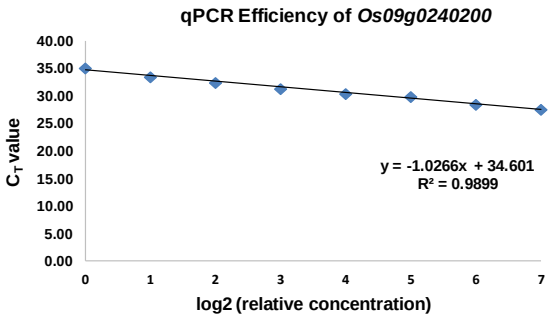


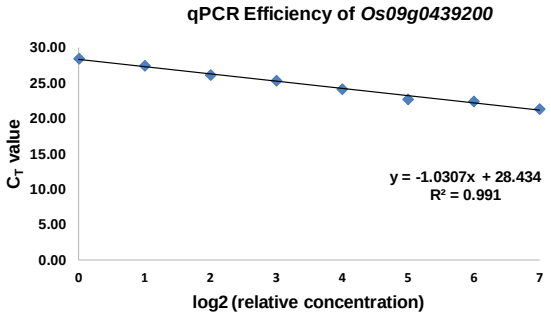


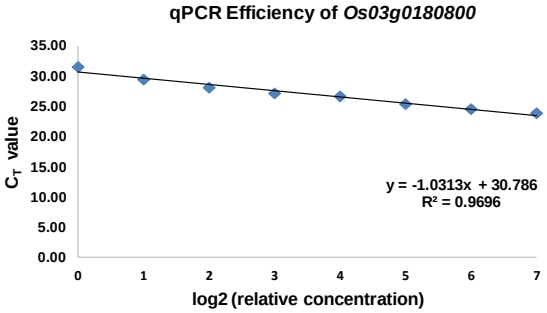


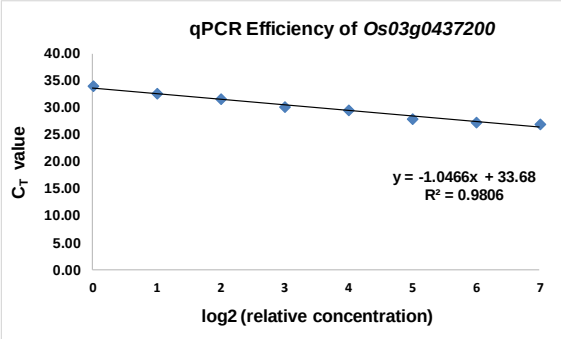


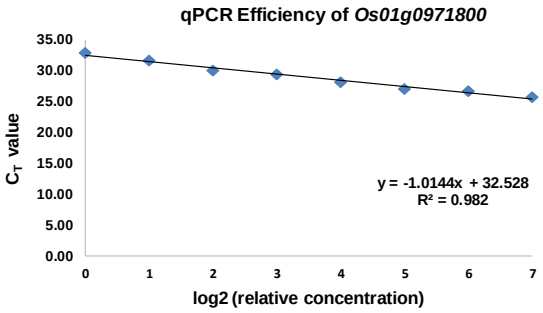


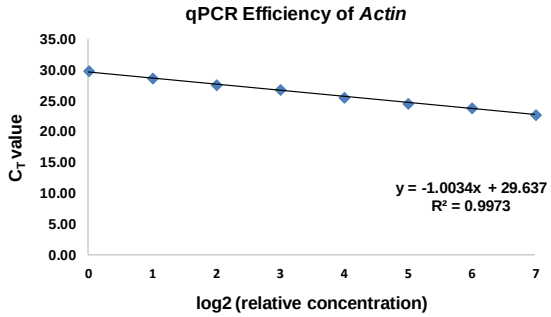


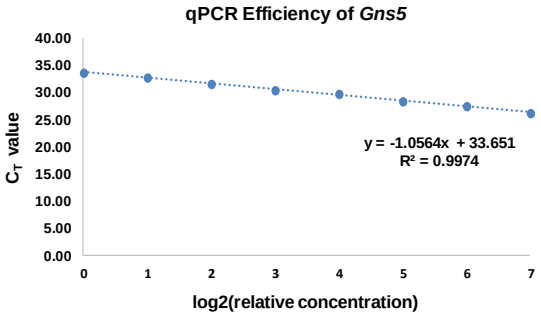

qPCR Efficiency of Os09g0439200
C_T value
log2 (relative concentration)
y = -1.0307x + 28.434
R² = 0.991


qPCR Efficiency of Os03g0180800
C_T value
log2 (relative concentration)
y = -1.0313x + 30.786
R² = 0.9696


qPCR Efficiency of Os03g0437200
C_T value
log2 (relative concentration)
y = -1.0466x + 33.68
R² = 0.9806


qPCR Efficiency of Os01g0971800
C_T value
log2 (relative concentration)
y = -1.0144x + 32.528
R² = 0.982


qPCR Efficiency of Actin
C_T value
log2 (relative concentration)
y = -1.0034x + 29.637
R² = 0.9973


qPCR Efficiency of Gns5
C_T value
log2(relative concentration)
y = -1.0564x + 33.651
R² = 0.9974