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Molecular Approaches to Understand Plant-Insect Interactions to Enhance Pest Control

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Doctor of Philosophy

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Abstract

Background: Phloem-feeding insects cause significant crop damage worldwide, but despite this little is understood about how plants protect and defend themselves from these threats. Phloem-feeding insects are very specific in their mode of feeding and present a unique stress on plant fitness. Not only do these insects feed for long periods of time on host plants, but they also act as vectors for plant viruses. The Brown planthopper (BPH)-Rice and Aphid-Arabidopsis systems provide good models for studying the induced responses in plants to phloem-feeding insects.

Results: In BPH-rice interaction studies, the results showed that 29% of differentially expressed genes in response to BPH feeding were involved in stress responses in plants. Of particular interest was the differential expression of genes encoding the pathogen related proteins β-1,3-glucanase 1, 2 and 5 genes and genes encoding callose synthase 1, 3 and 5. QRT-PCR results have shown that genes encoding callose synthase 1 and 5 (GSL1 and GSL5) were highly expressed in both the moderately resistant IR64 and the resistant IR70 rice cultivars; they were however down regulated in the BPH susceptible cultivar TN1. Similarly, genes encoding the GTP binding protein were expressed to higher levels in cultivars IR64 and IR70 in response to BPH feeding, compared to TN1. In contrast, genes involved in callose degradation, namely β-1,3-glucanase genes 1, 2 and 5 (Gns1/Osg1, Gns2 and Gns5) were highly expressed in the susceptible cultivar in response to BPH feeding; Osg1 and Gns2 were not expressed in either IR64 or IR70, while β- Gns5 was down regulated in both resistant cultivars, compared to the susceptible cultivar (TN1). This differential gene expression in response to BPH feeding might suggest an important role for these genes in plant defence against phloem-feeding insects. Further studies demonstrated that the exogenous application of hydrogen peroxide to the susceptible rice cultivar TN1 improved resistance of this cultivar to BPH to moderate. GTP binding protein, Callose synthase GSL1 and GSL5 genes were up-regulated, while β-1,3-glucanase genes Gns1, 2, 3 and 5 were down-regulated in response to BPH feeding, suggesting that reactive oxygen species generated under hydrogen peroxide treatment might play a role in bringing about the responses leading to resistance.

In aphid-*Arabidopsis* interaction studies, aphid bioassays showed that oxidative signal inducible protein kinases (Oxi1 serine-threonine MAPKs), β-1,3-glucanase Gns1, Gns2 and Gns3 mutants were resistant to aphid feeding and they could survive until the seeding stage when infested. However, Camta3-1, Camta3-2 (calmodulin binding transcription activators), and the Oxi1 null mutant (oxidative signal inducible with no-function) died in response to aphid infestation before reaching the seeding stage.

Furthermore, Col-0 (Columbia) and WS2 (Wisconsin) wild type backgrounds for Oxi1 and Oxi1 null mutant respectively, died quickly under aphid feeding. Gene expression analysis using QRT-PCR on the aphid resistant Oxi1 mutant and the susceptible parental line demonstrated that transcripts for callose synthase gene GSL5 were expressed at a higher level in the Oxi1 mutant compared to Col-0. Whilst β-1,3-glucanase Gns1, 2, 3 and 5 genes were down-regulated in the Oxi1 mutant in response to aphid feeding, β-1,3-glucanase Gns2 gene was induced in Col-0 to high levels in response to aphid feeding.

Application of hydrogen peroxide putatively induced the oxidative inducible signalling (Oxi1 serine-threonine) MAPKs. Induction of Oxi1 stimulated callose production probably via a Ca^{2+} signalling pathway. Application of hydrogen peroxide to Col-0 improved the resistance level of this susceptible line in response to aphid feeding. Transcript expression analysis demonstrated that GSL5 was expressed at high levels in response to aphid feeding, while β-1,3-glucanase Gns2 gene was down-regulated in response to hydrogen peroxide treatment. In addition Gns1, 3 and 5 genes were not expressed in response to aphid feeding. Interestingly, hydrogen peroxide increased the susceptibility of the Oxi1 mutant to aphid attack.

Conclusion: β-1,3-glucanase Gns2 gene might play an important role in plant susceptibility to phloem feeding insects in both monocots and dicots. Evidence from the present study suggests that callose synthase GSL5 plays an important role in plant defence against insects and may be a key gene in insect/wound response in plants. The application of hydrogen peroxide induces Oxi1 serine-threonine MAPKS and increased callose production via a Ca^{2+} signalling pathway and caused a down-regulation of β-1,3-glucanase Gns 1, 2, 3 and 5 genes. Over expression as well as down-regulation of Oxi1 may increase plant susceptibility to phloem feeding (BPH-aphids) insects suggesting that specific levels of Oxi1 are required.

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Dedication

This thesis is dedicated to the memory of my father, Mansour Menshawi Shoala, who died in the 11th of November 2008. May Allah have mercy upon his soul and grant him the highest above in Jannat (Paradise). I miss him every day.

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First and foremost, I have to thank my parents for their love and support throughout my life. Thank you both for giving me strength to reach for the stars and chase my dreams. My sisters, Aml, Yousrya and Mahasen; brothers, Medhat, Mohsen and Amr; auntie, Eetmad, my wife Dina Faruk Enany and Said Arafat deserve my wholehearted thanks as well.

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List of abbreviations

APXs: Ascorbate Peroxidase

BPH: Brown planthopper

Ca^{2+}: Calcium Pathway

Cals: Callose synthase

Camta3: Calmodulin-binding transcription activator

CATs: Catalase

CBLs: Calcineurin B-like proteins

CC: Companion cells

CDPKs: Calcium-dependent protein kinases

Col-0: Columbia-0 (*Arabidopsis* wild type)

E1: Salivation into sieve elments

E2: 2^{nd} salivation

EST: Expressed sequence tag

FM: Functional megaspore

Gns1: β-1,3-glucanase 1

Gns2: β-1,3-glucanase 2

Gns3: β-1,3-glucanase 3

Gns5: β-1,3-glucanase 5

GPx: Glutathione Peroxidase

GSL1: β-1,3-Glucan synthase 1 (callose synthase)

GSL3: β-1,3-Glucan synthase 3 (callose synthase)

GSL5: β-1,3-Glucan synthase 5 (callose synthase)

GTP: GTP binding protein (small protein regulating callose synthase)

H_2O_2: Hydrogen Peroxide

IR: Rice crosses made at IRRI are assigned a number with IR (international rice) as a prefix.

IR64: Moderate resistant rice cultivar IR64 to BPH

IR70: High resistant rice cultivar to BPH

IRRI: International Research Rice Institute

JA: Jasmonic acid

MAPK: Mitogen-Activated Protein Kinases

NADPH: Nicotinamide adenine dinucleotide phosphate

Oxi1: Oxidative inducible signalling (serine- threonine protein kinase)

PCR: Polymerase Chain Reaction

PD: Plasmodesmata

Pd: Potential drop

PI: Protease Inhibitors

QRT-PCR: Quantitative Real Time PCR

RAPD: Random Amplified Polymorphic DNA

RBOH: Plant Respiratory Burst Oxidase Homologs

RFLP: Restriction fragment length polymorphism

ROS: Reactive Oxygen Species

SA: salicylic acid

SE: Sieve elements

SODs: Superoxide Dismutase

SSH: Suppression Subtractive Hybridization

SSR: Simple Sequence Repeat

TN1: Taichung native 1

WS2: Wisconsin 2 (*Arabidopsis* wild type)

Chapter 1. General introduction

1.1 Food security

The State of Food Insecurity in the World 2011 highlights the differential impacts that the world food crisis of 2006-08 had on different countries, with the poorest being most affected (FAO, 2011). While some large countries were able to deal with the worst of the crisis, people in many small import-dependent countries experienced large price increases that, even when only temporary, can have permanent effects on their future earnings capacity and ability to escape poverty.

The above report focuses on the costs of food price volatility, as well as the dangers and opportunities presented by high food prices. Climate change and an increased frequency of extreme weather events, increased linkages between energy and agricultural markets due to growing demand for biofuels, and increased financialization of food and agricultural commodities all suggest that price volatility is here to stay. The report describes the effects of price volatility on food security and presents policy options to reduce volatility in a cost-effective manner and to manage it when it cannot be avoided. It will be important to provide improved market information, create gender-sensitive safety nets that are designed in advance and can be implemented quickly in times of crisis, and invest in agriculture for the long-term to make it more productive and resilient to shocks (FAO, 2011).

New and on-going driving forces are redefining the world food situation. Their combined effect, although impossible to quantify, stands to be a challenge for future food security. Scarcity is expected to define food production in the coming decades, and include scarcity of water, and energy, exacerbated by climate change. Competition for land will also be fierce, due to land degradation, urbanisation, the large-scale growing of biofuel crops and potential carbon sinks. Demand for food is growing in line with population and income growth. Globalisation and urbanisation are also contributing to dietary preferences switching them towards more resource-intensive food stuffs. Still we believe the growing population (estimated at approximately nine billion in 2050) can be fed, provided the right actions are taken. This requires sustained productivity growth in the agricultural sector in an environmentally and socially

sustainable manner. Innovation through a cross-sectoral approach is essential. Particularly promising are the fields of information and communication technology (ICT) and biotechnology, but also ecologically integrated approaches. The latter work with whole systems rather than individual crops and distributes knowledge, power and autonomy to farmers. While it is critical to boost food production, the world's systems for producing and distributing food will also need to change, so they can better cope with shocks and stresses, make more considerate use of resources and ensure more equitable access to food. Smallholder production is one important key; currently 1.5 billion people live in households depending on small farms (FAO, 2011). In order to move from subsistence to commercial farming, smallholder farmers need access to education, knowledge, assets, credit, markets and risk management. Reforms are essential in the areas of agricultural support, food aid, trade liberalisation, support regimes for biofuels and intellectual property rights. The possibility of better global governance mechanisms for food security should be examined.

1.1.1. Global food security Cereal crop production across the world

World cereal production in 2011 is expected to be more than sufficient to cover the anticipated utilization in 2011/12. Improved supply outlook resulted in declining prices during the second half of 2011 with a sharp fall in December. However, grain prices rebounded in January, mostly reflecting worries about weather conditions affecting 2012 crops in several major producing regions (FAO, 2011).

FAO has raised its forecast for 2011 world cereal production by 4.6 million tonnes since December to 2, 327 million tonnes, which would be 3.6 percent up from 2010 and a new record. Much of the upward revision is attributed to coarse grains, the production of which is likely to increase by 2.3 percent, to nearly 1, 152 million tonnes, 4 million tonnes more than reported in December. The forecast for global paddy production in 2011 has been upgraded by 800, 000 tonnes since the previous report, to a new high of 481 million tonnes (in milled terms) mainly on improved prospects for crops in Asia. If confirmed, world rice production would increase by 3.1 percent over the 2010 value. Following the

completion of 2011 wheat harvests, the forecast for world wheat output is now at a new record of 694.5 million tonnes, up 6.3 percent from 2010 and nearly unchanged from the December forecast.

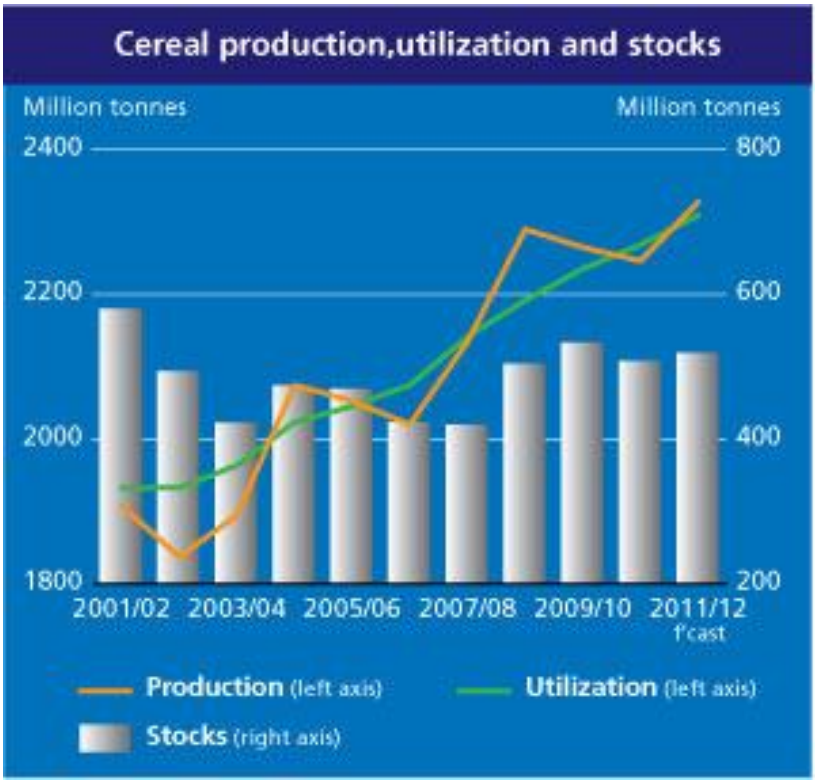

Fig 1.1. World Cereal production, utilization and stocks from 2001 up to 2012 (FAO, 2011).

The forecast for world cereal utilization in 2011/12 is lowered slightly from December, to nearly 2, 309 million tonnes, but still 1.8 percent higher than in 2010/11. Among the major cereals, total wheat utilization is anticipated to register the sharpest year-to-year growth, increasing by almost 3 percent to 682 million tonnes. At this level, wheat utilization would exceed its 10-year trend value by 2.6 percent. The main factor behind this faster growth is the expected sharp expansion in feed utilization of wheat, driven by large world supplies and competitive prices of wheat relative to maize. By contrast, total utilization of coarse grains is forecast to increase marginally (less than one percent) in 2011/12, to 1156 million tonnes, below its 10-year trend, as growth in the demand for feed is forecast to slow down due to higher intake of wheat and non-grain feed ingredients. The industrial use of coarse grains is also seen to rise at a much slower pace than in the past, mostly because of stagnating

maize-based ethanol production. On the other hand, the strong expected gains in world rice production are foreseen to result in an 11 million tonne, or 2.3 percent, increase of global rice utilization in 2011/12 to 471 million tonnes, with average per caput rice consumption up 1 percent, to 57.1 kg.

Based on the latest prospects for cereal production in 2011 and cereal utilization in 2011/12, the FAO's forecast for world cereal ending stocks by the close of seasons in 2012 has been raised by 5 million tonnes to 516 million tonnes. This would imply a 10.5 million tonnes gain from its revised opening level - raised by 4.5 million tonnes, following upward revisions to maize inventories, mostly in the United States. Based on the current forecast for ending stocks and anticipated utilization, the world cereal stocks-to-use ratio in 2011/12 would remain at the December estimate of 22 percent, nearly unchanged from the previous season and slightly higher than the 5-year average. World rice and wheat stocks are forecast to increase the sharpest, resulting in their stock-to-use ratio rising to 32 percent and 29 percent respectively, well above their 10-year averages. However, world coarse grain inventories are expected to be drawn down sharply (by 4 percent) this season, especially in the United States. Lower inventories would lead to a third consecutive drop in world stock-to-use ratio of coarse grains, to 14 percent, the smallest ratio registered since the start of record by FAO in 1980.

World cereal trade in 2011/12 is currently forecast to approach 288 million tonnes, up slightly since December and 2.6 percent higher than in 2010/11. Larger wheat exports account for most of the growth. At a 4-year high of 134 million tonnes, world wheat trade is boosted by the strong recovery in exportable supplies in the CIS countries more than offsetting sharp declines in shipments from the EU and the United States. However, world trade in coarse grains is anticipated to remain flat at 121 million tonnes, as much of the growing demand for feed is expected to be met by larger imports of wheat. At 32.8 million tonnes (milled basis), rice trade in 2012 would be 5 percent lower than in 2011, with much of the decline driven by a cut in purchases by Asian countries.

The improvement in global cereal supply situation in the face of largely subdued world demand put downward pressure on international prices of cereals during the second half of 2011, with the December FAO Food Price Index falling to its

lowest level since October 2010. In January, grain prices were up from their dip in December, supported by unfavourable weather in important growing regions as well as prospects for a decline in export supplies in the CIS. However, rice export prices remained on a downward trend, under seasonal harvest pressure and intense competition for market shares among exporters.

1.2. Rice

1.2.1. Origins

Rice is an ancient cereal crop and it has been grown for more than 10,000 years; it is a staple food of two third of the world's population (Isawa, 1996; Giri and Laxmi, 2000). Because of the long history of its cultivation and widespread cultivation, rice is the most genetically diverse of the world's cereal crops. Domesticated rice plants consist of two species – *Oryza sativa* and *Oryza glaberrima.* Among these two species, most of the research has been focused on *O. sativa* because it is the main cultivated rice in the majority of the rice growing regions (Isawa, 1996). *O. sativa* is an important cereal crop, which belongs to the grass family Poaecea in the Plant kingdom (Gnanamanickam, 2009). It is diploid, annual and a short day plant, which can self-fertilize. It is also one of the essential nutritional crops of mankind.

1.2.2. Distribution

Rice originated in China and Thailand. The crop further spread into other countries like Cambodia, Vietnam and southern parts of India, whilst the sub species expanded to other Asian countries like Korea, Pakistan, Japan, Myanmar, Sri Lanka, and Indonesia. After the middle of 15th century rice was grown in Italy, France and other European countries. During 18th century, the Spanish brought rice to South America. The domestication of rice is considered to be one of the most important developments in world history as it is the longest continuously grown cereal plant (Gnanamanickam, 2009).

1.2.3. Global importance

More than 3 billion people consume rice as a major form of daily calorie (50% to 80 %) intake; 150 million hectares of land are used for rice cultivation yearly and the annual world rice production was approximately 6 million tonnes in the last

few years (Delseny *et al*., 2001). It is estimated that by 2025, the annual rice production should increase by 60% to meet the future needs of the growing global population. Rice, thus being the staple food of around two-thirds of world population, plays a major role in eliminating poverty and malnutrition in rice cultivated countries (Gnanamanickam, 2009). Therefore there is a need to focus on rice productivity and cultivation to underpin food security. However, rice production is affected by many factors like environmental stress, diseases and pests. Around 200 million tons of rice is lost due to these factors annually.

1.2.4. World rice production

In 2011 world rice production was reduced by 1.7 million tons to 463.7 million tons, still a record, largely due to lower yields for Burma, Colombia, Egypt, and Indonesia, which are partially offset by increased productivity for Bangladesh, Thailand, and Vietnam. Global consumption was reduced by 4.1 million tons to 458.8 million, again a record, largely due to reductions for Burma, Egypt, India, Pakistan, and Thailand, partially offset by increases for China, EU-27, and Iran. Forecast global exports for 2011/12 are raised by 0.8 million tons to 33.9 million tons, down nearly a million tons from the record of 2010/11. Projected imports are raised for China, Egypt, EU-27, and Indonesia. Forecast exports are raised for India, Vietnam, and the United States, and lowered for Burma, China, and EU-27. Global ending stocks are projected at 103.3 million tons, up nearly 3.0 million tons from March 2012, an increase of almost 5.0 million tons from 2010/11, and the largest since 2001/02. Burma, Indonesia, EU-27, Pakistan, the Philippines, and Thailand account for the bulk of the upward revision in global ending stocks (WASDE, 2012).

1.2.5. Importance as a model species

Cereals are the most important group of crops as they account for approximately 60% of the world's agricultural production (Goff et al., 2002). Rice is one of the most widely grown cereals after maize and wheat and it accounts for approx. 30 % of the world's cereal production (Gnanamanickam, 2009). Although cereals have a long period of evolution from their common ancestral species, they have highly conserved genomes. The comparisons of physical and genetic maps of the cereals display conservation in their gene order and gene orientation. In spite of these similarities, they have varying genome sizes.

Among the different cereals, the rice genome is comparatively small (420 Mbp). Due to this small size and the high genome conservation of rice, it is an attractive target for the genomic studies of cereal crops (Goff et al., 2002). It thus used both as an important target crop and a model cereal for molecular studies.

The rice genome project (RGP), headed by a Japanese research group, constructed a genetic map of rice with 1383 DNA markers. This rice map is one of the most extensive DNA-marker maps produced for a higher plant. It contains a large number of expressed genes. A comparison of this rice map to other cereals showed that the order of genes in the rice chromosome is similar to wheat and maize (Isawa, 1996). The synteny among different genomes of cereal crops can be explained by the common ancestry of cereals from monocotyledonous plants. The rice genome also helped in the identification of genes in other cereals (Isawa 1996).

RGP also analysed 20,000 cDNAs from different rice tissues developed under varying conditions and has registered 10,988 ESTs. These ESTs provide significant help in identifying homology of other species. For instance, one EST of rice showed around 97% homology to the second AP2 domain, which suggested that the rice EST had homology to the AP2 gene in *Arabidopsis*, which determines the identity of floral organs (Isawa 1996).

1.3. Effect of pests on rice

Rice is one of the world's most important food crops. However, it also serves as an important food source for insects and is attacked by around 800 species, in both the field and during storage (Barrion and Litsinger, 1994). Biotic factors such as insect pests and microbes cause huge losses in rice productivity. Around the worldwide, the estimated rice production loss due to pests and diseases is 30%, of which 13% is due to insect damage (Gatehouse *et al.* 1992). Since rice grows in different types of soil and requires a humid environment, insect damage is highly probable in these regions as these conditions are favourable for the growth of many insects' species (Giri and Laxmi, 2000).

1.3.1. Rice Brown planthopper

The rice Brown planthopper (BPH) *Nilaparvata lugens* Stål is one of the most economically important insect pests, which can cause huge damage of rice plants. BPH can cause direct damage to rice plants by removing the phloem sap and also by transmission of viral disease during feeding such as ragged stunt virus and grassy stunt virus (Velusamy and Heinrichs, 1986; khush and Brar, 1991). Extensive usage of pesticides to control BPH cause serious problems including toxicity to natural enemies of BPH such as *Anagrus nilaparvatae* (Wang et al., 2008), harm to the environment, an increase in the evolution of resistance of BPH to pesticides, and an increase in total production costs, as well as possible long term damage to the agro-ecosystem and human health (Huang et al., 2001; Rola and Pingali, 1993). BPH, like as aphids are sap-sucking insects that have the ability to overcome many adaptations that plants have evolved as defence mechanisms. The interaction between sap-feeding insects and their host plants is complicated, but recent scientific advances have helped in the understanding of these dynamic interactions. Comparing feeding behaviour of BPH and aphid in susceptible and resistant plant cultivars allows the underlying defence mechanisms to be identified and may lead to new strategies to improve resistance in susceptible plants.

1.3.1.1. Feeding physiology

1.3.1.2. Structure of Mouth parts

The BPH as a homopterous insect has specialized mouth-parts for the abstraction of plant sap. The stylet is the most obvious element of the mouth, and functions as a piercing and sucking organ; it is about 650-700 µm long. It consists of an outer pair of mandibular and inner pair of maxillary stylets (Sogawa, 1973). The maxillary stylets are interconnected together to form two canals. The dorsal canal functions as a sucking canal for plant sap and it connects with the sucking pump through the pharyngeal duct, whilst the ventral canal is the excretion salivary canal for the insect saliva. The dorsal (food) canal is bigger than the ventral canal. The tip of the maxillary stylet is incurved and sharply pointed, whilst the mandibular stylet is pointed along its outer edges. Both the maxillary and mandibular stylets contain one or two nerves and hollow ducts.

1.3.1.3. Salivary Secretions

The electrical penetration graph (EPG) technique has been used to follow, discover and record the BPH feeding behaviour within plants (Tjallingii, 1978, 2006). Four stages of saliva secretion with two types of secreted saliva have been identified. Gelling salivation, which is the first stage, occurs during the pathway phase and forms a sheath of saliva enveloping the stylets in the plant tissue intercellularly; the other three stages comprise the watery salivation. Solid or sheath saliva is made of stable substances and remains in the plant tissues after removal of the stylet (Sogawa, 1973). The saliva is excreted by the salivary glands (Sogawa, 1967), which consists of eight different types of secretory sacs (Sogawa, 1965). The stylet sheaths play an important role in protecting and pushing the stylets beyond the labial tip and enable them to function as piercing and sucking mouthparts (Sogawa, 1971). They support the stylet during the penetration, by covering or sealing them into the sucking sites of the plant tissues. In addition to sheath salivation, watery salivation occurs during intracellular punctures that occur regularly throughout pathway activity and during phloem feeding. The soluble saliva contains digestive enzymes excreted by salivary glands. The enzymes α-glucosidase, which hydrolyses sucrose and trehalose, and β-glucosidase, which acts on phenolic glucosides such as arbutin and salicin, were detected in the salivary glands of BPH (Sogawa, 1968). It is thought that the watery saliva blocks the defence response in sieve elements when wounded (Knoblauch and Van Bel, 1998; Eckardt, 2001; Will and Van Bel, 2006).

1.3.1.4. Feeding

BPH predominantly sucks the plant phloem sap (sogawa, 1980). After landing on rice plants it starts to locate the weakest point in the plant and then inserts the stylet bundle with an accompanying salivary sheath into the plant (Spiller, 1990). BPH targets the sieve elements, which are the functional units of the sieve tubes and then starts to ingest the phloem sap (Sogawa, 1982; Seo et al., 2009). Thus BPH feeding has been divided into two main phases, the first phase includes the movement of the BPH stylet across the plant tissue and the second phase involves insect feeding (Hattori, 2001). The feeding process itself consists of both a sequence of behavioural responses to specific botanical

stimuli and responses released spontaneously or according to the internal needs of the insect. The feeding procedures have been divided into four major behavioural phases: orientation to host plants, labial exploration, stylet probing, and sucking.

1.3.1.5. Labial exploration

Before starting stylet probing, the BPH applies the labium horizontally to the plant epidermis and explores the surface by light labial dabbing. This is carried out to locate the areas alongside the leaf veins on the rice plant to identify the weakest point for the stylet to penetrate the vascular bundles. The leaf surface in susceptible rice plants is completely different from those of resistant plants. The leaves of susceptible rice varieties are easier to penetrate, although resistant rice varieties may have weak points under the cellular level or could lose resistance in response to a high number of insect attacks in the same area or position. In rice plants, the surface characteristics of the intervenal epidermis are very different from those of the epidermis over the veins. Rice plants with intervenal epidermis characters is coated with waxy materials and covered with thick layer, which make it hard to be penetrated by BPH, whereas the other one is smooth and easily penetrated by BPH. Thus the surface texture of the host plant plays an important role in plant resistance and susceptibility (Sogawa, 1977; 1982).

1.3.1.6. Stylet probing

BPH excretes small amounts of coagulable saliva during labial insertion into the plant epidermis. This salivary sheath seals the stylets at the site of insertion providing a tight connection for the stylet to act. The stylet leaves a circular mark after its removal, which is known as a feeding mark. Both male and female adults can produce an average of 16 feeding marks in one day on susceptible rice varieties, while in resistance rice varieties they can produce 30 and 50 feeding marks respectively, in one day. This demonstrates that BPH probes much more on resistant rice varieties (IR70, IR64) in comparison to susceptible ones (TN1), presumably as a result of failure to either penetrate the leaf tissues or due to unsuitable sap. The stylet is usually inserted at a slanting angle towards the vascular tissue. However, if stylet insertion occurs relatively far

from the vascular tissue, then the salivary sheath branches into a direction towards the vascular tissue by passing through some of the parenchyma cells. The stylets thus penetrate easily through the parenchyma cells and reach the phloem cells after which it stops penetrating. This movement of the stylet indicates that the phloem cells are its target tissue for sucking (Sogawa, 1982; Tjallingii, 1978, 2006).

1.3.1.7. Sucking and honeydew excretion

After stylet probing, the maxillary stylets protrude beyond the salivary sheath and initiate sucking activity. Female adults spend about 60-90% of their time on rice plants inbibing the sap, a process that lasts for at least 1-7 hours. During the sucking process, the BPH excretes small amounts of clear droplets of liquid excreta known as 'honeydew'. The honeydew is composed of sugars indicating that the BPH is ingesting the sap mainly from phloem. The daily excretion rate of honeydew by female adults on susceptible TN1 rice seedlings is about 13-14 µl. Excretion of honeydew is most active during the period 05:00-09:00, and declines sharply by 13:00 but increases again during the period from 17:00 to 21:00. Analysis of honeydew by paper chromatography has shown that it contains about 2-5% of carbohydrates, composed of glucose, fructose, sucrose, and a few oligosaccharides. The rate of honeydew excretion has been used as a measure of the suitability of different rice varieties to act as hosts for BPH i.e. a measure of resistance/susceptibility (Sogawa, 1970, 1982).

1.3.1.8. Feeding damage

Both the nymphs and adults of BPH accumulate and feed on the leaf sheaths at the basal region of rice plants. BPH causes severe damage on rice plants before and after the flowering stages. Hopper burn is the distinctive sucking damage caused by BPH (Bae et al., 1970). Insects that cause hopper burn symptoms are highly destructive agricultural pests worldwide, causing significant yield loss and control costs (Wilson and Calridge, 1991). The main symptom of hopper burn is an injury that appears as a yellowing of the older leaf blades, which gradually extends to all parts of the plant; in severe cases the whole plant turns brown and dies. Where many plants are affected, brown patches within the field are readily visible. Not only does BPH damage the vegetative tissues (Cagampang et al., 1974), but this subsequently significantly

impacts on the root development (Santa, 1959). Light infestation of rice by BPH is generally shown by fewer panicles and fewer grains per panicle, followed by a low percentage of ripened grain and lighter grain weight after the heading stage (Bae et al., 1970).

1.3.1.9. Brown planthopper biotypes

There are currently four distinct biotypes of BPH, classified according to their reaction on different rice cultivars (Chelliah and Bharathi, 1993). In 1976-1977, Biotypes 1 and 2 were identified as wild strains in the Philippines, Indonesia, and Vietnam, because they could damage varieties with no resistance genes (Khush, 1979); Biotype 3 was identified after rearing BPH on rice cultivar ASD7 (Pathak and Heinrichs 1982); Biotype 4 has been reported in South Asia, from where it originated (Khush, 1984). *Nilaparvata lugens* (BPH) population from Asia and Australia were separate, according to systematic study on the brown plant hoppers (Jones et al., 1996).

1.3.1.10. Brown planthopper control

Brown planthopper (BPH) is one of the most destructive insect pests of rice worldwide *(O. sativa L.)* and can cause severe yield losses. In addition to crop damage, BPH is one of the most important vectors for many viruses and phytoplasma (Velusamy and Heinrichs, 1986; khush and Brar, 1991). Chemical Insecticides are currently the main method to control brown planthopper together with host plant resistance as a part of an integrated pest management strategy. Chemical control is very expensive, destroys most of the natural predators of BPH, and can increase the chance of developing new BPH insecticide-resistant strains. Using induced resistance is a very promising approach, which could be both an economical and efficient method to control BPH as a part of Integrated Pest Management strategy (Way and Emden, 1999).

1.3.1.11. Insecticide resistance in Brown planthopper populations

Outbreaks of Brown planthopper can cause severe hopperburn and total loss of the rice crop if no effective control measures are used (Dyck and Thomas, 1979). In 1914, insects resistance to insectsides was reported by Melander

(Melander, 1914) and since that time the subject has received great attention due to the inability to control different pests (Forgash, 1984; Georghiou, 1986). In 1984, 1797 cases of resistance in arthropods were reported. In 1991, resistance to at least one insecticide was recorded for 504 species (Georghiou, 1986; Georghiou & Lagunes-Tejada, 1991). An insect population also can evolve cross-resistance to several closely related chemicals. In 1984, 17 insect species were reported to be resistant to all the major classes of insecticides (Georghiou, 1986). In Taiwan, Japan and the Philippines, BPH resistance to carbamates and organophosphates were reported as a result of the intensive usage of chemical sprays (Nagata *et al.,* 1979; Heinrichs, 1979). The repeated and indiscriminate application of insecticides is the main reason for the increases in BPH, changing its status from a secondary pest to a primary pest of rice (Soehardjan, 1973). Insect resistance to insecticides or the rapid breakdown of insecticides (Fernando, 1975; Sethunathan, 1971) could be a part of the problem. The other result of insecticide usage is the unintentional killing of natural enemies of the target pest (Alam, 1971; Fernando, 1975; Nishida, 1975a.b; Cheng 1976). In 1976, spraying parathion killed predators of BPH and caused an increase in the BPH population (Stapley, 1976). In 1977, BPH densities at IRRI were seen to increase after the application of methyl parathion (Dyck and Orlido, 1977), primarily as a consequence of killing of BPH predators resulting in an increase in the population of BPH (IRRI, 1977). In 1983, Chung and Sun observed that carboxylesterase hydrolysis was involved in BPH resistance to malathion (Chung and Sun, 1983). Subsequently, Dai and Sun (1984) proposed that enhanced esterase activity also conferred a major part of the observed resistance of BPH to permethrin and other pyrethroids of primary alcohol esters, such as phenothrin. Chang and Whalon (1987) resolved eight esterases with isoelectric points ranging between 4.3 and 5.3 from multi resistant BPH homogenate using isoelctric focusing; all eight forms were able to hydrolyze α- and β-naphthyl acetate, malathion, *cis*- and *trans*-permethrin at different rates *in vitro*.

1.4. Plant-Insect interactions

Plants-insect interactions are complex and dynamic. When insects attack plants, the plants respond with different strategies in order to reduce and stop

the insect damage. Plants possess both a constitutive defence system and an inducible defence system (Gatehouse, 2002). This induced response plays a very important role in conferring resistance against both biotic (including insects and pathogens) and abiotic stress (Maleck and Dietrich, 1999). The phytohormones like salicylic acid, ethylene and jasmonic acid are considered as important signalling molecules in plant-insect interactions that activate defensive genes (Remond and Farmer, 1998). Ethylene is involved in the down regulation of a subset of a wound-inducible, jasmonic acid dependent genes, and most of the genes that could be up regulated by plant pathogen interaction also could be induced by ethylene exposure (Deikman, 1997). In general plant-feeding insects can be classified as chewing or piercing/sucking insects according to their mechanism of feeding. Chewing insects cause extensive damage to the plant foliage, leafs, stems and roots. On the other hand piercing /sucking insects consume plant fluids as a nutrient source (Stotz et al., 1999, Karban and Baldwin, 1997). As a result of differences in insect feeding behaviour, different sets of plant genes are induced by chewing insects compared to those induced by sap sucking insects.

1.4.1. Signalling defence mechanisms

Plants are continuously challenged with various biotic and abiotic stresses and thus have evolved different defence mechanisms to cope with these different forms of stress. It is complicated to understand the biology of a single cell or cells in tissues in response to different stresses. Various pathways of complicated cellular signalling are activated to enable the systems to respond to stress. In plants, biotic and abiotic stress induce different signalling cascades that activate production of Reactive Oxygen Species (ROS), kinase cascades, and ion channels accumulation of hormones such as salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and abscisic acid (ABA). These signals switch on defence genes that lead to an overall defence reaction. The expression levels of defence genes vary at different growth stages of the plant and are influenced by different types of insect feeding and different environmental stimuli. The defence response is a well-controlled and complex signalling mechanism leading to host defence in response to abiotic and biotic stimuli. The defence response normally occurs at the site of infection/attack (local response), but the defence signalling is also triggered in the healthy tissues as

a systemic response. Mechanical wounding (abiotic stress) and chewing insects (biotic stress) trigger jasmonic acid and ethylene as part of the wounding response (Kessler and Baldwin, 2006), whereas sap sucking insects and pathogens trigger salicylic acid (Gatehouse, 2002).

In plants, Ca^{2+} and ROS are considered as important signalling molecules especially in the early response phases to both biotic and abiotic stresses. In plant cells, the expression levels of Ca^{2+} and ROS increase rapidly and within seconds in response to stress. Calcium is considered as the key signal transducer in the activated signalling cascades in the plant response to any stress and the Ca^{2+} cation is considered as an important key at which crosstalk between different pathways can occur (Dey et al., 2010; Takahashi et al., 2011). As a result of insect feeding, Ca^{2+} influx occurs dramatically followed by induction of Ca^{2+}-dependent signal transduction pathways, which include MAPK (mitogen-activated protein kinases) kinases downstream cascades (Arimura and Maffei, 2010). Ca^{2+} influx through membrane Ca^{2+} ion channels (Errakhi et al., 2008) and the calcium signals are transduced downstream through multiple pathways by calcium-interacting proteins such as CDPKs (calcium-dependent protein kinases) and CBLs (calcineurin B-like proteins) which all contain the 'EF-hand' calcium-binding motif (Kim et al., 2009). Reactive Oxygen Species (ROS) are also second messengers involved in the response to different stress stimuli. An oxidative burst is activated or induced in response to biotic stress such as a virulent microbial infection or abiotic stresses for example heat, cold drought, salinity and others (Wahid et al., 2007; Kwon et al., 2007; Miller et al., 2010). As a result of this response NADPH oxidases generate O^{2-} which is converted quickly to H_2O_2. Research studies demonstrated that Ca^{2+} signatures are the key to activating NADPH oxidase. In the plasma membrane, ROS are generated by NADPH oxidases.

In plants NADPH oxidase, ROS and Ca^{2+} are involved in positive feedback mechanisms in response to different stimuli. Reduction of ROS levels activates the Ca^{2+} influx into the cytoplasm, which in turn stimulates NADPH oxidase to generate ROS (Takeda et al., 2008). The NADPH oxidase is a complex component identified as the respiratory burst oxidase (RBO), first described in mammals (Lambeth, 2004). The transmembrane gp91phox protein is the RBO

enzymatic subunit that transfers electrons to molecular oxygen to produce superoxide (Lherminier et al., 2009).

MAPKs are a large family of serine/threonine protein kinases. They control different cellular activities, for instance gene expression, mitosis, differentiation, proliferation, and cell survival/apoptosis; they respond to extracellular stimuli. MAPKs work downstream of sensors/receptors and transfer the signals from extracellular stimuli into intracellular responses plus amplification of the transmuted signals. MAPKs act as the final product of protein cascade and transduce extracellular stimuli into transcriptional response in the nucleus (Wurzinger et al., 2011). MAPK signalling pathways are generated from dynamic protein complexes involving three cascades of MAPK kinases. In general these cascades involve three functionally linked protein kinases, a MAPK kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAPK. In MAPK modules, the MAPKKK, serine/threonine kinase, phosphorylates and activates MAPKKs, which, in turn, perform T and Y dual phosphorylation of MAPKs. In *Arabidopsis*, there are 20 MPKs, 10 MPKKs, and 80 MPKKKs (Colcombet and Hirt, 2008). In eukaryotes, MAPKs and CDPKs signalling cascades are widely induced in response to biotic and abiotic stresses. In a number of pathosystems, MAPKs and CDPKs signalling cascades can be stimulated in response to the same stressing factor, suggesting crosstalk between them (Wurzinger et al., 2011). On the other hand a specific CDPK or MAPK could be stimulated in response to diverse biotic and abiotic stresses. MPK3 and MPK6 are involved in the mitogen-activated protein kinase cascade. In *Arabidopsis,* it has been demonstrated that MPK3 and MAPK6 are activated in response to biotic and abiotic stresses (Gerold et al., 2009); furthermore, MKK2 plays an important role as a key regulator for cold- and salt-stress response (Teige et al., 2004) and also in the resistance response of *Arabidopsis* to *Pseudomonas syringae* (Brader et al., 2007). CDPKs, CDPK6 and CDPK3 control ABA regulation of the guard cell S-type anion, Ca^{2+} permeable channels and stomatal closure (Mori et al., 2006). CDPKs also play a major role in the adaptation to abiotic stress (Dat et al., 2010). In plants, calcium-dependent enzymes and the MAPK are involved in defence signalling pathways.

1.4.2. Phloem feeding insects

Phloem-feeding insects represent a special paradox in studies of plant resistance to biotic and abiotic stresses. Aphids, the largest group of phloem feeders, penetrate plant tissues by probing intercellularly with their stylet through epidermal and mesophyll cell layers to feed on photoassimilates translocated in the phloem sieve elements (Pollard, 1972), inflicting considerable fitness costs in many crop plants (Dixon, 1998); BPH similarly feeds on the phloem of the sieve elements. Aphids keep phloem sieve elements alive and sieve plates pores open by inhibiting clotting of the phloem proteins (p-proteins), which affect and prevent callose formation (Tjallingii and Hogen Esch, 1993; Prado and Tjallingii, 1994). Most insects with piercing mouthparts can suck phloem sap from a single sieve element for hours or even days. The electrical penetration graph (EPG) technique has been used to monitor plant penetration by aphids and other insects with sucking mouthparts (Tjallingii, 1988). This technique can record signal waveforms and help distinguish between different insect activities, such as mechanical stylet work, saliva secretion, and sap ingestion. Whilst chewing insects cause extensive damage in the plant foliage, leafs, stems and roots, piercing /sucking insects cause less damage, abstracting plant fluids as a nutrient source (Stotz et al., 1999, Karban and Baldwin, 1997). As a result of these differences in insect feeding behaviour, different sets of plant genes are switched on/induced by chewing insects compared to sap sucking insects.

Overall aim of the project

To better understand natural plant defence mechanisms to sap sucking insect pests, through characterisation of molecular and biochemical events in the plant.

These aims will be addressed by investigating the following:

1. Identification of deferentially expressed genes in a susceptible rice cultivar (TN1) following BPH infestation using suppression subtractive hybridization (Chapter 2).
2. Investigate the expression level of β-1,3-glucanase and callose synthase genes in response to BPH in both susceptible rice TN1, and varieties exhibiting moderate levels of resistance (IR64 and IR70) using QRT-PCR (Chapter 3) .
3. Induction of resistance genes by exogenous application of hydrogen peroxide to enhance resistance of TN1 to BPH (Chapter 4).
4. Investigate the activity of Superoxide dismutase activity in response to BPH feeding, hydrogen peroxide and salinity treatment in TN1 and IR64 rice cultivars (Chapter 5).
5. Investigation of *Arabidopsis* mutants (Oxi1, Camta3-1, Camta3-2, β-1,3-gluganase genes Gns1, Gns2 and Gns3) to confirm the role of β-1,3-glucanase in plant susceptibility in response to insect feeding by sap-sucking insects (chapter 6).
6. Investigation of the role of hydrogen peroxide in the induce defence system in response to sap-sucking insect feeding (Chapter 7).
7. Improve inherent levels of resistance of the susceptible rice variety TN1 using antisense to β-1,3-glucanase gene 5 (Gns5) (Ongoing and future work).

Chapter 2. Identification of brown planthopper-induced genes in the susceptible rice variety TN1 by suppression subtractive hybridisation

Abstract

The Brown planthopper (BPH), *Nilaparvata lugens* (Stål) (Homoptera: Delphacidae), is one of the most destructive phloem-sap-sucking insect pests of rice. However, rice plants have evolved both constitutive and induced mechanisms to protect themselves against insect attack. In this study, suppression subtractive hybridization was used to identify genes induced by BPH feeding in a susceptible rice cultivar (*Oryza sativa* TN1). Rice plants at the 3rd to 4th leaf stage were infested with BPH and shoots were collected at different time points (0, 6, 12, 35, and 72 hours) and pooled together. A suppression subtractive library was constructed with infested tissues and non-infested tissues forming the 'tester' and 'driver', respectively and 1000 clones were obtained. These clones were further analysed by differential gene expression screening, and 120 clones that were clearly induced in response to BPH feeding were identified. Of these 120 positive clones, 52 represented unique genes, 46 were duplicates and 22 had no matching results against the database. Of the 52 clones, 32 clones had high homology with plant genes of known function, whilst 20 were homologous to unknown proteins. Gene functions were identified by using NCBI and RICE Genome annotation database. Genes were grouped by function. Interestingly, those genes with functions concerned with the wound response accounted for the largest functional category (29%), while those involved in the stress response and oxidative stress accounted for 9% and 6%, respectively. Those in electron transport represented 9%, ABA/WDS (abscisic acid/ water deficit stress) induced proteins represented 6%, and those in signalling pathways accounted for 6%. Those involved in aromatic metabolism, ribonucleaseT2 and metabolic processes represented 6%, 6% and 3%, respectively. Of particular interest was the differential expression of genes encoding the pathogen related proteins β-1,3-glucanase1, 2 and 5 and genes encoding callose synthase 1, 3 and 5 .

2.1. Introduction

2.1.1. Brown planthopper (BPH)

Brown planthopper (BPH), *Nilaparvata lugens* (Stål) (Homoptera-Delphacidae) is one of the most destructive insect pests of rice worldwide (*O. sativa* L.) and can cause severe yield losses. In addition to crop damage, BPH is one of the most important vectors for viral diseases (Ling et al., 1970, 1978). Heavy infestation of rice by brown planthopper causes hopper burn characterized by the complete drying-out of plant followed by plant death (Sogawa, 1973). BPH have been categorized into several distinct biotypes depending on their ability to feed on different rice cultivars with different resistant genes (Chelliah and Bharathi, 1993; International Rice Research Institute, 1976). For example, biotype 1 cannot feed or infest rice plants with resistance genes Bph1 and 2, while biotypes that can attack rice cultivars with resistant genes Bph1 and Bph2 are called biotype 2 and 3 respectively. In 1976-197, Biotype 1 and 2 were identified as wild strains in the Philippines, Indonesia, and Vietnam, because they can damage varieties with no resistance genes (Khush, 1979). BPH biotype 3 was produced in the Philippines after being reared on rice cultivar ASD7 (Pathak and Heinrichs, 1982). BPH biotype 4 (also referred to as the South Asian Biotype) was first reported in South Asia (Khush, 1984). BPH biotype 4 cannot feed or infest rice cultivars that carry resistant genes Bph5, Bph6 and Bph7 (Khush, 1984). BPH population from Asia and Australia were separate, according to systematic study on the brown plant hoppers (Jones et al., 1996; Kawaguchi et al., 2001). Chemical Insecticides such as the neonicotinoids are the main method for control of brown plant hopper. However, chemical control is very expensive, destroys most of the natural predators of BPH, and can increase the chance of developing BPH insecticide resistant strains. The evolution of neonicotinoid-resistance was observed in selected laboratory strains of BPH, which showed mutations in the nicotinic acetylcholine receptor genes, the targeted molecules of neonicotinoid insectisides (Liu et al., 2005). Therefore using induced resistance may prove to be an economical and efficient method to control BPH as a part of Integrated Pest Management strategy. Understanding the mechanisms of rice–BPH interactions will help to develop resistant rice varieties and environmentally friendly insecticides.

2.1.2. Rice-BPH interactions

Rice is considered as a model monocot crop as well as one of the most important food crops worldwide. Global Rice production reached 476 million tonnes in 2011 (Fao, 2012). Brown planthopper (BPH) is the most serious pest of rice crops all over the world. Up to 60% of rice yield loss is common in susceptible rice cultivars attacked by BPH. In order to cope with the increasing demand for rice, improvements are needed to increase the resistance of rice plants to BPH attack. When BPH feeds on rice plants, it excretes small amounts of gelling saliva on the plant surface before stylet insertion, the site of feeding being the sieve elements. Gelling saliva is excreted continuously during the penetrating stage with the purpose of protecting the stylet. The stylet sheaths are made of stable substances, produced by specialized secretory follicles present in the salivary glands and remain within the plant tissues after withdrawal of the stylets. In addition to the sheath material, a watery or soluble saliva, which contains different proteins and enzymes (Tjallingii, 2006) such as β-glucosidases, phenoloxidase (Sogawa, 1967a,b), actins, tubulins, heat-shock proteins, protein disulphide isomerases and ATP synthase (Koinishi et al., 2009) is also present; this soluble saliva is responsible for digestion of the phloem sap of rice. BPH feeding causes an increase in sucrose, leaf ferri ion content and free amino acids, however, it causes a decrease in the leaf area, photosynthetic ratio, plant height, nitrogen concentration in the leaf and stem, dry weight and chlorophyll content (Rubia-Sanchez et al, 1999; Watanabe and Kitagawa, 2000). Feeding by a large number of BPH may result in drying of the leaves and wilting of the tillers resulting in a condition called ‘hopper burn’. In susceptible rice varieties, BPH cause hopper burn as a result of nutrient abstraction, a high level of infestation and oviposition, with a high survival ratio of eggs and nymphs. In contrast, in resistant varieties oviposition is significantly reduced/inhibited, development time is increased, and population growth rate is suppressed (Hao et al. 2000, Wang et al. 2000).

2.1.3. Induced defence mechanisms

Plant-insect interactions are complex and dynamic. When insects attack plants, extensive gene reprogramming occurs (Kaloshian, 2004) in an attempt to combat this damage. Plants possess both a constitutive defence system and an

inducible defence system (Gatehouse, 2002). This induced response plays a very important role in conferring resistance against both biotic (including insects and pathogens) and abiotic stress (Maleck and Dietrich, 1999). The phytohormones like salicylic acid, ethylene and jasmonic acid are considered as important signalling molecules in plant-insect interactions that activate defensive genes (Remond and Farmer, 1998). Ethylene is involved in the down regulation of a subset of a wound-inducible, jasmonic acid dependent genes, and most of the genes that could be up regulated by plant pathogen interaction also could be induced by ethylene exposure (Deikman, 1997). In general plant-feeding insects can be classified as chewing or piercing/sucking insects according to their mechanism of feeding. Chewing insects cause extensive damage to the plant foliage, leafs, stems and roots. On the other hand piercing /sucking insects consume plant fluids as a nutrient source (Stotz et al., 1999, Karban and Baldwin, 1997). As a result of differences in insect feeding behaviour, different sets of plant genes are induced by chewing insects compared to those induced by sap sucking insects.

In rice plants changes in the expression levels of specific genes linked to abiotic stress, pathogen invasion and phytohormone signalling pathways have been detected in response to BPH feeding (Zhang et al, 2004). In susceptible rice cultivars, genes involved in plant defence and macromolecule degradation have been shown to be up regulated including genes involved in carbohydrate and lipid degradation; however genes associated with photosynthesis and cell growth such as photosynthesis light reaction, carbon reduction cycle, pigment synthesis and cellulose synthesis were shown to be down regulated following BPH infestation (Yuan et al, 2005). A cDNA microarray containing 1920 suppression subtractive hybridization clones has been used to explore the differences of the transcript profiles between the susceptible (MH63) and resistant (B5) rice cultivars in response to BPH feeding. In total 160 unique genes were shown to be significantly affected by BPH feeding (Wang et al., 2008).

2.1.4. Molecular mapping of genes for BPH resistance

In cultivated and wild species of *Oryza sativa*, RFLP, RAPD and SSR markers have been used for mapping 21 genes for BPH resistance. Of these 21

resistance genes, 15 have been mapped to different chromosomal locations, 8 of which were strongly connected with molecular markers (Table 2.1). Athwal et al. (1971) discovered that a dominant *Bph-1* gene controls resistance in four different rice varieties 'Mudgo', 'MTU15', 'Co22', and 'MGL2', whereas *Bph-2*, confers resistance in 'ASD7' and 'Ptb18' rice varieties. Lakshminarayana and Khush (1977) reported that the Sri Lankan rice cultivar *Rathu Heenati* has a dominant and non-allelic gene for resistance which is independent of *Bph-1* and was identified as Bph-3. Babawee, another Sri Lankan cultivar, has a gene for brown plant hopper resistance, which is independent of *Bph-2* and is selected as Bph-4. Kabir and Khush (1988) reported three genes for resistance in three different Bangladesh rice varieties, Bph-5 in 'ARC10550', Bph-6 in 'Swarnalatha' and *Bph-7* in 'T12'. Nemamoto et al. (1989) reported a new recessive gene for resistance, *Bph-8,* in the following rice varieties 'Thai Co1.5', and 'Chin Saba' whilst *Bph-9* was identified as a dominant gene in the Sri Lankan cultivars, Balamavee, Kahramana, and Pokkali. Ishii et al (1994) discovered a new dominant gene for resistance, *Bph-10* that was present in an indica breeding line. Kawaguchi et al (2001) identified two further genes for BPH resistant, Bph-11, and Bph-12.

In a large scale screening programme at the International Rice Research Institute (IRRI) in the Philippines, Wu et al (1986) evaluated the resistance of 11, 000 wild rice accessions to BPH biotypes 1, 2, and 3. This screening programme identified that 19 accessions related to four wild species of *Oryza* were resistant to all BPH biotypes, thus highlighting wild species as potential sources of new genes for resistance to BPH. Velusamy (1988) studied the virulence of two different BPH biotypes (one from Tamil Nadu, South India and the other maintained at IRRI) on wild rice species. This study revealed that *O. officinalis* and *O. punctata* Kotschy ex Steud were resistant to southern Indian populations of BPH; furthermore, these two rice species were also reported to be highly resistant to all three previously described biotypes of BPH, as well as to the green rice leafhopper (*Nephotettix cincticeps* Uhler) and the white backed plant hopper (*Sogatella furcifera* Horvath) (Velusamy et al., 1984; Velusamy, 1988). Kawaguchi et al., (2001) identified and characterised 12 BPH resistant genes using DNA marker technology.

A systematic and in-depth search for genes linked to resistance to sucking insects is crucial to get a better understanding of the response of cells to

herbivore attack. The polymerase chain reaction (PCR)-based suppression subtractive hybridization (SSH) technique has been established for the quick and sensitive evaluation of mRNA expression profiles between 'tester' and 'driver' populations (Diatchenko et al., 1996; von Stein, 2001; Xiong et al., 2001). Work presented here uses molecular techniques to get a better understanding of the molecular mechanisms involved in rice in response to BPH feeding. Specifically it uses suppression subtractive hybridization to identify those genes in the susceptible rice cultivar TN1 that are differentially expressed in response to BPH feeding. These results provide an important initial step towards understanding the responsive mechanisms in rice to BPH.

Table 2.1. Examples of molecular mapping of BPH resistance genes in rice. Source (Brar et al., 2009).

genes	Cultivars	Chromosome	Markers	Reference
Bph1	*Mudgo*	12	RFLP	Hirabayashi and Ogawa (1995), Sun et al (2006)
Bph2	*Norin PL4*	12	RFLP	Murata et al (1998)
Bph3	*Rathu Heenati*	6	SSR	Jairin et al (2007)
Bph4	*Babawee*	6	RFLP	Kawaguchi et al (2001)
Bph5	*ARC 10550*	4	SSR	Khush et al (1985), Kabir and Khush (1988)
Bph6	*Swarnalata*	11	RABD	Jena et al (2002)
Bph7	*T12*	6	SSR	Kabir and Khush (1988)
Bph8	*Chinsaba*	7	SSR	Nemoto et al (1989)
Bph9	*Karahamana*	6	SSR	Su et al (2006)
Bph10	*O.australiensis*	12	RFLP	Ishii et al (1994)
Bph11	*O. officinalis*	3	RFLP	Hirabayashi et al (1998)
Bph12	*O. latifolia*	4	RFLP	Hirabayashi et al (1998)
Bph13	*O. eichingeri*	2	RABD	Rengawnayaki et al (2002)
Bph14	*O. officinalis*	3	RFLP	Yang et al (2004)
Bph15	*O. officinalis*	4	RFLP	Yang et al (2004)
Bph17	*O. officinalis*	4	SSR	Sun et al (2005)
Bph18	*O. australiensis*	12	SSR	Jena et al (2006)
Bph19	*AS20-1*	3	SSR	Chen et al (2006)
Bph20	*O. minuta*	4	STS	Rahman et al (2009)
Bph21	*O. minuta*	12	STS	Rahman et al (2009)

2.2. Materials and methods

2.2.1. Plant material

Oryza sativa L Taichung Native 1 (TNI), a susceptible variety for BPH, was obtained from The International Rice Research Institute, Philippines. TN1 seeds were sown in plastic trays (60 x 40 x 10cm) and on germination transferred to (25-cm) pots. The growth room used for growing rice plants was maintained at 28°C during the day and 21°C during the night with a 16-h day/8-h night photoperiod and with a relative humidity of 70%.

2.2.2. Insects

Rice brown plant hopper (*Nilaparvata lugens*) was reared on rice plants (TN1) in the insectary room at Newcastle University. The culture was originally obtained from The International Rice Research Institute, Philippnes. Insects were held under DEFRA Licence number (PHL 163A/6655).

2.2.3. Experimental design

Plants at the 3rd -5th leaf stage were used for this study. Susceptible rice (TN1) seedlings were each infested with 10 3rd-4th instar BPH nymphs; non-infested plants (control) were kept in a separate cage. Plants were 'harvested' at the following time points post infestation: 0, 6, 24, 35 and 72 hours; control plants were similarly collected at these same time points. Three individual plants were used as biological replicates for each time point.

2.2.4. Sampling

All the insects were taken from rice plants and the shoot and root material was harvested individually and frozen immediately in liquid nitrogen and kept in a freezer -80°C for RNA isolation. Sample treatment was essentially the same for the control plants.

2.2.5 RNA isolation and SSH

Total RNA was isolated from approximately 100 mg of frozen leaf tissue by using Trizol Reagent (Invitrogen), according to the manufacturer's protocols. The concentration and purity of the RNA samples was determined using a

Nanodrop (ND-1000 Spectrophotometer; Nanodrop Technologies). All samples had an absorbance ratio (absorbance at A_{260}/A_{280} nm) of between 1.9 and 2.2. Poly $(A)^+$RNA was isolated using MagneSphere® Magnetic Separation Products (Promega). The subtractive hybridization was carried out using a PCR-select cDNA subtractive kit according to the manufacturer's protocol (Clontech). Double-stranded cDNA was prepared from 3µg of poly $(A)^+$RNA (tester population) and leaf RNA (driver population). Forward subtraction was performed using cDNA synthesized from planthopper-infested plants as tester and that from non-infested plants served as driver to enrich genes that are induced by BPH infestation. The PCR products were inserted into the pCR®II-TOPO® (Invitrogen™) to clone differentially expressed genes. QIAprep® Miniprep (QIAGEN) was used for Plasmid DNA purification. The plasmid was digested by using *EcoR1* enzyme and the product was electrophoresed to detect different size of DNA.

2.2.6 Differential screening of the subtracted libraries

Individual clones from the suppression subtractive hybridization (SSH) library of BPH-fed on TN1 seedlings was used to screen for the corresponding full-length cDNAs. Total RNA was extracted by using Trizol reagent. Total RNA isolated after (6, 24, 35 and 72h) BPH feeding were pooled equally and converted to cDNA by using a cDNA synthesis kit (Invitrogen). Tester represented infested rice plants with BPH and driver represented non-infested plants with BPH. Both tester and driver poly $(A)^+$ RNA were isolated by using MagneSphere® Magnetic Separation Products. Double-stranded cDNA was produced from approximately 3 µg of poly $(A)^+$ RNA. The subpression subtraction hybridization libraries for differentially expressed cDNA were constructed by ligating the subtracted cDNAs into the the pCR®II-TOPO® vector. Individual recombinant white colonies were picked and cultured into LB medium Thecontaining ampicillin on 96-well microtitre plates. The cDNAs were used for probe labelling with [32P] dCTP. Hybridization buffer (Church buffer) was prepared according to the number of samples and incubated at 65°C overnight. Thereafter, 30 ml of Church buffer was used in the hybridization tube. RNA concentration was measured by using a NanoDrop spectrophotometer. The master mix was prepared according to the number of samples by using the components in table (2.2). Firstly, master mix was prepared from (primer + ¾ dNTPs) according to

the number of samples followed by pipetting 2 µl in each eppendorf tube. Eppendorf tubes were briefly centrifuged and incubated at the heating block 65°C for 5 minutes and then chilled in ice for 2 minutes. Secondly, master Mix (5X buffer + DTT (0.1M) was prepared and 6 µl added to Eppendorf tubes from step 1, then incubated at 42°C for 2 minutes and later chilled in ice. Approximately 3µl of 32P and 3µl Superscript RT (200 U/µl) were pipetted in each eppendorf tube and incubated at 42°C for 50 minutes. Thereafter, the eppendorf tubes were incubated at 100°C for 5 minutes, followed by adding 200µl of Church buffer (Table 2.3). All contents in the eppendorf tube were taken out and poured in the hybridization tube which contain the nylon membrane, then incubated in the rotator (slow rotation) at 65°C overnight. Following overnight hybridisation, the hybridisation solution was then discarded and the blots were washed under the following conditions:

2 x SSC (100 ml 20xSSC, fill up with H2O2 on 1000ml) room temperature 2 x 5 minutes

2 x SSC (100 ml 20x SSC + 10 ml SDS 10 %, fill up with H2O2 on 1000 ml) 65 °C 2 x 30 minutes.

1 x SSC (5 ml 20x SSC + 10 ml SDS 10 %, fill up on 1000 ml with H2O2) 65 °C 1 x 30 minutes.

0.5 X SSC, 0.1 SDS 65 °C 1 x 20 minutes.

0.2 x SSC room temperature 2 x 5 minutes.

All washes were carried out with gentle shaking.

Washing solution 1:

2x SSC (sodium chloride and sodium citrate solution)

(100 ml 20xSSC, fill up with di H2O on 1000ml)

Washing solution 2:

2x SSC, 0,1 % SDS

(100 ml 20x SSC + 10 ml SDS 10 %, fill up with di H2O on 1000 ml)

Pre-warm at 65°C.

Washing solution 3:

0,1x SSC, 0,1 %SDS

(5 ml 20x SSC + 10 ml SDS 10 %, fill up on 1000 ml with di H2O)

Pre-warm at 65°C.

Blots were washed until the signal from a Geiger counter dropped below ~ 5cps. Blots were wrapped in Saran Wrap and were then exposed to pre-flashed autoradiography film at –70 °C. Blots were stripped of any remaining hybridised probe by incubation in 5 mM Tris-HCl pH7.4 at 75 °C for 1 hour. Removal of the probe was confirmed by exposing the blots to pre-flashed autoradiography film for 1 week. About 200 clones of the cDNA library were transferred onto Hybond N+ membrane (Amersham). The blots were hybridized for more than 10h at 65◦C with the labelled probe, washed and then exposed to X-ray film for autoradiography.

Table 2.2. Master Mix components for probe labelling

Components	**Volum (µl)**
SDW	3 µl
RNA	10 µl
Primers (500 µg/ml)	1 µl
¾ dNTP (10mM, ATG)	1 µl
5X first strand buffer buffer	4 µl
DTT (0.1 M)	2 µl
^{32}P	3 µl
Superscript RT (200 U/µl)	1 µl

Table 2.3. Church hybridization buffer.

Stock solution	**Final concentration**	**Enough for 300ml**
SDS	7%	21g
0.5 M Na_2 HPO_4 (dibasic)	0.5M	150 ml
0.5 M $NHPO_4$ (monobasic)	0.5M	150 ml
0.5M EDTA	1mM	0.6 ml
Bovine serum albumin	1%	3 g
• Stir and heat (45-50° C) • Keep the buffer in oven at 65° C (buffer colour should be clear)		

2.2.7 Sequence analysis

DNA sequences were compared to those in the Gene Bank DNA and protein databases by using the blastn and blastx algorithms, at the DNA analysis web site maintained by the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and Rice genome annotation. The similarity scores between the cDNA clones and known sequences were represented by the blastx probability E-values.

2.3. Results

2.3.1. Detection and Identification of subtraction efficiency

The key to gaining successful SSH results are to eliminate effectively identical cDNA clones present in both testers and drivers (Diatchenko et al., 1996). PCR amplification shows the reduction in successfully subtracted mixtures. For the unsubtracted cDNA library, PCR product starts to appear after 18 and 24 cycles and increased after 33 cycles. Obviously, there are a number of distinctive bands between cDNA subtracted and unsubtracted libraries (Fig 2.1).

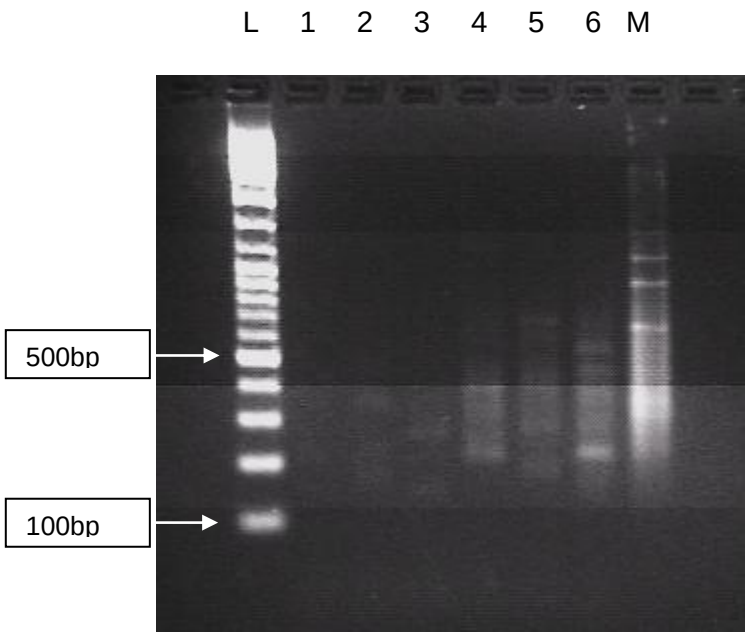

Fig 2.1. PCR analysis of subtraction efficiency. PCR was performed on subtracted lanes (1, 2, 3), unsubtracted lanes (4, 5, 6), (Gene Ruler DNA Ladder) and M (kit Marker). PCR product with generic primer for rice, Lanes 1 & 4: 23 cycles; Lanes 2 & 5: 28 cycles; Lanes 3 & 6: 33 cycles. Samples were electrophoresed on a 2% agarose/EtBr gel. The lower half of the gel image was adjusted to show the products more clearly.

2.3.2. Detection and identification of positive clones and differential screening

Subtractive cDNA libraries were constructed for both infested and non-infested (control) susceptible rice. Two mRNA populations were prepared. The first, extracted from the TN1 line after BPH feeding as the tester and the second, extracted from TN1 seedlings that had not been infested with BPH insects, as the driver population. The suppression subtractive hybridization library was

created by cloning the second PCR product into pCR®II-TOPO® (Invitrogen™) vector. Screening by using white and blue colonies showed that 95% of the transformants contained inserts. Of the 1000 positive clones, 180 clones were randomly selected and screened by using dot-blot analysis (Fig 2.2). At the end, 120 clones were selected for sequence analysis. QIAprep® Miniprep (QIAGEN) was used for Plasmid DNA purification. The plasmid was digested by using *EcoR1* enzyme and the product was electrophoresed to detect different size of DNA (Fig 2.3).

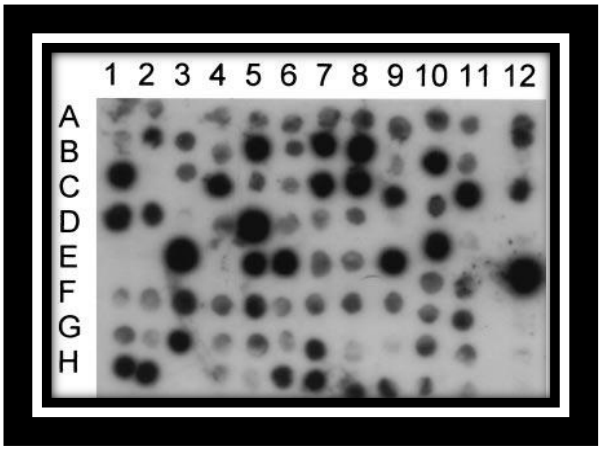

Fig 2.2. Differential screening of selected cDNAs using ^{32}P-labeled cDNA probes synthesized from poly (A)$^{+}$RNA. Fig (2.2) shows an Array of 96 putatively -expressed clones, shown after hybridization to probes derived from extracts of infested TN1 with BPH mRNA. Dark signals in the X-ray film indicate cloned loci that were expressed at high levels.

2.3.3. Sequence analysis

The libraries consisted of approximately 1000 positive clones. Around 120 recombinant clones with insertions longer than 100 bp induced by BPH were sequenced and analysed using Blast search in NCBI and Rice genome annotation data base. . The search results revealed that 36 out of 120 clones were differentially expressed in response to BPH feeding. Of these 120 positive clones, 52 represented unique genes, 46 were duplicates, 22 with no matching results. Of the 52 clones representing unique genes, 32 clones had high homology with plant genes of known function (Tables 2.2, 2.3), 20 were homologous to unknown proteins or cDNA clones from rice or other plants. Low-quality and repeated sequences were eliminated. Repeated sequences were appeared in clones (BPH01, BPH02, BPH03, BPH04, BPH05, BPH06,

BPH07, BPH08, BPH10, BPH11, BPH12, BPH13, BPH15, BPH25, BPH26, and BPH27). Tblastx and tblastn were used to analyse the sequenced clones. Gene functions were identified using NCBI and RICE GENOME ANNOTATION data base.

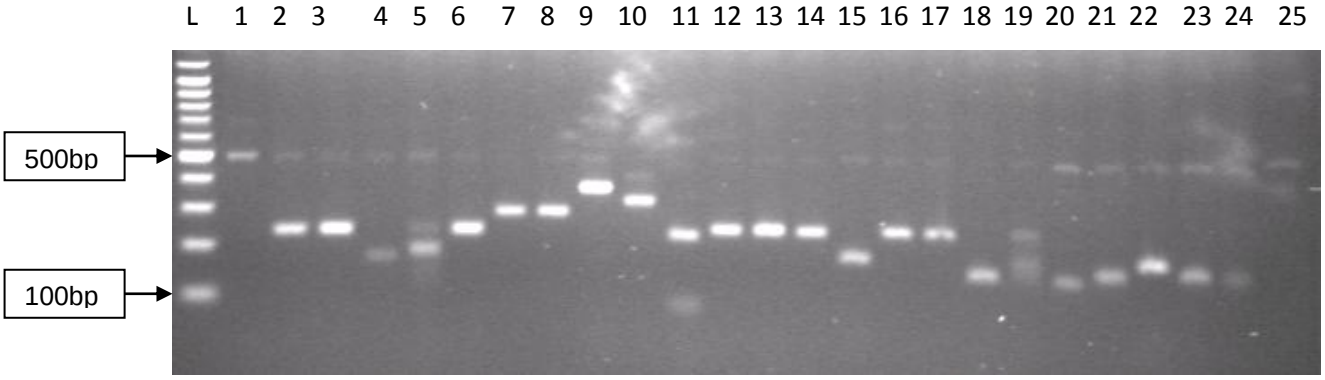

Fig 2.3. A number of inserts of subtracted cDNA clones. Lanes 1-25 shows 25 clones randomly selected from the subtracted library. L: DNA size marker.

2.3.4. Differential gene expression profiles in susceptible rice cultivar TN1 in response to BPH feeding

Sequence analysis of selected clones revealed that β-1, 3-glucanase genes 1, 2 and 5 were deferentially expressed in clones BPH01, BPH02 and BPH03 respectively. GTP-binding protein was deferentially expressed in clone number BPH04. Callose synthase 1, 3 and 5 were deferentially expressed in clones BPH05, BPH06 and BPH07 respectively. Clones BPH08 and BPH10 had inserts for ABA/WDS induced proteins. Genes involved in metabolism were deferentially expressed in clones BPH09 and BPH25. Delayed early response genes were shown to be deferentially expressed in clones BPH11, 15 and 23. Three genes known to be involved in the wound response were diferentially expressed in clones BPH12, 22 and 31. BPH13, 19, 21, 30, 33 and 34 clones contain inserts for genes with unknown function. Clones BPH14 and 16 contain the inserts for ribonuclease T2 family protein genes. Clone number BPH17 contains an insert for a gene involved in a signalling pathway. 60S ribosomal protein L39 gene differentially expressed in clone number BPH18. BPH20 and BPH24 clones contain inserts with gene function of RRM Response to stress. Oxidative stress genes were deferentially expressed in response to BPH feeding in clones BPH26 and 27. Genes with metabolic process, ATPase and transcription factors were deferentially expressed in BPH32, 35 and 36 clones.

Table 2.4. Identification of BPH-inducible genes in rice (*Oryza sativa*) matched with plant genes of known functions.

Clone	Accession number	Gene locus	Protein product	Group function	Chr	Expect
BPH01	AB070742.1	LOC_Os01 g713 0.1	β-1,3-glucanase 1	Wound response, drought-inducible and pathogen-related proteins	3	4e-71
BPH02	U72248.1		β-1,3-glucanase 2		1	5e-79
BPH03	U72251		β-1,3-glucanase 5		1	1.2e-60
BPH04		LOC_Os05 g48855	GTP binding protein,		5	1.6e-22
BPH05	AP001389.1	LOC_Os01 g55040	Callose synthase 1		6	4e-7
BPH06	AP003268.4	LOC_Os01 g55040.1	Callose synthase 3		1	7.4e-43
BPH07	AP008212		Callose synthase 5			
BPH08	PF02496	LOC_Os11 g06720	abscisic stress-ripening, putative, expressed	ABA/WDS induced protein	11	2.3e-37
BPH09	PF02469	FLA21	Putative fasciclin-like arabinogalactan protein 21	Aromatic metabolism	2	5e-32
BPH10	PF07876	LOC_Os07 g41820	Stress responsive A/B Barrel Domain	ABA/WDS induced protein	7	4.8e-48
BPH11	PF01733	LOC_Os07 g37100	Delayed-early response (DER)	Electron transport	7	1.8e-33
BPH12	PF00234	LOC_Os12 g02310	LTPL11 - Protease inhibitor/seed storage	Wound response, drought-inducible and pathogen-related proteins	12	8.6e-34
BPH13	PF07876	LOC_Os07 g41820	expressed protein	unknown	7	5.0e-48
BPH14	PF00445	LOC_Os09 g36680	Ribonuclease T2 family protein	Ribonuclease T2 activity and RNA binding	9	1.1e-23
BPH15	NM_001066 500	LOC_Os07 g0557100	Delayed-early response protein	Electron transport	7	6e-34
BPH16	PF00445	LOC_Os09 g36680	ribonuclease 3 precursor, putative, expressed	Ribonuclease T2 activity and RNA binding	9	1.1e-23
BPH17	PF02496	LOC_Os11 g06720	abscisic stress ripening protein 2, putative, expressed	Signalling pathway	11	1.4e-46
BPH18	PF00832	LOC_Os06 g08320	60S ribosomal protein L39, putative, expressed	Structural proteins and protein synthesis And Translation	6	2.7e-15
BPH19	AC124143	LOC_OSJN Bb0053D02	unknown protein	unknown	5	4e-21
BPH20	PF00076	LOC_Os12 g43600	RNA recognition motif containing protein, expressed	RRM Response to stress	12	1.6e-44
BPH21		LOC_Os09 g04460	retrotransposon protein, putative, unclassified	Unknown	9	9.4e-06
BPH22	PF00067	LOC_Os08 g16260	cytochrome P450 86A1, putative,expressed	Wound response, drought-inducible and pathogen-related proteins	8	2.3e-32

Table 2.5. Continued.

Clone	Accession number	Gene locus	Protein product	Group function	Chr	Expect
BPH23	PF02518.19	LOC_Os06g16260	OsSigP5 - Putative Type I Signal Peptidase homologue; employs a putative Ser/His catalytic dyad, expressed	Electron transport (ATPase, AAA family)	6	0.000
BPH24	PF00076	LOC_Os12g43600	Glycine-rich RNA-binding protein GRP1A, putative, expressed	RRM Response to stress	12	1.7e-21
BPH25	DQ073476	trnA	tRNA-Ala	Aromatic metabolism	3	6e-45
BPH26	PF00096	LOC_Os06g47840	zinc finger protein 622, putative, expressed	Oxidative stress/ Apoptosis	1	0.00
BPH27	PF00107	LOC_Os12g12590	NADP-dependent oxidoreductase P1,putative, expressed		12	0.00
BPH28	PF00004	LOC_Os02g53500.1	RFC5 - Putative clamp loader of PCNA, replication factor C subunit 5, expressed	Response to stress	12	0.00
BPH29	PF00560	LOC_Os06g38670	receptor-like protein kinase precursor, putative, expressed Leucine reach protein	Signal transduction Kinase activity	6	0.00
BPH30	PF01918	LOC_Os11g06760	Protein of unknown function	Nucliec acid binding Biological process	11	0.00
BPH31	PF00481.14	LOC_Os02g55560	DNA-binding protein phosphatase 2C, putative, expressed	Disease wound and stress response and catalytic activity	2	0.00
BPH32		LOC_Os04g43922	exosome complex exonuclease rrp4, putative, expressed	Metabolic process and nucleic activity	4	0.00
BPH33	AC124143	OSJNBb0053D02	unknown protein	Unknown function		5e-21
BPH34	AC120527	Clone OSJNBa0011J22	hypothetical protein		5	5e-48
BPH35	PF00004	LOC_Os02g53500	replication factor C subunit 3, putative, expressed	(ATPase, AAA family) Response to stress	2	0.00
BPH36	PF00176	LOC_Os06g01320	SNF2 family N-terminal domain containing protein, expressed	transcription regulation	6	0.00

2.3.5. Diferentially expressed gene functions

Differentially expressed genes (identified above and in Table 2.2) were classified into 10 functional categories. Percentages represent the percentages of genes belonging to a particular functional group, including those of unknown functions (Fig 2.4). Interestingly, those genes with functions concerned with the wound response accounted for the largest functional category (29%), while those involved in the stress response and oxidative stress accounted for 9% and 6%, respectively. Those in electron transport represented 9%, ABA/WDS induced proteins represented 6%, and those in signalling pathways accounted for 6%. Those involved in aromatic metabolism, ribonuclease T2 and metabolic processes represented 6%, 6% and 3%, respectively. Those of unknown functions represented 17% (Fig 2.4).

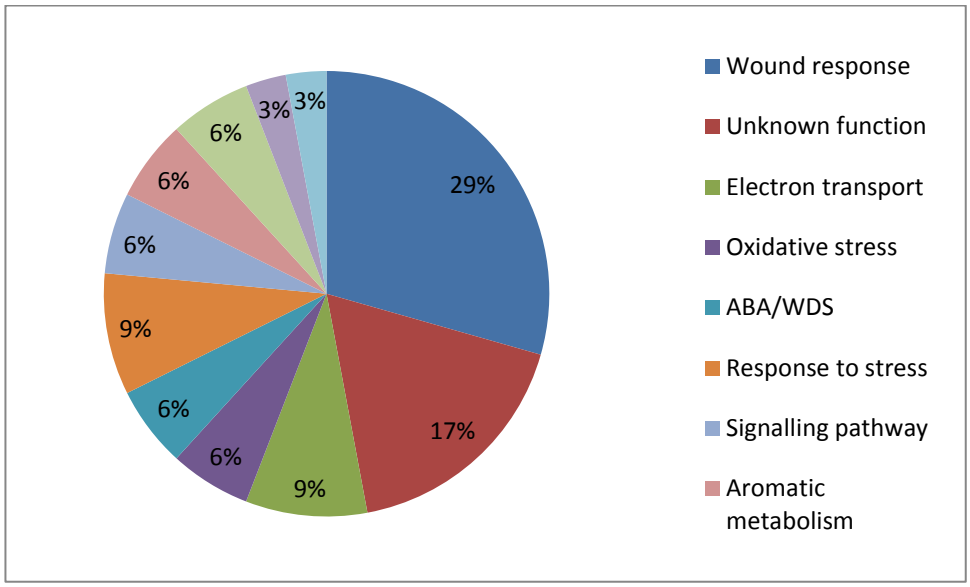

Fig 2.4. Specific expression of rice genes induced by brown planthopper classified by functionality. Differentially expressed genes were classified into 10 functional categories. Percentages represent the percentages of genes belonging to a particular functional group including genes with unknown functions and sequences that did not have any homology to known sequences in rice databases.

2.4. Discussion

2.4.1. Diferentially expressed genes in susceptible rice cultivar TN1 in response to BPH feeding

Suppression subtractive hybridization (SSH) can be successfully used to identify diferentially expressed genes. Undoubtedly, application of SSH provides a good way to find mRNA species that are differentially expressed in susceptible TN1 rice in response to BPH feeding, so as to get a better understanding of the molecular response to insect attack (Yang et al., 1999). This is an area of research that is currently receiving much attention, not least as a tool to developing rice cultivars with enhanced endogenous resistance. Although 21 resistance genes in rice plants against BPH have been identified to date, (Rahman et al., 2009), little is actually known about molecular mechanisms involved in the plant's resistance to insect feeding.

A protease inhibitor (PI) was one of the first wound-inducible proteins to be characterized and is considered as an important defensive component in plant responses to attack by chewing insects (Ryan, 1990; Pautot et al., 1991; Koiwa et al., 1997; Tamayo et al., 2000). PIs can inhibit proteases and elastases in the larval midgut forming inactive enzyme/inhibitor complexes (Gatehouse et al., 2000; Gatehouse, 2002; Tamayo et al., 2000), thus decreasing herbivore performance on some plants. Assembly of these inhibitors is greatly regulated by a signal transduction pathway that is initiated in response to insect damage and transduced as a wound response. In the present study a protease inhibitor was diferentially expressed in TN1 rice plants in response to BPH feeding. Another group of genes/proteins that are known to be involved in detoxification and form part of the induced wound response (Nelson et al., 2004) are the cytochrome P450s. They are haem-thiolate proteins involved in the oxidative degradation of various compounds. They are particularly well known for their role in the degradation of environmental toxins and mutagens. Data from the present study show that genes encoding cytochrome P450 were expressed in the susceptible rice in response to BPH infestation. ABA- and stress-inducible proteins were also differentially expressed in susceptible rice TN1 in response to BPH damage. Again, these finding are consistent with those reported previously in other plant/insect systems (Flors et al., 2009). Recently, ABA-

mediated resistance has also been highlighted in callose deposition and in the interplay between JA, SA, and ABA against some necrotrophs (Mauch-Mani and Mauch, 2005; Adie et al., 2007; Flors et al., 2008; Ton et al., 2009).

Reactive Oxygen species (ROS) genes are well known to be induced in response to both biotic and abiotic stress and could serve not only as protectants against the stress but could also act as signals activating the hyper sensitive reaction in plants (Tenhaken et al. 1995; Jiang et al., 2007). Zinc finger protein 622 (BPH26) and NADP-dependent oxidoreductase P1 are involved in oxidative stress/apoptosis (Torres et al., 2002). For example, absence of the NADPH oxidase genes AtrbohD and AtrbohF suppresses ROS production and the defense response of *Arabidopsis* against pathogen attack. The results reported in the present study for these two particular proteins are thus entirely consistent with their role in the inducible defence response in rice. Furthermore, the DNA-binding protein, shown to be differentially expressed in the present study is also known to play an important role in the defence mechanism in response to pathogens and changes in salicylic acid levels (Chen and Chen, 2000).

2.4.2. Wound response genes

Several research groups have identified numerous plant defence genes that are induced by insect feeding (Reymond et al., 2000). Different plant species exhibit a wide range of defence strategies in response to insect attack and damage. In the present study, a large number of genes identified are known to be involved in the wound response, drought response, and stress response, as well as pathogen-related proteins. Results from the subtractive library demonstrated that a number of wound response genes were differentially expressed. Interestingly, β-1,3-glucanase 1, 2 and 5 genes were differentially expressed in response to BPH feeding. These are classified as pathogen related genes (PR) (Frye et al., 2001; Senthilkumar et al., 1999) and play an important role in plant resistance in response to fungal infestation by hydrolysing fungal cell walls (Leubner-Metzger and Meins, 1999). They are also involved in callose hydrolysis (Hao et al., 2008). However, it is well known that sap-sucking insects often elicit a pathogen-like response due to their mode of feeding (Ferry et al.,

2011) and thus the over expression of these genes in the present study is perhaps not surprising.

The deposition of a linear β-1,3-glucan polymer, callose, in response to pathogen attack/wounding stress is a basic defense mechanism that enables the plant to arrest pathogen proliferation by reinforcing the cell wall in both monocots and dicots (Jacobs et al., 2003; Glazebrook, 2005; Hardham et al., 2007; Hao et al., 2008). In the *Arabidopsis* callose synthase-deficient mutant pmr4-1, which shows impaired pathogen-induced callose deposition, SA-dependent defense responses were strongly induced to augment the resistance to powdery mildew (Nishimura et al., 2003), whereas JA- dependent defense responses were down-regulated, resulting in its susceptibility to *A. brassicicola* (Flors et al., 2008). These findings indicate that pathogen-induced callose deposition plays an important role in resistance to the necrotroph fungus and is closely related to antagonistic interactions between JA-dependent responses and SA-related responses against fungal pathogens; cob-5 mutants showing the constitutive deposition of callose were found to overproduce JA and the JA-responsive defense genes, such as PDF1.2 (Ko et al., 2006), indicating that JA-dependent responses are positively involved in callose deposition against pathogen attacks. Callose deposition can be also induced by wounding (Hildmann et al., 1992). In a recent study by You et al. (2010) screening of the expressed sequence tag library of the wild rice species *Oryza minuta* revealed an unknown gene that was rapidly and strongly induced in response to attack by a rice fungal pathogen (*Magnaporthe oryzae*) and an insect (*Nilaparvata lugens*) as well as by wounding, abscisic acid (ABA), and methyl jasmonate treatments. Its recombinant protein was identified as a bifunctional nuclease with both RNase and DNase activities *in vitro*. This gene was designated *OmBBD* and is proposed by the authors to have a novel regulatory role in ABA-mediated callose deposition. Other studies have also demonstrated the role of β-1,3-glucanase 5, in plant defence in response to pathogen attack (Hao et al., 2008). These studies are thus in direct agreement with those reported in the present study where genes encoding β-1,3-glucanase 1, 2 and 5 were not only switched on in response to BPH, but expressed at very high levels (as detected by QRT-PCR). This is somewhat surprising since these enzymes are responsible for the hydrolysis of callose (β-1,3-glucan), which is formed in

response to BPH feeding as part of the plant's defence mechanism. Interestingly, in cultivars that are resistant to BPH (see Chapter 3), only β-1,3-glucanase 5 is expressed and only at very low levels. This may go part way to explaining why TN1 is highly susceptible i.e. BPH is able to induce enzymes that hydrolyse callose, which normally plugs the sieve elements, therefor preventing phloem feeding, and in so doing combats one of the plant's lines of defence. In addition to their proposed role in plant defence, these enzymes also play a role in pollen development. For example, β-1,3-glucanase (PR1) or Osg1 is required for callose degradation during pollen development in rice. Gene silencing of Osg1 by RNA interference resulted in rice male sterility (Wan et al., 2011). β-1,3-glucanase 1 and 2 hydrolyse β-1,3-glucans and β-1,3; 1,6-glucans (Akiyama and Pillai, 2001). In addition, β-1,3-glucanase is fully expressed in the paleae and lemmas of germinating seeds and before the pollination stage (Akiyama et al., 2004).

GTP binding protein is a small protein, which regulates callose synthase (Qadota et al., 1996) and in the present study was expressed in response to BPH attack in the susceptible cultivar TN1. In addition to expression of the β-1,3-glucanase genes (see above), callose/glucan synthase genes were also expressed in TN1 in response to BPH feeding. Callose is a polysaccharide, β-1, 3-glucan, with some β-1,6-branches and it occurs in the cell walls of a wide range of higher plants and as stated above plays an important role not only in plant defence in response to biotic and abiotic stresses, but also in a wide variety of processes during plant development. Callose is the final product of callose synthases and it is normally degraded by β-1,3-glucanases. Callose deposition in sieve plates is increased in resistant rice cultivars compared to susceptible ones (Hao et al., 2008). According to gene structure modelling, most *β*-1,3-glucans genes have 40–50 exons; and the exceptions include only two genes callose synthase 1 and *5*, which have two and three exons, respectively. A single *β-1,3-glucans* gene can also have different functions; for example, callose synthase 5 is normally induced in response to wound- and pathogen attack in leaf tissue; and it also plays an important role in exine formation and pollen wall patterning (Jacobs et al., 2003; Enns et al., 2005). According to these findings, genes have overlapping functions in both insect attack and pathogen responses.

Chapter 3. Response of susceptible (TN1) and resistant (IR64 and IR70) rice cultivars to BPH infestation: expression of genes encoding GTP binding protein, Callose synthase 1, 3 and 5 and β-1,3-glucanase 1, 2, 3 and 5

Abstract

The Brown planthopper (*Nilaparvata lugens Stål;* BPH) is one of the most economically important insects pests causing high levels of damage to rice plants. BPH causes damage both by abstraction of phloem sap, and by transmission of viral diseases during feeding such as ragged stunt virus and grassy stunt virus. Previous studies have shown that BPH spend longer periods of time wandering over the surface tissues of the resistant cultivars IR64 and IR70 and less time feeding, than on the susceptible cultivar TN1. In the present study the role of genes involved in callose synthesis and deposition (callose synthase and GTP binding protein) and callose degradation (β-1,3-glucanases) were investigated in susceptible (TN1), partially resistant (IR64) and resistant (IR70) cultivars in response to BPH feeding. The results demonstrated that genes encoding callose synthase 1 and 5 (GSL1 and GSL5) play an important role in plant defence in response to BPH feeding being highly expressed in both the moderately resistant/resistant cultivars IR64 and IR70, but down regulated in the susceptible cultivar TN1. Similarly, genes encoding the GTP binding protein were more highly expressed in cultivars IR64 and IR70 in response to BPH feeding, compared to TN1 where expression was low. In contrast, genes involved in callose degradation, namely β-1,3-glucanase genes 1, 2 and 5 (Osg1, Gns2 and Gns5) were highly expressed in the susceptible cultivar in response to BPH feeding; Osg1 and Gns2 were not expressed in either IR64 or IR70, while β- Gns5 was down regulated in both these resistant cultivars, compared to the susceptible cultivar (TN1). These findings are in agreement with the higher levels of callose deposition observed on the sieve elements in the resistant cultivars compared to the levels of deposition in the susceptible cultivar. These results support the hypothesis that callose deposition plays an important role in preventing BPH feeding, interfering with phloem transportation, forming the basis of the observed resistance in IR64 and IR70. β-1,3-glucanases genes (Gns2 and Gns5), in contrast, encode enzymes involved in

callose degradation, which are induced by BPH, and thus play a role in the susceptibility of TN1.

3.1. Introduction

Previously, Suppression subtractive hybridization technique was used to detect differentially expressed genes in rice cultivar TN1 in response to BPH feeding. Callose synthase genes GSL1, 3 and 5 genes were differentially expressed post BPH feeding and detected many times in number of clones in the SSH liberary. Callose deposition is the basic defense mechanism that enables the plant to stop insect or pathogen attack by reinforcing the cell wall in both monocots and dicots. GSL1, GSL3 and GSL5 were considered as important finidings in TN1 in response to BPH feeding. Another important finindings was genes encoding GTP binding protein. GTP binding protein is a small protein, which regulates callose synthase and in the present study was expressed in response to BPH attack in the susceptible cultivar TN1. So that, detecting the expression level of those genes in susceptible, moderat resistant and resistant rice cultivars may help to understand the main role of those genes in the plant defense system. On the other hand, genes encoding β-1,3-glucanase 1, 2 and 5 were differentially expressed in response to BPH feeding and also detected several times in SSH liberary (chapter 2). This is somewhat surpricing since these enzymes are responsible for the callose hydrolysis. So that, studing the transcript level of β-1,3-glucanase 1, 2 and 5, callose synthase (β-1,3-glucan) GSL 1, 3 and 5 and GTP biniding protein. Also studing the interaction between these genes in susceptible, moderate resistant and resistant rice cultivars may give us better understating to BPH-rice interaction.

3.1.1. Rice and its interaction with Brown planthopper

Rice is one of the world's most important food crops and it is attacked by 800 insect pests' species, in both the field and storage (Barrion and Litsinger, 1994). Brown planthopper (BPH) is one of the most economically important insect pests of rice and can cause devastating levels of damage. Not only can BPH cause direct damage to rice plants by removing phloem sap, but they can also transmit viral diseases during feeding such as ragged stunt virus and grassy stunt virus (Velusamy and Heinrichs, 1986; khush and Brar, 1991). The interaction between sap-feeding insects and their host plants is complicated but is currently receiving much attention. Comparisons of the feeding behaviour of BPH in susceptible and resistant plant cultivars, and in turn the different responses of these cultivars to infestation, will provide better insight into the

induced defence mechanisms and should lead to new strategies to improve resistance in susceptible plants. After landing on a rice plant BPH probes the surface to locate the weakest point in the plant and then inserts its stylet bundle with an accompanying salivary sheath into the plant (Spiller, 1990); the insect targets the sieve elements which are the functional unit of sieve tubes and from where they ingest the phloem sap (Sogawa, 1982; Seo et al., 2009). BPH feeding is divided into two main phases, the first phase includes location of the sieve elements and the second phase involves active feeding (Hattori, 2001). The electrical penetration graphs (EPG) have been used to investigate and record BPH feeding behaviour within the plants (Tjallingii, 1978, 2006).

3.1.2. Callose Structure and Callose Synthase

Callose is a polysaccharide β-1,3-glucan with some β-1,6-branches and occurs in the cell walls of a wide range of higher plants. Callose normally uses uridine diphosphate glucose (UDP-glucose) as a substrate during biosynthesis. Molecular and biochemical studies in many different plant species have shown that callose synthases are involved in the synthesis of callose (Verma and Hong, 2001; Brownfield et al., 2008). Callose plays important roles during a variety of processes in plant development and/or in response to multiple biotic and abiotic stresses. It is now generally believed that callose is produced by callose synthases and that is degraded by β-1,3-glucanases. Despite the importance of callose in plants, it is only recently that the molecular mechanisms of its synthesis have begun to be elucidated (Chen and Kim, 2009). Recent molecular and genetic studies in *Arabidopsis* have identified a set of genes that are involved in the biosynthesis and degradation of callose.

Callose is a widespread component in higher plants. In addition to its major role in a wide variety of processes during plant development, it occurs at particular stages of growth and differentiation in the cell walls or cell wall-associated structures (Stone and Clarke, 1992). As a component of the cell wall callose is synthesized at certain developmental stages such as during cell plate formation (Verma and Hong, 2001; Samuels et al., 1995), and formation of the pollen tube walls (Dumas and Knox, 1983) in response to wound, pathogen, and insect attack (Aidemark et al., 2009). Callose deposition occurs in the plasmodesmata

(PD) (Radford et al., 1998; Northcote et al., 1989) and sieve plates (McNairn et al., 1967) so as to regulate intercellular transport, often as a response to developmental cues or environmental signals, e.g., wounding and pathogen attack (Kauss, 1996; Köhle et al., 1985; Aidemark et al., 2009). Callose deposition strengthens the cell wall at the location of attack (Aist, 1976; Bell, 1981); resistant cultivars its deposition in the plasmodesmata (PD) helps prevent the spread of fungal infections (Trillas et al., 2000). Callose deposition can also be induced by treating the plants with aluminium (Bhuja et al., 2004; Schreiner et al., 1994) to seal the PD (sivaguru et al., 2000; Levy et al., 2007). Callose deposition can also be synthesized by abscisic acid, and other physiological stresses (Stone and Clarke., 1992). The plant defence hormone, abscisic acid influences both callose-dependent and -independent resistance against the fungal pathogen *Leptosphaeria maculans* (Staal et al, 2007)

3.1.3. Callose Deposition in Response to Stress

Callose deposition occurs at the plasma membrane and cell wall interface in response to a wide range of wound stresses. Callose deposition is induced within minutes in response of mechanical damage, chemical damage or ultrasonic treatments. Callose deposition can also be induced by plasmolysis in response to physiological or biotic stress, including microbial infection, and high and low temperatures (Stone et al., 1992). Several research groups reported that callose mutants GSL5/PMR4/CalS12 are responsible for callose synthesis in sporophytic tissue in response to wounding and/or pathogen. GSL5/PMR4/ CalS12 Mutants failed to synthesize callose at papillae. Surprisingly, reduction/depletion of callose in *gsl5* mutants makes the plants more resistant to pathogens, not more susceptible. Callose has a negative effect on plant defence in response to pathogen attack, possibly by delaying the plants' defence machinery against pathogen attack (Jacobs et al., 2003). The other possibility is the lack of callose in GSL5/PMR4/ CalS12 mutants may enhance the SA signalling, which results in increased resistance to pathogens (Nishimura et al., 2003). Recently two independent laboratories reported that callose synthase 7 (Cals7) is responsible for callose deposition in the sieve plates. Furthermore, mutants deficient in this enzyme (cals7) were unable to

produce callose in sieve pores in response to mechanical wound stresses (Bo and Zonglie, 2011).

3.1.4. β-1,3- glucanase genes

β-1,3-glucanases are a class of hydrolytic enzymes that catalyse the cleavage of 1,3-β-D-glucosidic linkages in β-1,3-glucans, which are found in the cell walls of various plant tissues and plant fungal pathogens (Bachman and McClay, 1996; Wessels and Sietsma, 1981; Stone and Clarke, 1992). They are widely used as molecular markers for resistance response to pathogens and systemic acquired response (SAR).

β-1,3-glucanase genes have been detected and identified in a wide range of plants, including rice (Romero et al., 1998). In plants, β-1,3-glucanases have been classified as pathogenesis-related (PR) proteins. They play a major role in plant defences in response to fungi by hydrolysing fungal cell walls and displaying antifungal activity (Leubner-Metzger and Meins 1999). Another interesting role of β-1,3-glucanase related to PR function is their involvement in response to cold (Griffith and Yaish, 2004; Yaish et al., 2006); they also play critical roles in normal developmental plant processes. There are a number of genes belonging to the β-1,3- glucanase family expressed in roots and floral tissues of healthy plants that are involved in complex hormonal and developmental regulation (Lotan et al., 1989; Memelink et al., 1990). The PR proteins include all pathogen-induced proteins and their homologs, and are routinely classified into 17 families (PR-1 to PR-17) based on their biochemical and molecular biological properties. β-1,3-glucanase belongs to PR2 group of pathogen-related (PR) proteins, they are induced in response to pathogen infection (van Loon et al., 2006). In rice plants, fourteen β-1,3-glucanase genes have been identified to date. These genes, together with other monocot *β*-glucanases, are classified into four subfamilies, endo-1,3-β-glucanases (subfamily A), endo-1,3;1,4-*β*- glucanases subfamily (B), and subfamilies (C) and (D) (Romero et al. 1998). Subfamily (A) hydrolyse β-1, 3-glucan linkages, but vary widely in their requirements for β-1,6-glucan branch linkages nearby on the polymer chain. Polymers of β-1,3-glucan are found in both plants and fungi, but polymers of β-1,3;1,6- glucan are found only in fungi. β-1,3-glucanase genes in subfamily (A) play important roles in plant defence and development.

Two tandem gene clusters, *Gns2–Gns3–Gns4* and *Gns5– Gns6*, have been detected and identified in subfamily (A) (Romero et al. 1998). β-1,3-glucanase Gns4 and Gns5 proteins have been purified from rice bran (Akiyama et al., 1997; Yamaguchi et al., 2002). *Gns4* is expressed in large quantities in germinating seedlings and highly induced by treatments with salicylic acid (Romero et al. 1998). *Gns5* is assumed to encode a PR-2 protein involved in defence against pathogen attack (Shimono et al., 2007); it is also induced in the susceptible rice cultivar TN1 in response to brown planthopper (Hao et al. 2008; Du et al. 2009). Moreover, *OsGLN1* (Akiyama and Pillai, 2001), *OsGLN2* and *Osg1* (Yamaguchi et al., 2002) have been allocated to subfamily (A) based on their substrate specificity to hydrolyse 1,3 and 1,3;1,6-*β*-glucans. In rice seedlings, *OsGLN1* was found to be up-regulated in response to drought stress and abscisic acid treatment in both root and shoot tissues. The recombinant protein GST-OsGLN1 can hydrolyse the cell wall β-glucan of the fungus *Pyricularia oryzae* that causes rice blast disease (Akiyama and Pillai 2001). β-1,3-glucanase *Gns1* gene belongs to the subfamily (B) based on sequence similarities to the *EI* and *EII* genes of barley (Romero et al. 1998). It is regulated in response to ethylene, cytokinin, wounding, salicylic acid, and fungal elicitors (Simmons et al., 1992). Over expression of β- glucanase 1 (Gns1) show significantly higher resistance to rice blast than wild type plants (Nishizawa et al. 2003). β-1,3-glucanase genes *Gns7* and *Gns8* belong to the subfamily (C) and they are induced only in growing tissues such as germinating seedlings, roots, and etiolated shoots (Romero et al. 1998). *Gns9* is a member of the subfamily (D), based on its low sequence similarity to the other subfamilies (Romero et al. 1998). The promoter of β-1,3-glucanase Gns9 gene was actively regulated in rice calli, but not in other tissues of transgenic rice plants (Huang et al., 2001). Distinct functions for subfamilies (C) and (D) genes have not yet been characterized. β-1,3-glucanase *Osg1* is essential for callose degradation in tetrad dissolution; hence, its silencing results in male sterility (Wan et al., 2011). β-1,3-glucanase *Osg1 and Gns5* genes have been induced in the leaf sheaths of susceptible rice plants (TN1) in response to brown planthopper feeding (Hao et al., 2008).

The overall aim of this study was to investigate differential gene expression in susceptible (TN1), moderately resistant (IR64) and resistant (IR70) rice cultivars in response BPH feeding. Bioassays were carried out for all three rice cultivars to confirm their resistance levels. Anatomical features of infested plants compared to control were examined to discover induced callose deposition in response to BPH feeding. Furthermore, quantitative real-time PCR was performed to investigate the transcript level of selected β-1,3-glucanase and callose synthase genes.

3.2. Material and methods

3.2.1. Plant Materials and Growth Conditions

Three rice varieties were used in this study, TN1 (Taichung Native 1), IR64 and IR70, which are susceptible, moderately resistant and resistant to the Brown planthopper, respectively. These varieties were kindly supplied by IRRI (International Rice Research Institute), Philippines. Plants were maintained at 28°C during days and 21°C during nights with a photoperiod 16h day: 8h night, 70% RH.

3.2.2. Insects

A culture of rice Brown planthopper (*Nilaparvata lugens* Stål; BPH) previously obtained from IRRI were reared on rice cultivar TN1 before starting the bioassays and subsequent work presented here. Insects were held under DEFRA Licence number (PHL 163A/6655).

3.2.3. Brown planthopper bioassay

Rice plants at 4^{th}-5^{th} leaf stage were used for the bioassay. Each plant was infested with 10 3^{rd} -4^{th} instar BPH nymphs. Plant damage caused by BPH was categorized into 6 levels, 0 - 9, with 0 as no damage (Table 3.1). Symptoms ranged from partial to obvious yellowing and low to high population density of BPH (Huang et al., 2001). The bioassay was scored independently by an observer 'blind' to the experimental treatment. The main reason for running the bioassay was to determine the exact resistant aand susceptible level foe each rice variety.

Table 3.1. Scoring system of rice plant damage caused by Brown planthopper (BPH) (Huang et al., 2001).

Resistance Score	Plant symptoms
0	None of the leaves shrank and the plant was healthy
1	One leaf was yellowing
3	One to two leaves were yellowing or one leaf shrank
5	One to two leaves shrank or one leaf shrivelled
7	Three to four leaves shrank or two to four leaves shrivelled, the plant was still alive
9	The plant died

3.2.4. Infestation of rice tissues for subsequent gene expression analyses

Rice cultivars TN1, IR64 and IR70 at the 4^{th}-5^{th} leaf stage were each infested with 10 3^{rd}-4^{th} instar BPH nymphs. Infested plants (after removal of BPH) and their respective non-infested control plants were immediately flash frozen in liquid nitrogen at the following time points post infestation: 0, 3, 6, 12, 24, 48h. Three individual plants were used as biological replicates for each time point and each variety.

3.2.5. RNA extraction

Total RNA was isolated from approximately 100 mg of frozen leaf tissue using Trizol Reagent (Invitrogen) according to the manufacturer's protocols. The concentration and purity of the RNA samples was determined using Nanodrop (ND-1000 Spectrophotometer; Nanodrop Technologies). All samples had an absorbance ratio (absorbance at A_{260}/A_{280} nm) of between 1.9 and 2.2. Following quantification, all RNA samples were normalized to 100ng μl^{-1}.

3.2.6. Primer design

To ensure maximum specificity and efficiency during qPCR amplification under a standard set of reaction conditions, Allele ID 7.7 software was used to design QRT-PCR primers. Actin1 (ACT1) was used as a reference gene in this study (Hao et al., 2008) (see table 3.2). ACT1 gave consistant expression level at different time points.

Table 3.2. Callose synthase and β-1, 3-glucanase designed primers plus the reference gene (ACT1) for QRT-PCR.

Gene	Accession number	Specific Primers for Real-Time PCR		Expected Size (bp)
		Forward Primer (5`-3`)	Reverse Primer (5`-3`)	
ACT1	AB047313	CAGCACATTCCAGCAGAT	GGCTTAGCATTCTTGGGT	**108bp**
GTP	NC_008398	AAGGATGCTGTATGTAAG	GTAGACTCTCAAGAACTT	**127bp**
GSL1	AP001389	TGAGGACCTGCCACGATT	CACGCTGATTGCGAACAT	**120bp**
GSL3	AP003268	TGGCAAGCGACCACATAG	AGACCTTAGCACGGACTG	**285bp**
GSL5	AP008212	GTGGTGTCCCTGCTATGA	GTTGTTTGCTATTCTCCC	**187bp**
β-Gns1	AB070742	GGCGTATGGGACAAAGGA	TTCAGAGGCGAAGGATGG	**240bp**
β-Gns2	U72248	GATTCAGAGGTTGGCATTGGTA	GCTACTTGTTGGACGGTTCT	**80bp**
β-Gns3	U72249	ATGAACATTGGTTGGATT	AGATGAGACTGAATAGGT	**125bp**
β-Gns5	U72251	TTGCGGCCATTCCTACAGT	TGGTGAGGGCGATGCTTG	**185bp**

3.2.7. Quantitative real time PCR (QRT-PCR)

One-step brilliant ll sybr green master mix (Agilent) was used for QRT-PCR (CHROMO 4 continuous fluorescence detector, PTC-200 Peltier Thermal Cycler). Gradient PCR was used to identify the annealing temperature. White QRT-PCR tube (0.2 ml) capped strips were used. A 25-µl reaction volume PCR was employed using 100ng RNA, 12.5µl of one step Master Mix (Agilent), 2pmol each gene-specific primer, and Ultrapure DNase/RNase-free distilled water (Qiagen) to 25µl. Amplification of RNA employed the following conditions: 30 minutes incubation at (50°C -60°C) to allow the reverse transcription, initial 10 min of denaturation at 94°C; followed by 39 cycles of denaturation 94°C for 30 s, annealing at (50°C -60°C) for 30 s, and extension at 72°C for 45 s; followed by a final extension for 5 min at 72°C (see table 3.3). Standards were included to allow results from different PCR runs to be compared. Melting curve analysis was performed at the end of the QRT-PCR cycles (Fig 3.1).

Table 3.3. Protocol setup for Real Time PCR runs.

Steps	Protocol Setup for Real-Time PCR
0	Temperature Control: Sample Calculation Lid Mode: Constant 100.0C; Shutoff < 30.0C
1	Incubate at 50-60 C for 00:30:00 minutes
2	Incubate at 94.0 C for 00:10:00 minutes
3	Incubate at 94.0 C for 00:00:30 seconds
4	Incubate at 50-60 C for 00:00:30 seconds
5	Incubate at 72.0 C for 00:00:45 seconds
6	Plate Read
7	Go to line 3 for 39 more times
8	Incubate at 72.0 C for 00:05:00 seconds
9	Melting Curve from 45.0 C to 90.0 C read every 0.5 C hold 00:00:05
10	END

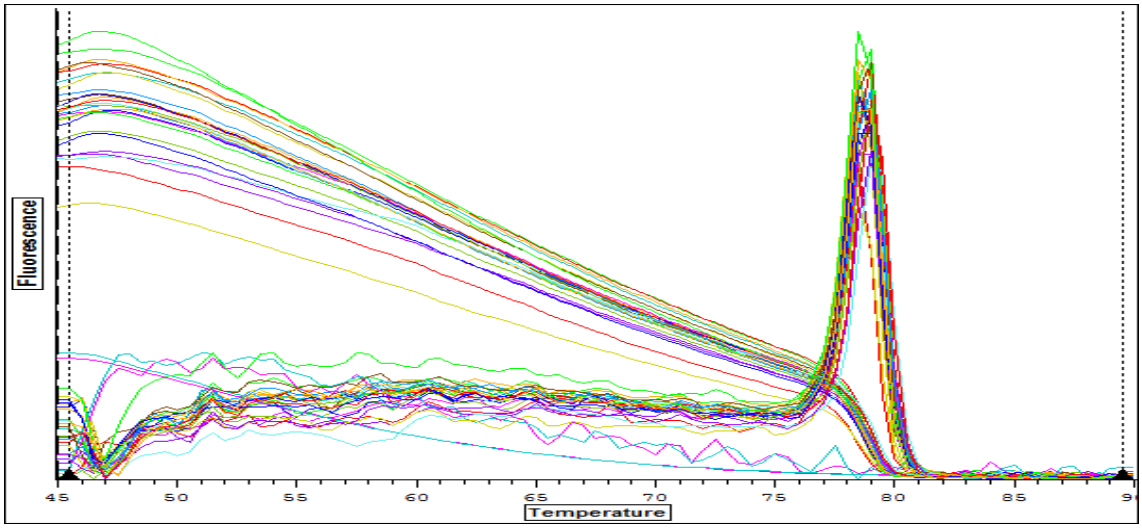

Fig 3.1. A representitative example of melting curve analysis.

3.2.8. QRT-PCR calculations

Efficiency was calculated from the slopes of the calibration curve according to the equation: $E = 10^{[-1/slope]}$.

Relative expression has been calculated according to Pfaffl equations (Pfaffl, 2001).

1- Relative expression = $2^{[\Delta CT\ control\ -\ \Delta CT\ Target\ gene]}$

2- Relative expression = $2^{\Delta\Delta CT}$

Relative quantification determines the changes in steady-state mRNA levels of a gene across multiple samples and expresses it relative to the levels of an internal control RNA. This reference gene was actin1 (ACT1) gene which was amplified in a separate tube. Therefore, relative quantification does not require

standards with known concentrations. Relative quantification is based on the expression levels of a target gene versus a reference gene and in many experiments is adequate for investigating physiological changes in gene expression levels. Two equations were used to calculate the expression of a target gene in relation to ACT1 reference gene. Calculations were based on the comparison of the distinct cycle determined by various methods, e.g., crossing points and threshold values (Ct) at a constant level of fluorescenc. Threshold cycle of the triplicate reactions were determined by using the Opticon Monitor software.

3.2.9. Histochemistry and Microscopy

Rice plants were each infested with 10 BPHs. Leaf sheaths were collected, fixed in FAE (formaldehyde: acetic acid: 70% ethanol, 5:5:90 [v/v/v]), dehydrated, embedded in paraffin, and cut into 10-µm-thick sections using a microtome. The sections were mounted on microscope slides, dewaxed, and rehydrated for staining at room temperature.

For callose observations, 10-µm-thick sections were mounted on glass slides (5 sections / slide). Callose staining was performed as described by Dietrich et al. (1994) with some modifications. Rehydrated sections were stained with 0.1% (w/v) aniline blue in 0.15 M K_2HPO_4 for 5 min and examined under a UV epifluorescence microscope. Callose deposition on individual sieve plates was classified as either faint or bright: Faint types included clearly visible plates with a thin, green-yellow appearance, whereas bright was used to describe all thickly callosed sieve plates with bright blue fluorescence (McNairn and Currier, 1967).

3.2.10. Statistical methods

Data were subjected to analysis of variance (Two ways ANOVA). The Tukey HSD (Honestly Significant Diffrence) was used to analyse the output of ANOVA. Two ways ANOVA performs an analysis of variance for testing the equality of populations means when classification of treatments is by two variables or factors. The standard error of the mean was also calculated and presented in the graphs as error bars. Where applicable: * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$.

3.3. Results

3.3.1. Performance of Brown planthopper on rice cultivars IR70, IR64 and TN1

The levels of plant damage were scoring system recorded according to IRRI Guidelines (Huang et al., 2001; IRRI, 1996; see Table 3.1). One week after BPH infestation there were clear visible differences in the level of damage, with those recorded for the susceptible rice cultivar TN1 being significantly higher (3.2) compared to the previously classified moderately resistant line IR64 (1.3) and the resistant line IR70 (0.9). Two weeks after infestation, the damage levels had increased, with TN1 (5.8), being highly significantly different $F_{2, 6}$=4.9, ($p< 0.001$) compared to IR64 (1.6) and IR70 (1.4). Resistant rice cultivars IR70 and IR64 scored (2.8), (3.2) restrictively at time point 3 (three weeks after infestation), while the susceptible rice cultivar TN1 was again significantly ($p< 0.001$) more damaged (7.8) compared to IR70 and IR64. Four weeks after BPH infestation, susceptible rice cultivar TN1 scored 9 at time point four and all plants were dead. However, the damage scores for IR70 and IR64 were 2.9, 3.6 respectively; at this stage both cultivars had started showing some symptoms (Fig 3.1).

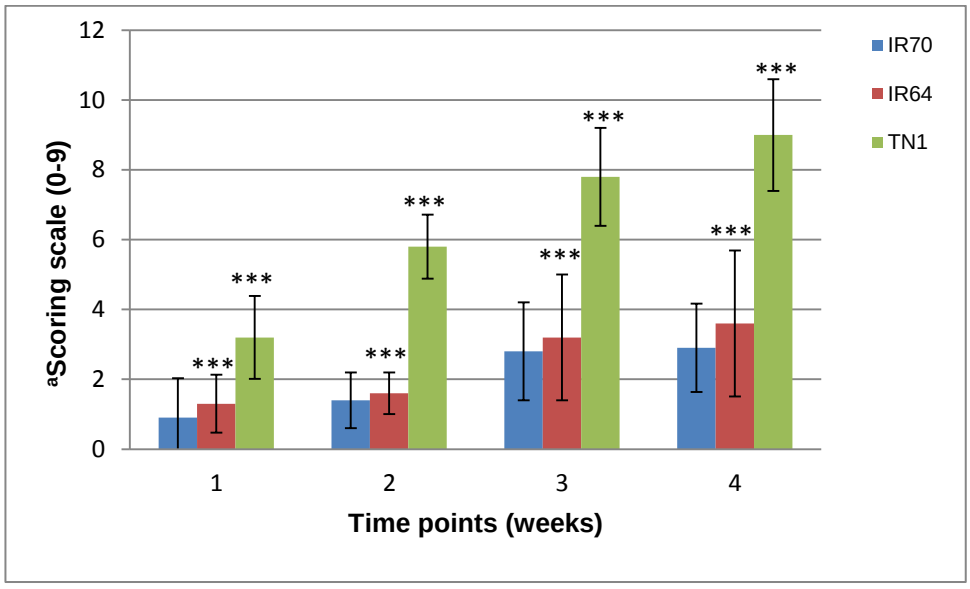

Fig 3.1. Brown planthopper Bioassay with Rice cultivars IR70, IR64 and TN1. BPH bioassays were carried out on rice cultivars previously classified as BPH resistant, moderatly resistant and susceptible, IR70, IR64 and TN1 respectively. Plant damage to BPH feeding was scored based on the degree of seedling damage. The scoring criteria were based on the Standard Evaluation System for Rice (IRRI, 1996); with 1 indicating very slight damage and 9

indicating that the seedling was dead. Ten BPH nymph instars were introduced to each plant on day 0 and plant symptoms were recorded weekly for 4 weeks. Data represent the means for 10 replicates for each cultivar. Data are means ±SE (*n* =10). Significant differences were indicated with, * p<0.05, ** p< 0.01, *** p<0.001. Two-way ANOVA was used to generate the *p*values.

[a] Data represent scoring scales for resistant (0-<3), between (3-<4) moderate resistant, (4-<7) moderate susceptible, (7-<8) susceptible, and (8-9) highly susceptible.

3.3.2. Relative expression levels of genes encoding GTP binding protein and callose synthase 1, 3 and 5 (GSL 1, 3, 5) in the susceptible rice cultivar TN1

At time point 3h+control, the expression levels of GTP and callose synthase 1, 3 and 5 genes i.e GSL 1, 3, 5 were 1.1, 1.08, 1.3 and 1.1 fold respectively relative to the zero time point (control). Three hours post infestation with BPH, the expression level of GSL5 increased by 1.5-fold compared to 1.1-fold in the control; however genes encoding GTP binding protein and GSL1, 3 were down regulated (Fig 3.2a). Six hours post infestation with BPH, the expression levels of GTP binding protein gene significantly increased to the highest level of 2.9-fold compared to 1.1-fold in the control, however GSL 1, 3 and 5 genes were down regulated. Gene expression of the GTP binding protein peaked at a level of 3.2-fold 12 hours after infestation with BPH compared to 1.5-fold for GSL1; at this time point expression of GSL3 and GSL5 was down regulated. The expression level of GSL1 increased slightly to 1.7 fold compared to previous time points 24 hours after feeding at BPH, while GTP and GSL5 were expressed at the same level of 1.3-fold, but again GSL3 remained down regulated. The expression level of GTP binding protein increased to 1.5-fold 48 hours after feeding at BPH; however GSL1, GSL3 and GSL5 genes were all down regulated (Fig 3.2). In the non-infested susceptible rice line TN1, the expression levels of GTP binding protein, GSL1, GSL3, and GSL5 were 1.06, 1.03, 1.0 and 1.1-fold at 48 hours, respectively.

3.3.3. Relative expression levels of genes encoding GTP binding protein and callose synthase 1, 3 and 5 (GSL 1, 3, 5) in the moderately resistant rice cultivar IR64

At time point 3 hours in non-infested control plants (IR64, BPH free); the expression levels of GTP binding protein, GSL1, GSL3 and GSL5 were 1.06,

1.1, 1.2 and 1.3-fold respectively. In the corresponding BPH infested plants at this same time point the expression levels of GTP binding protein increased significantly ($p<0.001$) to 9.4-fold compared to 1.1 and 1.5-fold for GSL1 and GSL3 respectively, but GSL5 was down regulated (Fig 3.2b). The expression level of GTP binding protein gene then decreased to 2.9-fold 6 hours post feeding at BPH, while the expression levels of GSL1 and GSL5 were 1.1 and 1.7 fold respectively, but GSL3 was down regulated. Twelve hours post feeding by BPH, GTP binding protein gene expression remained at similar levels (2.7-fold), while GSL1, GSL3 and GSL5 expressed to 1.0, 1.2 and 1.8-fold, respectively. After infestation with BPH for 24 hours, the GSL5 gene showed an increase in the expression levels (4.8-fold) compared to 1.5-fold for the GTP binding protein gene, and GSL1 and GSL3 showed an expression level of 1 fold. By 48 hours after feeding at BPH, GTP binding protein gene showed the highest level of expression (11.5-fold; $p<0.001$) compared to 4.2 fold for GSL1, whilst expression levels for GSL3 and GSL 5 remained at similar levels (1.5 and 1.3-fold respectively) (Fig 3.2b). In this moderately resistant rice line (IR64) after 48 hours, the expression levels for GTP, GSL1, GSL3 and GSL5 were 1.1, 1.2, 1.2 and 1.4-fold for the non-infested plants (i.e. control).

3.3.4. Relative expression of GTP binding protein and Callose synthase 1,3 and 5 genes (GSL1, 3, 5) in the resistant rice cultivar IR70 in response to BPH

At time point 3 hours for the non-infested plants (control), relative expression of genes encoding GTP, GSL1, GSL3 and GSL5 were 1.2, 1.0, 1.2 and 1.6-fold, respectively. At this same time point post feeding at BPH, the expression level of GSL5 increased to 2.8 fold compared to 2.0 fold for GTP and GSL3, while GSL1 showed only 1.0 fold increase in expression levels. The expression level of GSL1 increased significantly ($p<0.05$) to 3 fold, 6 hours after BPH feeding, compared to 1.0, 1.3 and 1.5-fold for GTP, GSL3 and GSL5 respectively. After 12 hours BPH infestation, GTP and GSL1 genes showed a ($p<0.05$) significant increase of 3 fold compared to 2.4 and 1.5 fold for GSL3 and GSL5, respectively (Fig 3.2c). Relative expression of GSL5 then increased to 2.3-fold compared to 1.9 fold for GTP binding protein gene 24 hours after feeding at BPH, while expression of GSL1 and GSL3 was 1.0 and 1.2 fold respectively. After 48 hours BPH feeding GSL5 expressed at the highest level (2.8 fold)

compared to 2.5, 2.2 and 1.0-fold in GSL3, GSL1 and GTP genes respectively. In this resistant rice line (IR70) after 48 hours, the expression levels for GTP, GSL1, GSL3 and GSL5 in the absence of BPH infestation were 1.3, 1.0, 1.2 and 1.4-fold respectively.

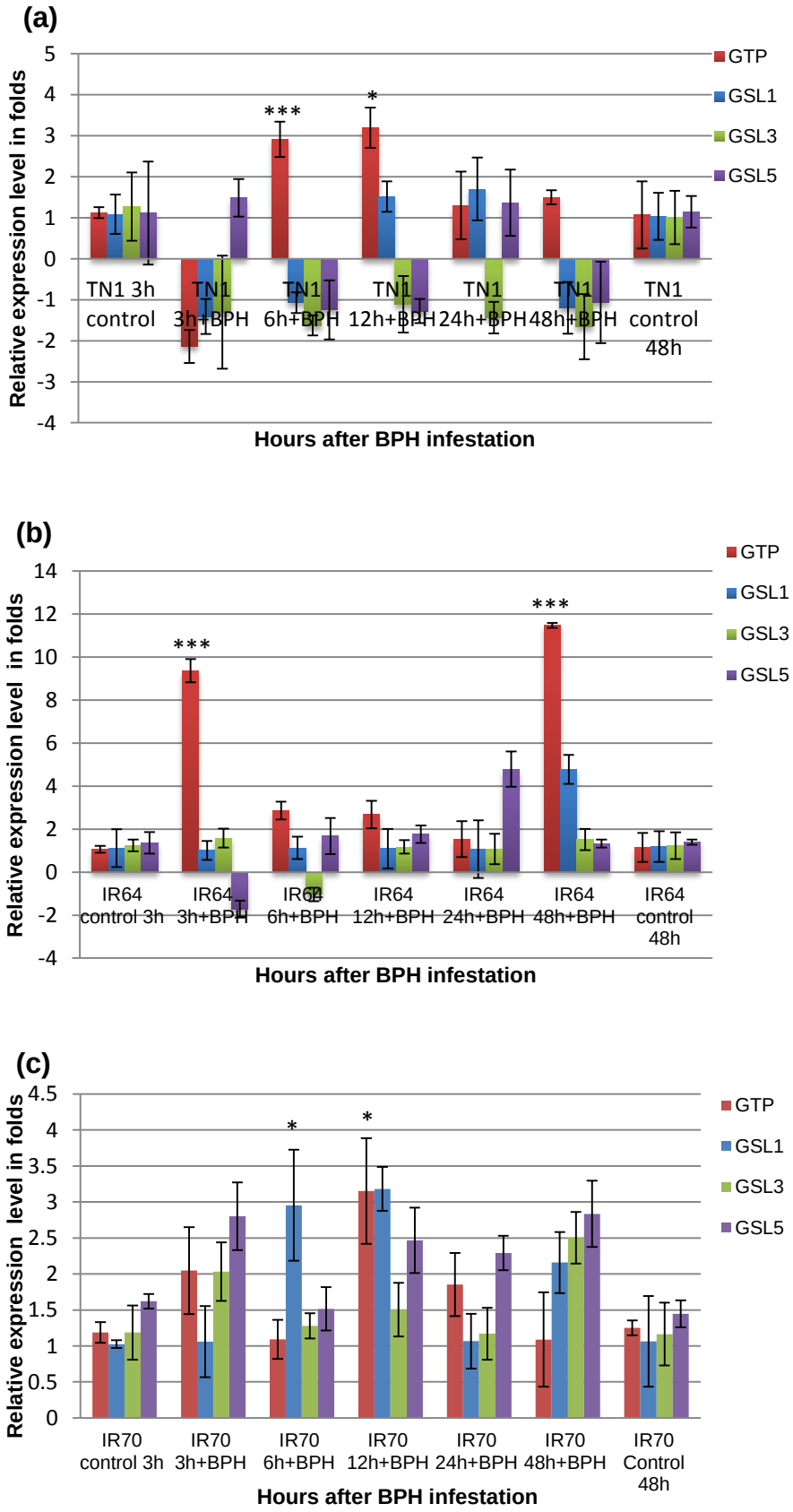

Fig 3.2. Relative expression of GTP and callose synthase 1, 3 and 5 genes (GSL1, 3, 5) in susceptible, moderately resistant and resistant rice cultivars TN1, IR64 and IR70 respectively in response to BPH feeding. Figure (a) represented the expression analysis of GTP binding protein, GSL1, GSL3 and GSL5 in susceptible rice cultivar TN1 in response to

BPH feeding. Figure (b) represented the expression level of GTP, GSL1, GSL3 and GSL5 in moderately resistant IR64 in response to BPH feeding. Figure (c) represented the QRT-PCR analysis of GTP, GSL1, GSL3 and GSL5 in resistant rice cultivar IR70 in response to BPH feeding. Total RNA was extracted from rice leaf sheaths after different BPH feeding times (3h, 6h, 12h, 24h, and 48h) plus BPH-free times (0, 3h, 48h); expression of genes was quantified relative to the value obtained from 0 (h) samples (BPH-free plants). Each RNA sample was extracted from approximately 200 mg of fresh leaf sheaths of rice plants. Rice Actin1 gene was used as reference control. Data are means ±SE (n =9). Significant differences in gene expression were indicated with, * $p<0.05$, ** $p< 0.01$, *** $p<0.001$. One-way ANOVA was used to generate the p values.

3.3.5. Relative expression of β-1, 3-glucanase 1, 2, 3 and 5 genes (Gns 1, 2, 3, 5) in susceptible rice cultivar TN1 in response to BPH

In the non-BPH infested susceptible variety TN1 (control) after 3 hours, relative expression of Gns1, Gns2 and Gns5 genes were all 1-fold. In the corresponding BPH infested plants at this time point, the expression levels of Gns5 was 2-fold, compared to 1.1-fold for both Gns1 and Gns2 (Fig 3.3a). Six hours after infestation with BPH, Gns2 increased significantly ($p<0.05$) to 2.9-fold compared to 1.8 and 1.3-fold for Gns1 and Gns5 genes, respectively. By 12 hours, the expression levels of Gns2 and Gns5 were both 1.5-fold compared to 1.2 fold for Gns1 and 24 hours after infestation, expression of genes encoding Gns2 increased to 2.8-fold compared to 1.9-fold for Gns1 and 1.5-fold for Gns5. By 48 hours after feeding at BPH, Gns2 increased to its highest level (3.1-fold) compared to 2.8 and 2.5-fold in Gns5 and Gns1 respectively. However, none of these differences in expression levels were significant, except for that of Gns 2 after 6 hours (Fig 3.3a). In this susceptible rice line (TN1) after 48 hours, the expression levels in the absence of BPH feeding for Gns1 was 1.1 fold compared to 1 fold for both Gns2 and Gns5 genes. At none of the time points was Gns3 detected, irrespective of whether the TN1 plants had been subjected to BPH feeding or not.

3.3.6. Relative expression of β-1,3-glucanase 1, 2, 3 and 5 genes (Gns 1, 2, 3, 5) in the moderately resistant rice cultivar IR64 in response to BPH

Genes encoding β-1,3-glucanase 1, 2 and 3 did not appear to be expressed in either the BPH infested or non-infested plants of this moderately resistant cultivar (IR64), irrespective of the time points analysed. Gns 5 was down

regulated in response to BPH feeding at all time points taken except for 6 hours after infestation when its expression was significantly ($p<0.001$) increased 2-fold (Fig 3.3b). Expression of this gene in the non-infested IR64 cultivar appeared to remain constant.

3.3.7. Relative expression of β-1,3-glucanase 1, 2, 3 and 5 genes (Gns 1, 2, 3, 5) in resistant rice cultivar IR70 in response to BPH

As with the moderately resistant cultivar, genes encoding β-1,3-glucanase 1, 2 and 3 were not expressed in the BPH resistant cultivar, irrespective of whether the plants had been infested or not. Interestingly, expression of Gns5 three hours after infestation was the same as in the non-infested plants. However, thereafter expression levels of this gene was down regulated in response to BPH feeding, with the lowest levels being observed after 48 hours, although this was not significant (Fig 3.3c). As in IR64, expression of Gns5 appeared to remain constant in the non-infested plants.

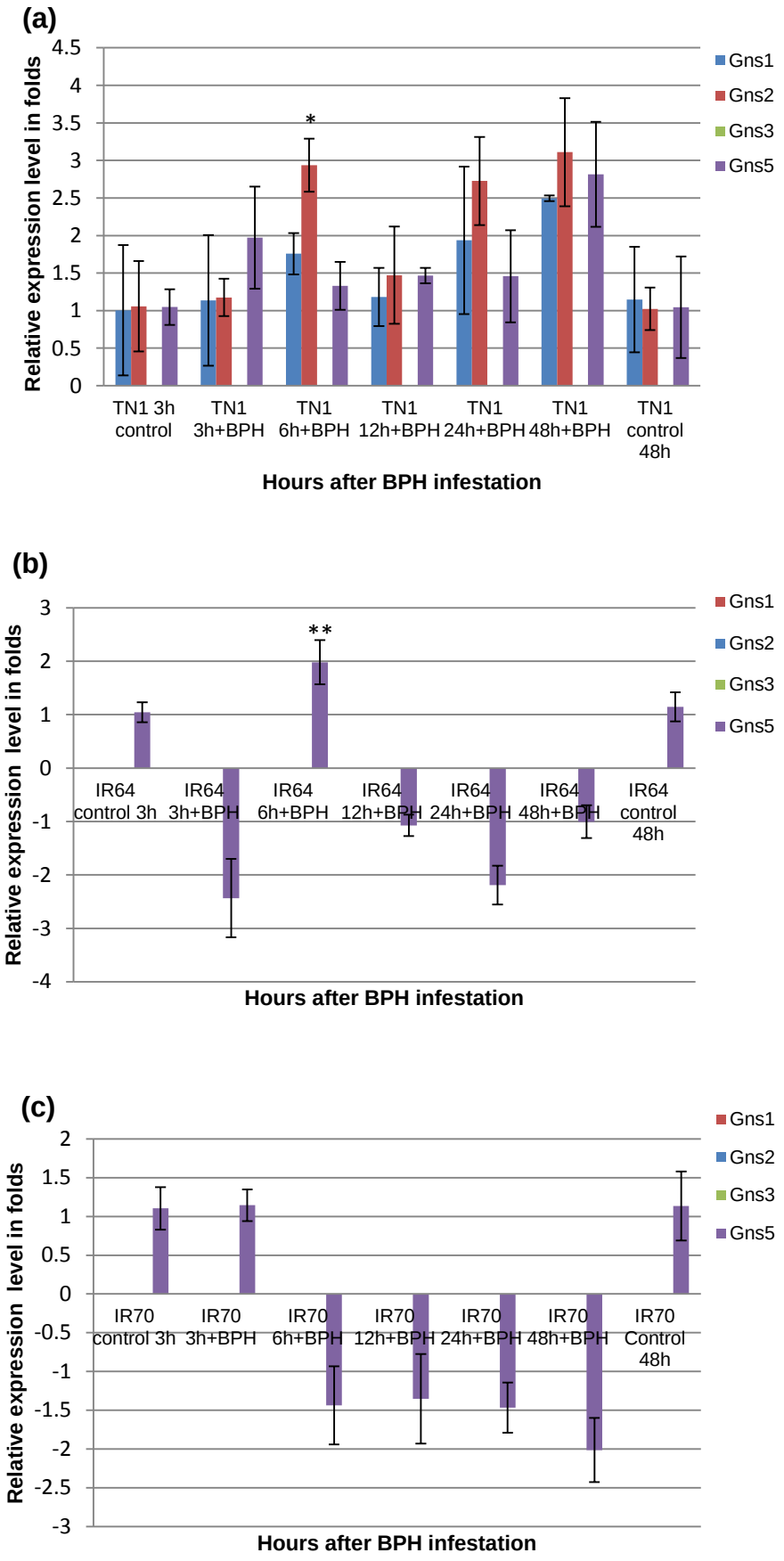

Fig 3.3. Relative expression of β-1,3-glucanase 1,2,3 and 5 genes in susceptible, moderately resistant and resistant rice cultivars TN1, IR64 and IR70 in response to BPH

feeding. Figure (a) represented the expression analysis of β-1,3-glucanase Gns1 (OSG1), Gns2, Gns3 and Gns5 genes in susceptible rice cultivar TN1 in response to BPH feeding. Figure (b) represented the QRT-PCR analysis of β-1,3-glucanase Gns1 (OSG1), Gns2, Gns3 and Gns5 genes in moderately resistant rice cultivar IR64 in response to BPH feeding. Figure (c) represented the expression analysis of represented the expression analysis of β-1,3-glucanase Gns1 (OSG1), Gns2, Gns3 and Gns5 genes in resistant rice cultivar IR70 in response to BPH feeding. Total RNA was extracted from rice leaf sheaths after different BPH feeding times (3h, 6h, 12h, 24h, and 48h) plus BPH-free times (0, 3h, 48h); expression of genes was quantified relative to the value obtained from 0 (h) samples (BPH-free plants). Each RNA sample was extracted from approximately 200 mg of fresh leaf sheaths of rice plants. Rice Actin1 gene was used as reference control. Data are means ±SE (*n* =9). Significant differences in gene expression were indicated with, * p<0.05, ** p< 0.01, *** p<0.001. One-way ANOVA was used to generate the p values.

3.3.8 Callose deposition in the infested and non-infested susceptible cultivar TN1

Callose deposition was greater in the sieve elements of the non-infested TN1 plants (Fig 3.4a) than in infested plants (Fig 3.4b). Furthermore, the fluorescence signal for callose was fainter in the susceptible cultivar TN1 for both infested and non-infested than was observed for resistant IR70 (see below).

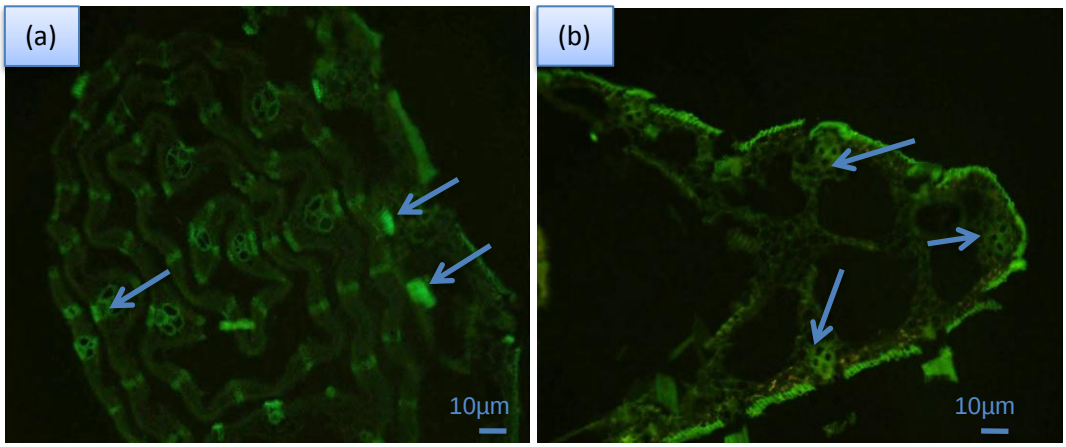

Fig 3.4. Callose deposition in BPH-infested and non-infested leaf sheath of susceptible rice cultivar TN1. An Image (a) representing transitional section of non-infested rice leaves TN1 (control). Figure (a), Induced callose deposition (blue arrows) on the sieve plates with bright green florescence in the susceptible cultivar TN1 post BPH feeding. An image (b) representing transitional section of BPH-infested rice leaves TN1. Figure (b) showing callose deposition (blue arrows) on the sieve plates with faint green florescence. Leaf sheaths were collected, fixed in FAE (formaldehyde: acetic acid: 70% ethanol, 5:5:90 [v/v/v]), dehydrated,

embedded in paraffin, and cut into 10-µm-thick sections using a microtome. The sections were mounted on microscope slides, dewaxed, and rehydrated for staining at room temperature. For callose observations, 10-µm-thick sections were mounted on glass slides (5 sections / slide). Callose staining was performed as described by Dietrich et al. (1994) with some modifications. Rehydrated sections were stained with 0.1% (w/v) aniline blue in 0.15 M K_2HPO_4 for 5 min and examined under a UV epifluorescence microscope. Blue arrows indicate deposited callose on the sieve plates of the sieve tubes. Scale bar =10 µm.

3.3.9. **Callose deposition in infested and non-infested plants of the resistant rice cultivar IR70**

There were greater levels of callose deposition on the sieve plates of BPH infested IR70 plants (Figs 3.5b and 3.5c) compared to the non-infested plants (control; Fig 3.5a). Similarly all sieve tubes targeted by BPH showed more fluorescence in the infested IR70 plants than in the controls. The fluorescence signal was very strong in infested plants compared to control plants (Fig3.5a).

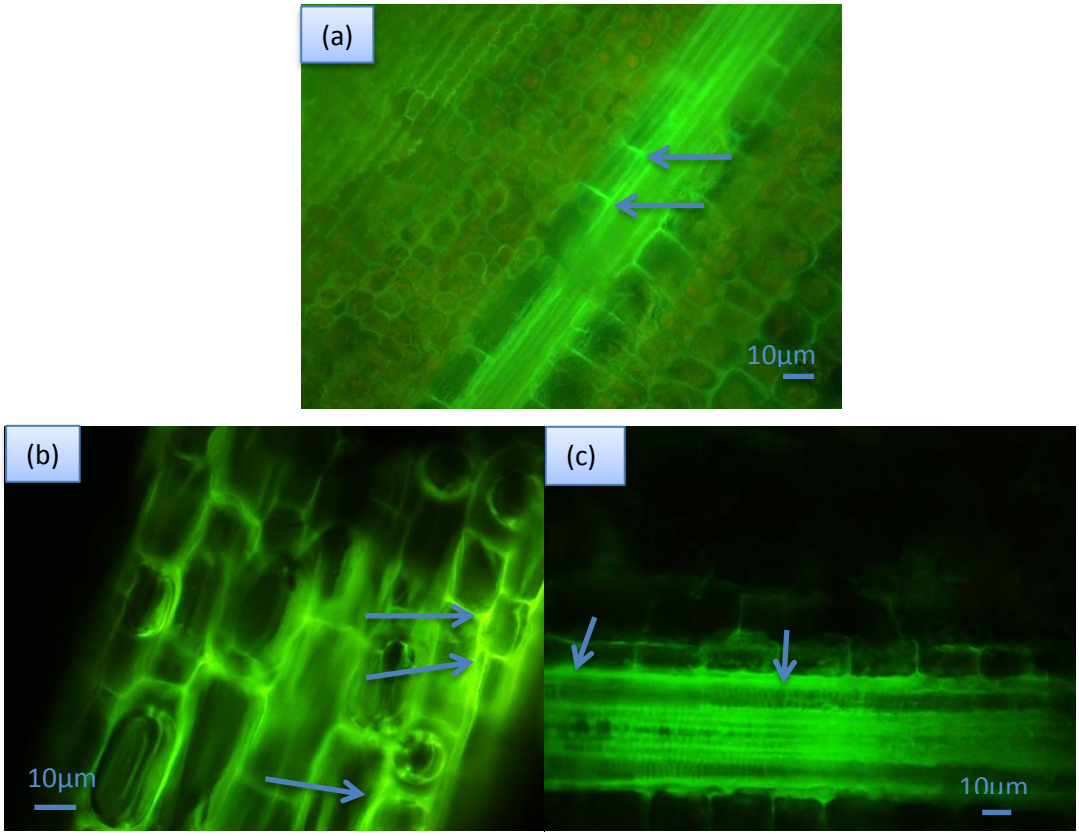

Fig 3.5. Callose deposition in BPH-infested and non-infested leaf sheath in the resistant rice cultivar IR70. Figure (a) representing induced callose deposition in longitudinal sections of leaf sheaths from non-infested (control) resistant rice cultivar IR70 plants. The image representing longitudinal section of non-infested rice leaves (control). Callose deposition (blue arrows) deposited on the sieve plates with bright green florescence in the resistant rice cultivar IR70. Figures (b) and (c) are representing longitudinal sections of induced callose deposition in the leaf sheath of the resistant rice cultivar IR70. Induced Callose deposition (blue arrows) on

the sieve plates with brighter green florescence in the BPH-infested resistant rice cultivar IR70 compared to the control. Images b and c represent the longitudinal section of infested rice leaves with 10 BPH insects. Leaf sheaths were collected, fixed in FAE (formaldehyde: acetic acid: 70% ethanol, 5:5:90 [v/v/v]), dehydrated, embedded in paraffin, and cut into 10-µm-thick sections using a microtome. The sections were mounted on microscope slides, dewaxed, and rehydrated for staining at room temperature. For callose observations, 10-µm-thick sections were mounted on glass slides (5 sections / slide). Callose staining was performed as described by Dietrich et al. (1994) with some modifications. Rehydrated sections were stained with 0.1% (w/v) aniline blue in 0.15 M K_2HPO_4 for 5 min and examined under a UV epifluorescence microscope. Blue arrows are pointing to deposited callose in sieve tubes on the sieve plates. Blue arrows are pointing to deposited callose in sieve tubes on the sieve plates. Scale bar =10 µm

3.4. Discussion

Enhancing host plant resistance in response to biotic stress is an important part of integrated pest management. Although there are rice varieties with enhanced resistance to BPH, and in some cases the genes responsible have been identified, the mechanisms involved still remain unclear. The first identified BPH resistance gene to be induced in response to BPH feeding was Bph1. In this example resistance is thought to be associated with many metabolic processes such as flavonoid and polyphenol production, including salicylic acid; it is also associated with amino acids and organic acids, such as succinic acid, and malic acid (Sogawa and Pathak, 1970; Sogawa, 1976). This gene has now been fine mapped enabling development of sequenced tagged Site (STS) markers for marker-assisted selection (Cha et al., 2008). There is now significant evidence to suggest that induced defences are effective and have low fitness costs. Numerous studies have identified gene expression patterns in rice in response to BPH feeding (Zhang et al., 2004; Yuan et al., 2005; Park et al., 2007; Wang et al., 2008).

In plants, the phloem tissues consist of sieve tubes and companion cells. BPH insects target the sieve elements, which are the functional units in the sieve tubes (Will et al., 2007). The sieve element/companion cell components are very sensitive to biotic and abiotic stresses. When stressed, these sieve elements become sealed by a range of mechanisms, such as callose formation and protein plugging (McNairn and Currier, 1967; Will and Bel, 2006). Callose plays important roles in many processes such as plant growth and development. Also, callose is deposited at the plasma membrane and cell wall interface in response to a wide range of wound stresses; this deposition can occur within minutes. It has been demonstrated that callose synthesis is Ca^{2+} dependent (King and Zeevaart, 1974). Phloem feeding insects induce calcium pathway Ca^{2+}, which activates callose synthesis and production (Arsanto, 1986; Volk and Franceschi, 2000). The high production of callose in the sieve plates either reduces the rate of phloem translocation or blocks it completely (McNairn and Currier, 1967). Electro penetration graph data have confirmed that BPH wander over the tissue surfaces for longer periods of time on the resistant rice cultivars compared to susceptible ones. Previous studies suggest that the

induced sealing of the sieve tubes in rice by callose plays an important role in the inhibition of BPH feeding (Hao et al 2008). In cotton (*Gossypium hirsutum*), callose deposition was shown to completely inhibit phloem translocation (McNairn and Currier, 1967).

Results from the present investigation show that BPH feeding affects the expression of genes related to the synthesis and hydrolysis of callose in rice plants, that is genes encoding GTP binding protein, callose synthases (GSL), and β-1,3-glucanases (Gns); furthermore, the results also demonstrate that expression of these genes varied between BPH susceptible (TN1) and BPH resistant (IR64, IR70) cultivars. The results confirm that BPH feeding on the susceptible cultivar TN1 induces the expression of genes encoding β-1,3-glucanase 1, 2 and 5 (Gns 1, 2, 5). It is known that β-1,3-glucanases lead to the hydrolysis of callose, as indeed suggested by the epifluorescence images and that susceptibility is due, at least in part, to decreased callose deposition on the sieve elements. The increased levels of β-1,3-glucanase genes in response to BPH infestation is thus consistent with this cultivar being highly susceptible to BPH. This hypothesis is further supported by the finding that genes encoding β-1,3-glucanases 1 and 2 were not detected either in the partially resistant cultivar (IR64) nor the resistant cultivar (IR70), irrespective of whether the plants had been subjected to BPH feeding or not . Furthermore, Gns5 was down regulated in the highly resistant cultivar at all time points analysed, and also down-regulated in the partially resistant cultivar except for 6h post feeding. It would appear that in the absence of these callose degrading enzymes, callose deposition occurs on the sieve plates in the resistant rice cultivars plugging the pores, so preventing BPH from continuously ingesting phloem sap.

Thus in the present study genes encoding β-1,3-glucanase 1, 2 and 5 (Gns1, Gns2 and Gns5) are likely to have been involved in callose degradation induced by BPH, and responsible for the susceptibility of TN1 plants. In addition to their role in defence, β-1,3-glucanase gene Gns1 or Osg1 is required for callose degradation in pollen development. Hao et al. (2008) found that the Osg1 gene was only expressed in susceptible TN1 and not in the resistant cultivar B5. The silencing of Osg1 gene caused male sterility in rice plants (Wan et al., 2011). Gns5 also plays an important role in plant susceptibility. Hao et al., (2008) also

found the Gns5 gene to be expressed at high levels in TN1, but only at low levels in the resistant cultivar B5 in response to BPH feeding. This suggests that in the present study Gns2, which is expressed in the susceptible cultivar TN1, but not in the moderately resistant and resistant rice cultivars IR64 and IR70, respectively, plays a key role in susceptibility. β-1,3-glucanase genes Gns2 and Gns5 are the key genes in TN1 susceptibility in response to BPH feeding. If this hypothesis is correct, then gene silencing of β-1,3-glucanase genes Gns2 and Gns5 might increase the resistance of TN1 BPH.

β-glucanase encoding genes have been classified into four subfamilies according to their structure and function. Two tandem gene clusters, Gns2–Gns3–Gns4 and Gns5–Gns6, have been recognized as a defence-related subfamily A (Romero et al., 1998); the novel Osg1 gene of rice has also been allocated to subfamily A (Tomoya et al., 2002). Isozymes of glucanase subfamily A vary widely in their requirements, requiring 1,6-β-glucan branch linkages nearby in the polymer chain for activity. Polymers of β-1,3; 1,6-glucans are found only in fungi, whilst polymers of β-1, 3-glucan are found in both plants and fungi. This suggests that β-1,3-glucanase Gns4 plays an important role in antifungal defence rather than callose decomposition because it hydrolyses β -1,3;1,6-glucans (Akiyama et al., 1997; Tomoya et al., 2002). On the other hand, isozymes that mainly hydrolyse β-1,3-glucans have been proposed to play important roles in the hydrolysis of callose and in defence against pathogen attack (Akiyama et al., 1997).

Results from the present study also demonstrated that more callose deposition occurred on the sieve plates in both susceptible and resistant rice plants in response to BPH compared to un-infested plants, so supporting the proposed role of callose deposition in induced plant defence. However, in the resistant rice cultivar IR70, most of the target sieve tubes showed strong fluorescence, representing more deposited callose within them. In contrast, in the susceptible rice cultivar TN1, the callose signals were faint suggesting much lower levels of deposited callose in many sieve tubes.

Callose (β-glucan) is composed of glucose residues linked together through β-1,3-linkages; callose synthases are involved in the synthesis of callose. Previous studies have shown that the gene encoding callose synthase 5 (GSL5)

is normally induced in response to wounding especially, insect wounding, and is also required for papillary callose formation (Andrew et al., 2003). Based on gene structure modelling, GSL1 and GSL5 have two and three exons respectively, while most GSL genes have 40–50 exons (Verma and Hong, 2001; Enns et al., 2005). GSL1 and GSL5 are involved in the formation of the callose wall that separates the microspores of the tetrad. They also play an important role in pollen grain germination (Enns et al., 2005). A single GSL gene can have several different functions; for example, GSL5 is responsible for the synthesis of wound- and pathogen inducible callose in leaf tissue; it also plays an important role in exine formation and pollen wall patterning (Jacobs et al., 2003; Enns et al., 2005). Several research groups independently demonstrated that GSL5/PMR4/CalS12 is responsible for callose synthesis in sporophytic tissue in response to wounding and/or pathogen attack; mutants of GSL5/PMR4/ CalS12, failed to synthesize callose in the papillae (Jacob et al., 2003).

In the present study expression of callose synthase genes was seen to be complex. In the resistant cultivar IR70, these genes increased in overall expression, particularly GSL1 and GSL5 in response to BPH feeding. The response was less marked in the moderately resistant cultivar (IR64) although expression levels for GSL5 and GSL1 were notably higher 24h and 48h following BPH feeding. In the susceptible cultivar there was no consistent trend in gene expression levels. Whilst callose synthase enzymes are directly involved in the synthesis of callose, the GTP binding protein is involved in the regulation of callose synthase and hence plays an important role in callose synthesis, activating defence signalling in plants in response to insect attack (Blumwald *et al*., 1998). In the present study the gene encoding this protein was up-regulated in all three cultivars, irrespective of their tolerance levels, in response to BPH feeding, thus supporting the observation that callose deposition was greater in these cultivars following infestation. Interestingly the highest fold changes in expression occurred in the moderately resistant cultivar IR64 rather than in the highly resistant cultivar. What is clear from the present study is that the expression of these genes involved in callose synthesis and degradation is dynamic and complex. However, their relative expression levels in the 3 cultivars investigated in response to BPH infestation supports the

hypothesis that callose deposition plays a major in role in resistance of rice to Brown planthopper. Selective gene silencing will provide further insight in to their respective roles in induced defence.

Chapter 4. Hydrogen peroxide plays an important role in improving ther esistant level of susceptible TN1 rice: mechanism of action

Abstract

Reactive Oxygen Species (ROS), an early response to different stimuli, control many different processes in plants. The reactive oxygen species hydrogen peroxide (H_2O_2) has been reported as a toxic cellular metabolite and functions as a key signalling molecule produced in response to different stimuli in plants. Also hydrogen peroxide is involved in numerous processes such as, cell wall rigidification, transcription of defence-related genes and programmed cell death. Hydrogen peroxide plays a dual role in plants: at low levels it acts as a messenger molecule involved in mediating signalling pathways which trigger tolerance against various biotic and abiotic stresses. However, at high concentration it coordinates programmed cell death.

BPH-susceptible rice cultivar TN1 seedlings treated with 10mM H_2O_2 exhibited enhanced resistance in response to BPH infestation with a significant decrease in BPH feeding damage ($p<0.01$). TN1 seedlings exposed to dual stressors (i.e to both 10mM H2O2 and BPH) for 24 hours showed significant increase in the transcript level of GSL5 by 5.08 fold ($p<0.01$). However genes encoding GTP, GSL1 and 3 were relatively expressed to 1.1, 1.07, and 1.6 fold, respectively. After 48 hours treatment with 10mM hydrogen peroxide, the expression level of genes encoding GTP, GSL 1, 3 and 5 were 1.4, 1.1, 1.4, and 1.06 fold, respectively. After 48 hours of treatment with water (control), the expression level of genes encoding GTP, GSL1, 3 and 5 were 1.03, 1.09, 1.3 and 1.4 fold respectively.

In TN1 seedlings exposed to 10mM H_2O_2 for 24h, the expression level of β-1,3-glucanase 5 (Gns5) was significantly downregulated by 7.1-fold ($p<0.001$) compared to 5.4 and 6.9 in Gns1 and Gns2 respectively. Plants exposed to dual stressors (i.e. to both 10mM H_2O_2 and BPH infestation) for 24h showed that the expression levels of β-1,3-glucanase Gns1 and 5 were down regulated by 4 and 8-fold respectively, however the expression level of Gns2 was highly significant ($p<0.001$) and the decrease in the expression was 14.9-fold. After 48 hours of

water treatment (control), the transcript level of Gns1, 2 and 5 were 1, 1 and 1-fold respectively.

Exogenous application of hydrogen peroxide induces Oxi1 serine –threonine MAPKs which are important for plant defence. Callose synthase plays an important role in plant resistance, especially callose synthase GSL5. β-1,3-glucanase genes, especially Gns1, 2 and 5, play key roles in plant susceptibility against BPH feeding.

4.1. Introduction

Brown planthopper (BPH) feeding affects the expression level of genes related to synthesis and hydrolysis of callose (GTP binding protein, callose synthase (GSL) and β-1,3-glucanases Gns) in susceptible, moderately resistant and resistant rice cultivars, TN1, IR64 and IR70 respectively. Furthermore, the expression level of GTP, Callose synthase (GSL 1, 3 and 5) and β-1,3-glucanases (Gns1, 2, 3 and 5) genes varied between Susceptible TN1 , moderately resistant IR64 and resistant IR70 (chapter 3). Also results in chapter 3 confirmed that BPH feeding on the susceptible rice cultivar TN1 induced β-1,3-glucanase 1, 2 and 5 (Gns 1, 2, 5). It is known that β-1,3-glucanases cause the hydrolysis of callose and that susceptibility is due, at least in part, to decreased callose deposition on the sieve elements. Genes encoding β-1,3-glucanase 1, 2 and 5 were expressed to high levels in susceptible rice cultivar TN1 in response to BPH feeding. However genes encoding β-1,3-glucanases 1 and 2 were not detected either in the partially resistant cultivar (IR64) nor the resistant cultivar (IR70) in response to BPH feeding. Additionally, Gns5 was down regulated in both partially resistant and resistant rice cultivars IR64 and IR70 respectively. It would appear that in the low level or absence of callose degrading enzymes, callose deposition occurred on the sieve plates of the resistant rice cultivars plugging the pores, so stopping BPH from ingesting phloem sap. Also, more callose deposition occurred on the sieve plates in both susceptible and resistant rice cultivars in response to BPH compared to non-infested plants (chapter 3). However, in the susceptible rice cultivar TN1, BPH manage to induce genes encoding β-1,3-glucanase 1, 2 and 5 to cause callose hydrolysis and re-attack the plant. So that inducing callose synthase may increase the resistance level in susceptible rice cultivar TN1. To mimic the mutant Oxi1 serine-threonine MAPK protein kinase, hydrogen peroxide were applied in plants to induce Oxi1 mutant. Spraying TN1 plants with low level of hydrogen peroxide induce Oxi1 serine-threonine MAPK kinase protein which in turn stimulates calcium pathway. Therafter, calcium trigger callose synthesis followed by callose production. Callose deposition increases the resistance in susceptible plants in response to insect attack.

4.1.1. Rice-BPH interaction

Rice, one of the world's most important food crops is attacked by insect pests totalling around 800 species, in both field and storage (Barrion and Litsinger., 1994). Brown Plant Hopper (BPH) is one of the most economically important insects which can cause huge damage of rice plants. BPH causes direct damage to rice plants by removing the phloem sap and also can transmit viral disease during feeding such as ragged and grassy stunt viruses (Velusamy and Heinrichs, 1986; khush and Brar, 1991). Extensive chemical control of BPH on rice can cause serious problems including toxicity to the natural enemies of BPH such as *Anagrus nilaparvatae* (Wang et al., 2008), harm the environment, increase the BPH resistance to pesticides, increase total production cost, and possible long term agro-ecosystem and human health damage (Huang et al., 2001; Rola and Pingali., 1993). BPH and aphids, as sap sucking insects have the ability to overcome many adaptations that plants have evolved to protect themselves from the insect damage. The interaction between sap-feeding insects and their host plants is complicated but most of the recent studies have revealed key results to help in understanding this interaction. Comparing feeding behaviour of BPH in susceptible and resistance rice cultivars allowed underlying insect attack mechanism to be identified and lead to new strategies to improve resistance in susceptible plants. BPH feeding processes are complex but the use of the electrical penetration graph (EPG) technique provides an opportunity for detailed cataloguing of stylet activities during feeding. The electrical penetration graph (EPG) technique has been used to follow and record the BPH feeding behaviour inside the plants (Tjallingii, 1978, 2006). BPH feeding process have been divided into two main phases according to (EPG), the first phase include the movement of the BPH stylet a cross the plant tissue and the second phase involves insect feeding (Hattori, 2001). The mouthparts of BPH, like other phloem feeding insects, consist of a stylet bundle which forms the piercing and sucking organ (Sogawa, 1982). BPH insects start the feeding process by screening the leaf area of targeted plant followed by searching for the weakest point in the leaf surface. Thereafter, BPH inserts the stylet bundle with an accompanying salivary sheath into the leaf (Spiller, 1990). BPH targets the sieve elements which is the functional units of the sieve tubes and then starts ingestion of the sap phloem (Sogawa, 1982; Seo et al., 2009).

4.1.2. Reactive Oxygen Species (ROS)

Reactive Oxygen Species (ROS) and especially hydrogen peroxide (H_2O_2) are formed during normal cell metabolism in plants. High levels of ROS that cause oxidative damage are commonly linked with opposing environmental conditions (Mittler, 2002; Noctor et al., 2002). The progression of aerobic metabolic processes for instance respiration and photosynthesis, lead to the continuous production of reactive oxygen species (ROS) in mitochondria, chloroplasts and peroxisomes, endoplasmic reticulum and in the cytosol (Gill and Tuteja, 2010). Therefore, ground state oxygen is changed to different ROS either by energy or electron transfer reactions. The former leads to the creation of singlet oxygen (O_2), whereas the latter results in the serial reduction to superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$) (Foyer and Noctor, 2000). Different ROS have a common feature which is their ability to react with wide range of biomolecules such as lipids, proteins, and nucleic acids that are necessary for cells activity and integrity. ROS is scavenging different antioxidant defence mechanisms under unstable conditions. The balance between ROS production and scavenging may be disturbed by different abiotic and biotic stress conditions, leading to a quick and transitory increase of the intracellular level of ROS (Gill and Tuteja, 2010).

ROS is known as signalling and regulatory molecules rather than harmful products of metabolic imbalance (Apel and Hirt, 2004; Mittler et al., 2004; Pastori and Foyer, 2002). Also ROS is controlling the regulation of defence responses and cell death (Alvarez et al., 1998; Zhang et al., 2003), stomatal aperture (Kwak et al., 2003; McAinsh et al., 1996; Murata et al., 2001; Pei et al., 2000), cell expansion and polar growth (Coelho et al., 2002; Foreman et al., 2003; Liszkay et al., 2004; Rodrıguez et al., 2002, 2004; Schopfer et al., 2002) and leaf and flower development (Sagi et al., 2004). In addition, ROS produced in response to biotic and abiotic stresses regulate signal change and gene expression (Baxter-Burrell et al., 2002; Desikan et al., 2001; Mittler et al., 2004; Pastori and Foyer, 2002; Shin and Schachtman, 2004; Shin et al., 2005). Accumulation of ROS occurs in different cells in response to pathogen attack (Trujilo et al., 2004). In addition to the reverse effects in a single cell type, for example, hydrogen peroxide inhibits hair growth of roots while hydroxyl radical stimulates root hair growth (Jones et al., 1998; Foreman et al., 2003). The

mechanism mediating such distinct responses rely in part on the complement of enzymes for productions and scavenging of ROS in a given cell or organelle (Mittler et al., 2004) plus the proteins and lipids lying upstream or downstream of the ROS, for example phospholipase D and phosphatidic acid (Zhang et al., 2003), ROP GTPases (Baxter-Burrell et al., 2002) and MAP kinases (Kovtun et al., 2000; Rentel et al., 2004). In the control of stomata opening, cell expansion and polar growth, plasma membrane (PM) Ca^{2+} channels appear to be the downstream of ROS production (Coelho et al., 2002; Foreman et al., 2003; Kwak et al., 2003; Murata et al., 2001; Pei et al., 2000). The resultant elevation of cytosolic Ca^{2+} could act as a second messenger or regulator of exocytosis and the cytoskeleton. ROS activation of Ca^{2+} channels probably forms the basis of a regulatory network in which specificity of 'output' is determined by the input combination of an individual ROS (superoxide anion, hydroxyl radical or H_2O_2) and a target Ca^{2+} channel in any given cell type. This would permit cell specificity and spatio-temporal heterogeneity in ROS/Ca^{2+} mediated signalling reactions.

Sensitivity of root PM Ca^{2+} channel activity to hydroxyl radicals (OH) declines from the epidermis to the pericycle and from the elongation area (epidermis) to the mature epidermis (Demidchik et al., 2003). An additional key revealed from root studies is obvious insensitivity of root epidermal PM Ca^{2+} channels to H_2O_2 (Demidchik et al., 2003; Foreman et al., 2003), tending to support the concept of differential ROS effects. Thus a distinctive pattern emerges differential ROS effects and differential channel activation that may be involved in growth and environmental sensing. Root-cell PM Ca^{2+} influx conductance have been proved to be persistent to exogenous application of hydrogen peroxide H_2O_2 (Demidchik et al., 2003; Foreman et al., 2003; Kohler et al., 2003; Kwak et al., 2003; Murata et al., 2001; Pei et al., 2000). While, guard cell PM Ca^{2+} channels react to micro molar extracellular H_2O_2 (Kohler et al., 2003).

4.1.3. Hydrogen peroxide (H_2O_2)

In plants, biotic and abiotic stresses are known to raise the concentrations of reactive oxygen species (ROS) such as hydrogen peroxide, super oxide and hydroxyl ions. The increase of ROS leads to an oxidative damage at the cellular level (Zhang et al., 2001). Exogenous hydrogen peroxide (H_2O_2) application

induces the plant defence signals in response to pathogen attack (Levine et al., 1994; Alvarez et al., 1998) abiotic (Prasad et al., 1994; VanCamp et al., 1998) and oxidative stresses (Morita et al., 1999). Hydrogen peroxide plays a dual role in plants. At low concentration it acts as a messenger molecule involved in acclimatory signalling and triggering tolerance against various biotic and abiotic stresses (Karpiniski et al., 1999; Dat et al., 2000). High concentrations of hydrogen peroxide lead to programmed cell death (Dat et al., 2003). High extracellular hydrogen peroxide (representative of apoplastic (H_2O_2) under stress conditions) stimulated a PM hyperpolarization-activated Ca^{2+} conductance in elongation zone epidermal protoplasts. Molecular mechanisms of H_2O_2 signal transduction in plants still mysterious. More studies are needed to answer this question; How H_2O_2 can trigger two extreme responses (Kovtun et al., 2000; Neill et al., 2002).

Hydrogen peroxide has been reported to stimulate germination of seeds and growth of shoots (Narimanov & Korystov, 1997). Also it can be used to reduce root and leaf diseases caused by different soil born bacteria and fungi. For example, surface sterilization and disinfestations of pine (Barnett, 1976; James & Genz, 1981) and lettuce seeds (Pernezny et al., 2001) decrease the bacterial and fungal infection. High concentrations of hydrogen peroxide cause some problems such as seedling toxicity and reduced seed germination (Edwards and Sutherland, 1979; James & Genz, 1981; Pernezny et al., 2001), indicating that cautious application as a seed disinfectant is required.

4.1.4 Hydrogen peroxide (H_2O_2) and Superoxide (O_2^-) as two reactive oxygen species (ROS) involved in root growth and differentiation

Oxygen supply is essential for roots, not only for cell respiration, but also for the formation of reactive oxygen species (ROS). Reactive Oxygen Species are the key factors for oxidative burst and also play important role in the physiological process in plants (Mittler et al., 2004). They all mainly produced in the apoplast by several enzymes. At the surface of the cells, the plasma membrane NADPH oxidase is responsible for the one-electron reduction of oxygen, yielding superoxide anion ($O_2^{\bullet-}$), an important key factor for root growth and the development of root hair (Foreman et al., 2003). The superoxide ion may be converted into H_2O_2 spontaneously or by superoxide dismutase. Some other enzymes can produce Hydrogen peroxide such as apoplastic oxalate oxidase

(Caliokan & Cuming, 1998), diamine oxidase (Federico & Angelini, 1986). Hydrogen peroxide is necessary for many developmental and physiological processes (Gapper & Dolan, 2006; Kwak et al., 2006), such as root hair growth (Foreman et al., 2003), the peroxidase-mediated formation of lignin (Ros Barceló, 1997). Hydrogen peroxide is essential for hydroxyl radical ($OH^{\bullet}$) formation by peroxidases (Chen & Schopfer, 1999). Hydroxyl radicals play important role for cell elongation because it has a loosening effect on cell walls (Liszkay et al., 2004). The tip of roots is a zone of active ROS production (Liszkay et al., 2004). It contains cells in different states within a short distance including meristematic and elongating cells, and cells at different states of differentiation (Scheres et al., 2002). Root growth of stressed plants with different stimuli can be controlled by exogenous application of hydrogen peroxide. For example, applications of hydrogen peroxide in low concentration inhibit root development in alpine larch (Shearer, 1961). Under stress conditions, high extracellular H_2O_2 stimulated a Plasma membrane (PM) hyperpolarization-activated Ca^{2+} conductance in elongation zone epidermal protoplasts. This conductance differed from that stimulated by extracellular hydroxyl radical OH and may function in stress signalling. In rice root cells, an increase of hydrogen peroxide concentration leads to root growth reduction caused by abscisic acid (ABA) (Lin & Kao, 2001). By contrast, low concentration of hydrogen peroxide lead to an increase in mass and length of roots (Narimanov & Korystov, 1997).

4.1.5. Hydrogen peroxide and Oxidative signal Inducible1 (Oxi1)

Oxidative signal Inducible1 (Oxi1) is a serine/ threonine kinase necessary for oxidative burst-mediated signalling in plant roots. Oxi1 protein kinase is a key player connecting ROS accumulation to disease resistance (Anthony et al., 2004; Rentel et al., 2004). Oxi1 is a member of AGC of protein kinase family and it is induced by exogenous application of H_2O_2. Furthermore, Oxi1 is required for full activation of MPK3 and MPK6 in response to treatment with H_2O_2 and cellulase, mimicking pathogen attack (Rentel et al., 2004). Both MPK3 and MPK6 are involved in the mitogen-activated protein kinase following recognition of bacterial flagellin by the receptor-like kinase FLS2 (Asai et al., 2002) which initialises the induction of defence genes such as WRKY22/29 and GST genes and is effective in defence responses against both biotic and abiotic

stresses (Gomez-Gomez et al., 2001; Asai et al., 2002; Chinchilla et al., 2006). NADPH-produced ROS is shown to drive expression of Oxi1 during plant-pathogen interaction and increase the plant immunity in response to Pseudomonas syringae. Regulation of Oxi1 expression levels is important in mediating an appropriate defence response but down regulation and overexpression of Oxi1 results in enhanced susceptibility to biotrophic pathogens.

4.1.6. The link between hydrogen peroxide and second messengers Ca^{2+} and ROS

Hydrogen peroxide acts as a signal molecule in plants. In plants, hydrogen peroxide generation mediated a plasma membrane bound NADPH oxidase complex (Yang et al., 2007; Lherminier et al., 2009). In *Arabidopsis* seedlings, exogenous application of 10mM hydrogen peroxide triggered a biphasic Ca^{2+} elevation (Rentel and Knight, 2004).

In plants, Ca^{2+} and ROS are considered as important signalling molecules especially in the early response to both biotic and abiotic stresses. In plant cells, the expression level of Ca^{2+} and ROS increase rapidly and within seconds in response to biotic or abiotic stresses. Calcium is considered as the key signal transducer in the activated signalling cascades in response to both biotic and abiotic stresses. Calcium is considered as an important key at which crosstalk between pathways can occur (Dey et al., 2010; Takahashi et al., 2011). Reactive Oxygen Species (ROS) are also second messengers involved in the response to different stimuli. An oxidative burst activated or induced in response to biotic stress, such as a virulent microbial infection and to abiotic stresses for example heat, cold drought, salinity and others (Lamb and Dixon, 1997; Wahid et al., 2007; Kwon et al., 2007; Miller et al., 2010). ROS production occurred in plants by plasma membrane NADPH oxidases and apoplastic oxidases as an early response to pathogen infection (Allan and Fluhr, 1997; Lamb and Dixon, 1997; Bolwell et al., 2002; Torres and Dangl., 2005; Galletti et al., 2008). In plants, NADPH oxidase, ROS and Ca^{2+} are involved in positive feedback mechanism in response to different stimuli. Reduction of ROS levels activates Ca^{2+} influx into the cytoplasm which in turn stimulates NADPH oxidase to generate ROS (Takeda et al., 2008). The co-occurrence and the levels of the

induction of Ca^{2+} and ROS vary greatly and are dependent to pathosystem and environmental conditions. For instance, callose deposition in *Arabidopsis* is connected with the levels of hydrogen peroxide production in response to the flagelin epitope Flg22 and the polysaccharide chitosan, environmental variability that imposes differential growth conditions. This shows that callose deposition is a multifaceted response controlled by several signalling pathways, depending on the environmental conditions and the challenging pathogen-associated molecular pattern (Luna et al., 2011). In pea plant, crosstalk between Ca^{2+} and ROS signals in response to abiotic stress has been reported through research in the cellular response to long-term cadmium exposure (Rodriguez-Serrano et al., 2009). In *Arabidopsis thaliana*, Cytoplasmic Ca^{2+} and ROS level increase similarly with the same kinetics in response to mechanical stimulation (Monshausen et al., 2009). Calcium stimulates ROS production (especially H_2O_2); however ROS stimulates the Ca^{2+} concentration in the cytoplasm through the activation of Ca^{2+} channels in the plasma membrane (Takeda et al., 2008; Mazars et al., 2010).

The present study was designed to characterize the influence of the exogenous application of hydrogen peroxide on the expression levels of selected genes encoding GTP, callose synthase (GSL1, 3 and 5) and β-1, 3-glucanase genes (Gns1, 2, 3 and 5) in the BPH-susceptible rice cultivar (TN1). The main theory of this work, exogenous application of hydrogen peroxide induce Oxi1 (serine - threonine MAPK) which in turn trigger callose synthase via Ca^{2+}. Bioassay was performed in 24 hours post treated TN1 seedlings with 10mM hydrogen peroxide followed by BPH infestation and symptoms were recorded. QRT-PCR was performed to investigate the expression level of Callose synthase GSL1, 3 and 5; β-1,3-glucanase Gns1, 2, 3 and 5 genes to confirm the suggested theory.

4.2. Material and methods

4.2.1. Plant Materials and Growth Conditions

Rice seeds (*Oryza sativa*) TN1 were soaked in distilled water for 24 h and germinated in the dark for 45 h at 37°C. Then the rice seedlings were grown in the growth room at 28/21°C (16-h day/8-h night) with a relative humidity of 70%. TN1 was kindly supplied by IRRI (International Rice Research Institute), Philippines.

4.2.2. Insects

Insects were cultured according to the procedures outlined in section 3.2.2.

4.2.3 Brown planthopper bioassay

Brown plant hopper bioassay was conducted according to the procedures outlined in section 3.2.3.

4.2.4. Experimental design

The rice cultivar TN1 was used for this study. Three plants were used per treatment as an individual biological replicates. The treatments are summarised in table (4.1)

Table 4.1. Summary of TN1 rice cultivar treatments.

Plant	Treatment	Time point
TN1	Water as a contact spray (TN1+water)	0h
TN1	10mM H_2O_2 as contact spray and systemic application (TN1+ 10mM H_2O_2 (24h))	24h
TN1	BPH infestation post H_2O_2 treatment (TN1+10mM H_2O_2 (24h)+ BPH (24h))	24h
TN1	10mM H_2O_2 as contact spray and systemic application (TN1+10mM H_2O_2 (48h))	48h
TN1	BPH infestation post H_2O_2 treatment (TN1+10mM H_2O_2 (48h)+ BPH(48h))	48h

4.2.5. RNA extraction

RNA was extracted according to the procedures outlined in section 3.2.5.

4.2.6. Primer design

Primers were designed according to the procedures outlined in section 3.2.6.

4.2.7. Quantitative real time PCR (QRT-PCR)

QRT-PCR preparation and protocol setupt were run according to the procedures in section 3.2.7.

4.2.8. QRT-PCR calculations

QRT-PCR results were calculated according to the procedures in section 3.2.7.

4.2.9. Statistical methods

Data were subjected to analysis of variance (Two ways ANOVA). The Tukey HSD (Honestly Significant Diffrence) was used to analyse the output of ANOVA. Two ways ANOVA performs an analysis of variance for testing the equality of populations means when classification of treatments is by two variables or factors. The standard error of the mean was also calculated and presented in the graphs as error bars. Where applicable: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.3. Results

4.3.1. Performance of Brown planthopper on susceptible rice cultivar TN1 in response to hydrogen peroxide treatment and BPH infestation

The levels of plant damage were scoring system recorded according to IRRI Guidelines (Huang et al., 2001; IRRI, 1996; see Table 3.1). One week after BPH infestation, in TN1 plants treated with 10mM hydrogen peroxide there were clear visible differences in the levels of damage, with those recorded for control plantsbeing significantly higher (3.2) compared to post treatment TN1 plants with 10mM hydrogen peroxide (0.9). Two weeks after infestation, the damage levels had increased, with TN1 (5.8), being highly significantly different ($p< 0.001$) compared to (1.2) in post treatment TN1 with hydrogen peroxide. Three weeks post BPH infestation, post treatment TN1 seedlings with 10mM hydrogen peroxide scored (3.7), while the susceptible rice cultivar TN1 was again significantly ($p< 0.001$) more damaged (7.8). Four weeks after BPH infestation, susceptible rice cultivar TN1 scored 9 and all plants were dead. However, the damage scores for post treated TN1 with 10mM hydrogen peroxide was (4). At this stage both susceptible TN1 and post treated TN1 with hydrogen peroxide had started showing some symptoms (Fig 4.1).

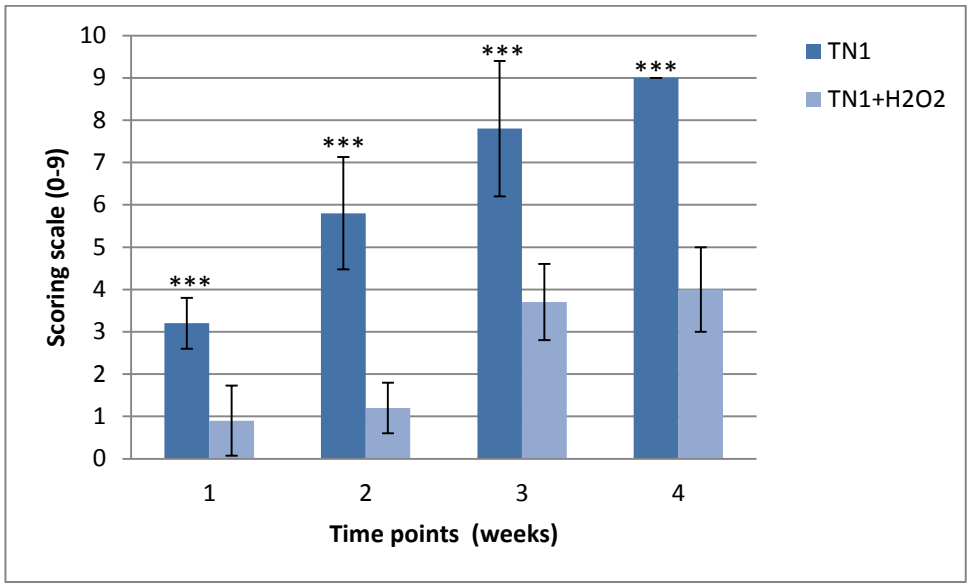

Fig 4.1. Brown planthopper Bioassay with Rice cultivar TN1 in response to hydrogen peroxide treatment and BPH infestation. BPH bioassays were carried out on susceptible rice cultivar TN1 and susceptible at the 3rd – 5th leaf stages. Plant damage to BPH feeding was scored based on the degree of seedling damage. The scoring criteria were based on the Standard Evaluation System for Rice (IRRI, 1996); with 1 indicating very slight damage and 9

indicating that the seedling was dead. Ten BPH nymph instars were introduced to each plant on day 0 and plant symptoms were recorded weekly for 4 weeks. Data represent the means for 10 replicates for each cultivar. Data are means ±SE (*n* =10), *, p<0.05, **, p< 0.01, ***, p<0.001. One-way ANOVA was used to generate the *p* values.

[a] Data represent scoring scales for resistant (0-<3), between (3-<4) moderate resistant, (4-<7) moderate susceptible, (7-<8) susceptible, and (8-9) highly susceptible.

4.3.2. Relative expression levels of genes encoding GTP binding protein and callose synthase 1, 3 and 5 (GSL 1, 3, 5) in susceptible rice cultivar TN1 in response to hydrogen peroxide treatment and BPH infestation

The results showed that the expression levels of genes encoding GTP binding protein, callose synthase 1, 3 and 5 i.e GSL 1, 3, 5 were 1.07, 1.1, 1.5 and 1.3 fold respectively, 24 hours post treatment with water (control), relative to the zero time point (control). Twenty four hours post treatment with 10mM H_2O_2, the expression level of GTP increased by 1.5-fold compared to 1.07-fold in the control; however genes encoding GSL1, 3 and 5 were 1.09, 1.05 and 1.05-fold respectively (Fig 4.2). Plants exposed to the dual stress (i.e to both 10mM H_2O_2 and BPH) for 24 hours showed significant increase in the transcript level of GSL5 by 5.08 fold (p<0.01), however genes encoding GTP, GSL1 and 3 were relatively expressed to 1.1, 1.07, 1.6-fold respectively. Forty eight hours post treatment with 10mM hydrogen peroxide, the expression level of genes encoding GTP, GSL 1, 3 and 5 were 1.4, 1.1, 1.4, and 1.06-fold respectively. Plants exposed to the dual stress (i.e to both 10mM H2O2 and BPH) for 48 hours showed significant increase in the transcript level of GSL5 by 3.2-fold (p<0.01), however genes encoding GTP, GSL1 and 3 were relatively expressed to 1.1, 1.07, 1.6-fold respectively. Forty eight hours post treatment with water (control), the expression level of genes encoding GTP, GSL1, 3 and 5 were 1.03, 1.09, 1.3 and 1.4 fold respectively (Fig 4.2).

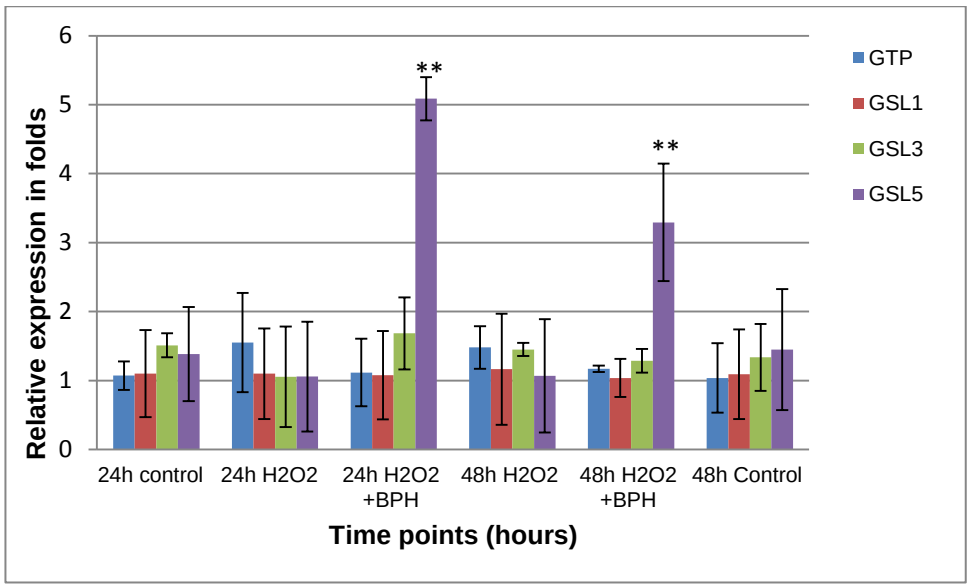

Fig 4.2. Relative expression levels of genes encoding GTP binding protein and callose synthase 1, 3 and 5 (GSL 1, 3, 5) in susceptible rice cultivar TN1 in response to hydrogen peroxide treatment and BPH infestation. Results of real-time PCR represent the expression analysis of GTP binding protein gene and Callose synthase genes GSL1, 3 and 5 in susceptible rice cultivar TN1 in response to 10mM hydrogen peroxide treatment and BPH feeding (Table 4.2). Total RNA was extracted from TN1 sheath leaves (3^{rd}- 5^{th} leaf stage) of treated plants with 10mM H_2O_2 for 24h and 48h, post treated plants with 10mM H_2O_2 for 24h and 48 h followed by BPH infestation for (24 and 48 hours) and non-treated plants (control) (Table 4.2). Expression level of genes was quantified relative to the value obtained from 0 (h) samples (BPH and hydrogen peroxide -free plants). Each RNA sample was extracted from approximately 200 mg of fresh leaf of TN1 leaves. Rice ACT1 gene was used as reference control. Significant differences in gene expression were indicated with * $p<0.05$, ** $p< 0.01$, *** $p<0.001$. Data are means ±SE (n =9). Two way Anova were used to generate the p values.

4.3.3. Relative expression of β-1,3-glucanase 1, 2, 3 and 5 genes (Gns 1, 2, 3, 5) in susceptible rice cultivar TN1 in response to hydrogen peroxide treatment and BPH feeding

Following BPH and/or H_2O_2 treatment, no transcripts were detected for the β-1,3-glucanase gene 3 (Gns3). Twenty four hours post treatment with water (control) the transcript level of Gns1, 2 and 5 were 1, 1.1 and 1.2-fold respectively. Under conditions of either the abiotic stress i.e. H_2O_2, or biotic stress (BPH infestation), transcripts for Gns1, 2, and 5 were down-regulated for both time points. Similarly, these transcripts were also down regulated in plants receiving both stresses compared to the non-stressed plants. Plants exposed to 10mM H_2O_2 for 24h showed that the expression level of Gns5 was significantly downregulated by 7.1-fold ($p<0.001$) compared to 5.4 and 6.9 in Gns1 and

Gns2 respectively. Plants exposed to dual stress (i.e. to both 10mM H_2O_2 and BPH infestation) for 24h showed that the expression level of β-1,3-glucanase Gns1 and 5 were down regulated by 4 and 8-fold respectively, however the expression level of Gns2 was highly significant ($p<0.001$) and the decrease in expression was 14.9-fold. Forty eight hours post water treatment (control), the transcript level of Gns1, 2 and 5 were 1, 1 and 1-fold respectively (Fig 4.3).

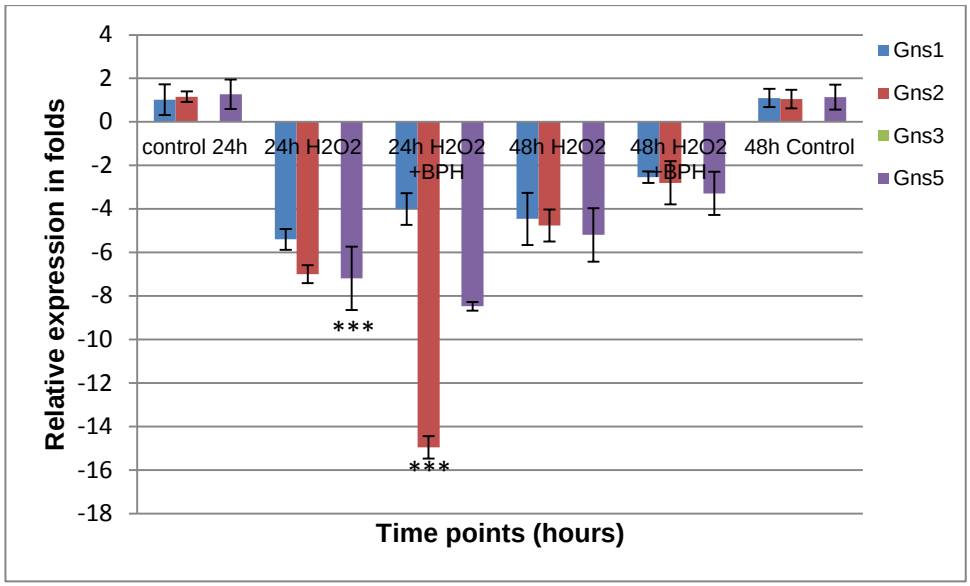

Fig 4.3. Relative expression of β-1,3- glucanase genes (Gns1, 2, 3 and 5) in response to hydrogen peroxide treatment and BPH feeding. Results of real-time PCR represent the expression analysis of Gns1, 2, 3 and 5 genes in susceptible rice cultivar TN1 in response to 10mM hydrogen peroxide treatment and BPH feeding (Table 4.2). Total RNA was extracted from TN1 sheath leaves (3^{rd}- 5^{th} leaf stage) of treated plants with 10mM H_2O_2 for (24 and 48 hours), post treated plants with 10mM H_2O_2 for (24 and 48 hours) followed by BPH infestation for (24 and 48 hours) and non-treated plants (control) (Table 4.2). Expression of genes was quantified relative to the value obtained from 0 (h) samples (aphid and hydrogen peroxide -free plants). Each RNA sample was extracted from approximately 200 mg of fresh leaf of TN1 leaves. Rice ACT1 gene was used as reference control. Significant differences in gene expression were indicated with * $p<0.05$, ** $p< 0.01$, *** $p<0.001$. Data are means ±SE (*n* =9). Two ways Anova were conducted to generate the *p* values.

4.3.4. The effect of exogenous application of 10mM H_2O_2 in the phenotype of TN1 seedlings compared to control (water treatment)

Fig 4.4 Image (A) and (B) show that there are significant difference in the phenotype characters (shape, size, and leaf length and leaf width) between treated and non-treated TN1 with 10mM H_2O_2. Twenty four hours post

application of hydrogen peroxide, TN1 leaves length, width and size was bigger compared to control (water treatment) (B). Figure 4.4 (C) TN1 seedling 72 hours post treatment with 10mM hydrogen peroxide, the plant size was significantly bigger than the control but leaves showed yellowing symptoms (Fig 4.4 (C) and (D)).

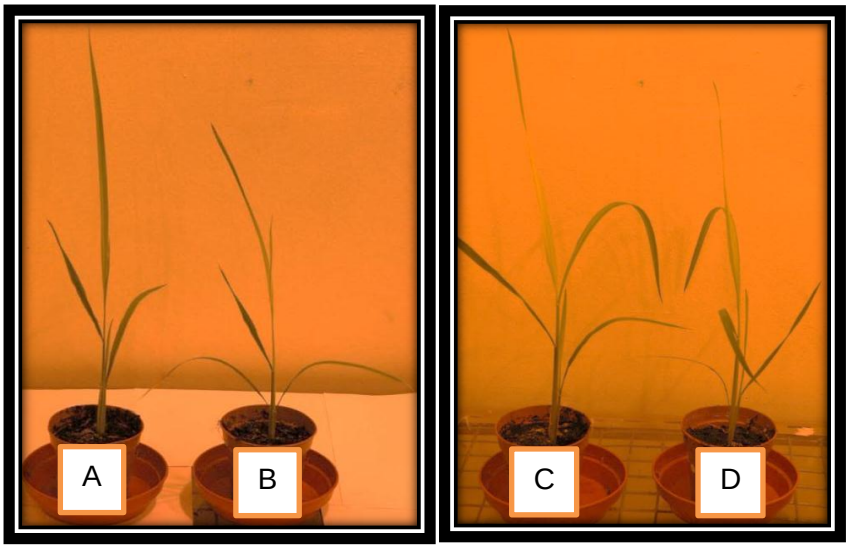

Fig 4.4. The effect of exogenous application of hydrogen peroxide in the phenotype of susceptible rice cultivar TN1 compared to control plants (treated with water). (A) TN1 seedlings, twenty four hours post treatment with 10mM H_2O_2 (spray and systemic application); (B) TN1 seedlings, Twenty four hours post treatment with water (spray and systemic application); (C) TN1 seedlings, seventy two hours post treatment with 10mM H_2O_2 (spray and systemic application); (D) TN1 seedlings, seventy two hours post treatment with water (spray and systemic application).

4.4. Discussion

Reactive Oxygen Species (ROS), an early response to different stimuli, control lots of different processes in plants. ROS such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) are regularly produced by normal cellular metabolic process as well as photosynthesis and respiratory electron flow (Halliwell and Gutteridge, 1989). Hydrogen peroxide (H_2O_2) production is a key response and also involved in numerous processes such as cell wall rigidification, transcription of defence-related genes and programmed cell death (Levine et al., 1994; Neill et al., 2002). The reactive oxygen species hydrogen peroxide (H_2O_2) has been reported as a toxic cellular metabolite and also functions as a signalling molecule that stimulates response to different stimuli in plants. Up regulation of hydrogen peroxide in response to various stimuli indicating that it is the key factor for tolerance induction in stressed plants (Neill et al., 2002). Hydrogen peroxide plays a dual role in plants**:** at low levels it acts as a messenger molecule involved in mediating signalling pathways which trigger tolerance against various biotic and abiotic stresses. Hydrogen peroxide at high concentrations coordinates programmed cell death (Dat et al., 2003).

Results from the present investigation showed that exogenous application of hydrogen peroxide improved the resistance in BPH-susceptible rice cultivar TN1 in response to BPH feeding. Bioassay with TN1 treated with 10mM followed by BPH infestation showed that the BPH-susceptible rice cultivar TN1 became moderately resistant to BPH (Fig 4.1). Also, hydrogen peroxide treatment resulted in marked differences in the phenotype of TN1 plants compared to non-treated plants (control) within 24-48 hours (Fig 4.4). Hydrogen peroxide treatment in TN1 affects the expression level of genes related to the synthesis and hydrolysis of callose in rice plants, thus are genes encoding GTP binding protein, callose synthases (GSL), and β-1,3-glucanases (Gns). The results showed that the expression level of genes encoding β-1,3-glucanase 1, 2 and 5 (Gns 1, 2, 5) were down-regulated in response to hydrogen peroxide treatment. Callose synthase gene GSL5 was up-regulated in response to exogenous application of hydrogen peroxide followed by BPH infestation.

Exogenous application of hydrogen peroxide induces Oxi1 serine-threonine MAPK kinase. MAPK Protein kinase (serine-therionine) stimulates Ca^{2+} pathway which in turn trigger callose synthase followed by callose production (Fig 4.5). Callose deposition especially GSL5 (Jacobs et al., 2003) occurred in the sieve elements of the sieve phloem in response to insect wounding.

In higher plants, callose synthesis and accumulation are well controlled during plant growth and development such as, cell division, cell growth and differentiation. Callose deposition can also be induced in response to biotic or abiotic stress (Bacic et al., 2009; Verma, 2001; Hong et al., 2001). Under normal growth conditions callose is present in the sieve plate at normal level. It accumulates rapidly and drastically in response to stress. Callose is also deposited in the plasmodesmata and at sieve plates to limit intercellular transport, often as a response to developmental cues or environmental signals, e.g., wounding and pathogen attack. Genes encoding callose synthases (GSL) have now been identified in several plant species **(**Aidemark, 2009).

Application of hydrogen peroxide triggers the second messengers Ca^{2+} and ROS signalling molecules. In plant, Ca^{2+} and ROS are considered as important signalling molecules especially in the early response to both biotic and abiotic stresses. In plant cells, Ca^{2+} and ROS signalling increase rapidly and within seconds in response to biotic or abiotic stresses. The exogenous application of hydrogen peroxide improves signal transduction of ROS which subsequently improve the resistance of the cellular level and in the whole plant. An oxidative burst activated or induced in response to biotic stress such as insect feeding, avirulent microbial infection and to abiotic stresses for example heat, cold drought, salinity and others (Lamb and Dixon, 1997; Wahid et al., 2007; Kwon et al., 2007; Miller et al., 2010). An increase in ROS or hydrogen peroxide in the cells in response to exogenous application of hydrogen peroxide could be toxic to the insect and affect insect feeding and subsequently insect reproduction.

Application of 10mM hydrogen peroxide to the rice cultivar TN1 followed by BPH infestation, induced Oxi1 protein kinase. Induction of Oxi1 protein kinase is required for full activation of MAPK3 and MAPK6. Thereafter, MAPKs trigger Ca^{2+} signalling pathway which In turn stimulate callose synthase followed by

callose deposition in response to BPH feeding. As a result of hydrogen peroxide application, BPH-susceptible rice cultivar TN1 became moderate resistant.

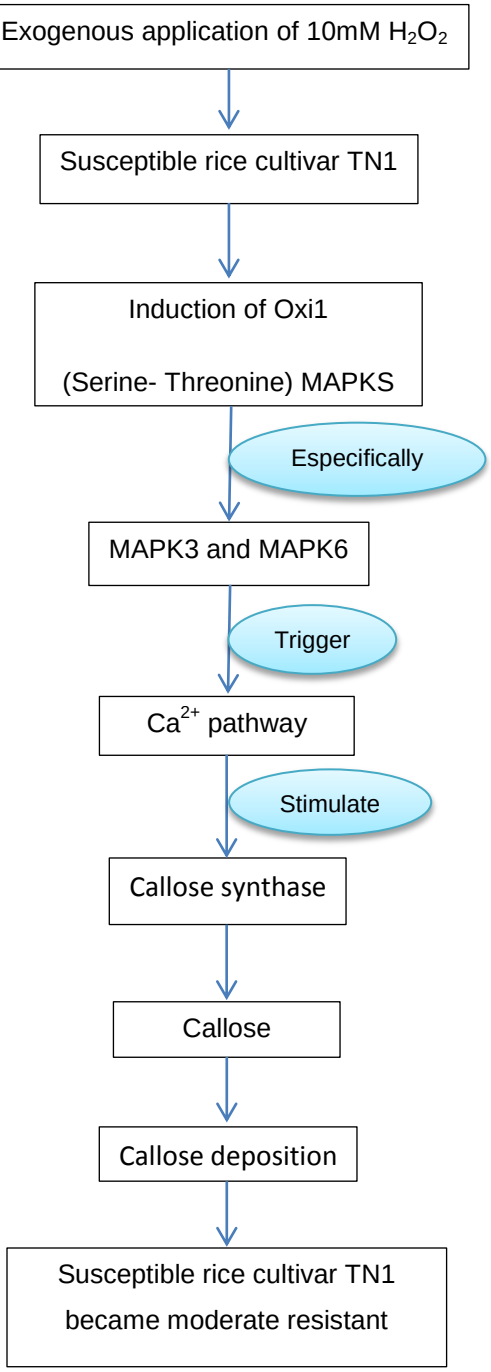

Fig 4.5. Schematic diagram showing predicted signalling pathway in susceptible rice cultivar TN1 in response to hydrogen peroxide treatment.

Chapter 5. Superoxide dismutase activity in rice plants in response to biotic (BPH) and abiotic stress (NaCl and hydrogen peroxide)

Abstract

Plants have developed antioxidant systems to protect cellular membranes and organelles from damaging effects of AOS. Tolerance to biotic and abiotic stresses varieties have been correlated with increased activity of antioxidant enzymes and levels of antioxidant metabolites. Plants protect the cellular and sub-cellular systems from the cytotoxic effects of these ROS in the form of enzymes such as superoxide dismutase, ascorbate peroxidase, peroxidase, glutathionereductase and catalase and metabolites such as glutathione, ascorbic acid, α- tocopherol and carotenoids.

At zero time point (control) SOD activity in TN1 was 0.106 unit/mg^{-1} compared to 0.119 unit/mg^{-1} activity in moderately resistant IR64. Three hours post BPH feeding, the SOD activity for moderate resistant IR64 increased significantly to 0.19 unit/mg^{-1} ($p<0.01$) compared to 0.13 unit/mg^{-1} activity in susceptible TN1. Thereafter, SOD activity in IR64 was significantly higher 0.17 unit/mg-1 compared to 0.099 unit/mg^{1-} in TN1 six hours post BPH feeding. Twelve hours post BPH feeding; SOD activity for IR64 increased significantly 0.19 unit/mg^{1-} compared to 0.077 unit/mg^{1-} in TN1 twelve hours post BPH feeding. Interestingly, the SOD activity in IR64 increased significantly ($p<0.01$) to the highest level 0.21 unit/mg^{1-} compared to 0.70 unit/mg^{1-} in TN1 twenty four hours post BPH feeding. SOD activity isoforms showed connected decrease with the total activity in TN1 in response to BPH feeding. However, SOD activity isoforms showed connected increase with the total activity in IR64 in response to BPH feeding.

The total SOD activity in both control shoots and roots was not significantly different. However, the total SOD activity in salt stressed TN1 shoots was significantly higher ($p<0.05$) with 0.21 unit/mg^{1-} compared to 0.165 unit/mg^{1-} in stressed roots. Twenty four hours post treatment with hydrogen peroxide, total activity in shoots was significantly (0.01) high 0.203 unit/mg^{1-} compared to 0.123 unit/mg^{1-} in the roots. SOD isoforms showed connected increase with the total activity in TN1 in response to salt stress. However, SOD activity isoforms

showed connected decrease with the total activity in TN1 in response to hydrogen peroxide treatment compared to salt stress.

SOD is the important free radical scavenger in the plants. SOD can clear the excessive free oxygen radicals such as O_2, H_2O_2 and OH^- which are dangerous to plant cells. An increase in the SOD activity improved the resistance of rice plants in response to biotic and abiotic stress. Increased antioxidant enzyme activity was one of the BPH/NaCl-stress tolerance mechanisms of rice. However, low activity of SOD may lead to an increase in the free radicales which in turn cause cell toxicity followed by cell death. Exogenous application of hydrogen peroxide in TN1 may increase SOD activity in rice plants in response to BPH feeding. SOD plays an important role in the plant defence in response to different stimuli.

5.1. Introduction

Reactive oxygen species (ROS) have a cell signalling role in many biological systems, especially in plants. ROS induce programmed cell death or necrosis, induce or suppress the expression of many genes, and activate cell signalling cascades, such as those involving mitogen-activated protein kinases. ROS, in particular hydrogen peroxide, were recognized as important signalling molecules in plant kingdom. The reactive oxygen species hydrogen peroxide (H_2O_2) has been reported as a toxic cellular metabolite and also functions as a signalling molecule that stimulates response to different stimuli in plants.

Exogenous application of hydrogen peroxide improved the resistance in BPH-susceptible rice cultivar TN1 in response to BPH feeding. Treated susceptible rice cultivar TN1 with 10mM followed by BPH infestation showed that the BPH-susceptible rice cultivar TN1 became moderately resistant to BPH. The application of hydrogen peroxide in suceptible rice cultivars affects the expression level of GTP binding protein, calosse synthase (GSL1 and GSL5) and β-1,3-glucanases (Gns1, 2 and 5). Treated susceptible rice TN1 with 10mM H_2O_2 showed and increase in the expression level of callose synthase GSL5 and downregulation of β-1,3-glucanases (Gns1, 2 and 5). Exogenous application of hydrogen peroxide induced Oxi1 serine-threonine MAPK kinase and triggered signal transduction of ROS. An induction of MAPK protein kinase stimulated calcium pathway which in turn triggered callose synthase followed by callose production (chapter 4).
Hydrogen peroxide treatment may increase the activity of superoxide dismutase in response to BPH feeding and lead to resistance enhancement in susceptible rice cultivar TN1.

5.1.1 Reactive oxygen species (ROS)

Reactive oxygen species (ROS) play a central role in plant defence against various pathogens (Mittler et al., 2004). The superoxide anion (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radicals are the three major forms of ROS. These molecules are highly reactive and toxic and can lead to the oxidative destruction of cells (Asada and Takahashi, 1987). The rapid accumulation of plant ROS at the site of pathogen attack site is a phenomenon called oxidative burst which

is toxic to pathogens directly (Lamb and Dixon, 1997) and can lead to a hypersensitive response (HR) that results in a zone of host cell death, which prevents further spread of biotrophic pathogens (Heath, 2000; Gechev et al., 2006). In addition to the described direct effects, ROS can also serve as signals that lead to the activation of other defence mechanisms (Dat et al., 2000; Grant and Loake, 2000).

Plants produce Reactive Oxygen species (ROS), in response to certain environmental stresses, such as salinity (Hernandez et al., 1995), drought (Price et al., 1989), desiccation (Senaratna et al., 1985a, b), extreme temperatures (Kendall and McKersie 1989; McKersie et al., 1993), high light intensity (Fryer et al., 2002) and ozone (Van Camp et al., 1994). Chloroplasts are particularly susceptible to ROS due to high concentration of oxygen that reacts with escaped electrons from the photosynthetic electron transfer system (Foyer et al., 1994). ROS affects a variety of biological macromolecules, cause severe cellular damage, and inhibit photosynthesis resulting inreduction in the yield of the crops.

5.1.2 Super Oxide Dismutase (SOD)

Plants have developed antioxidant systems to protect cellular membranes and organelles from damaging effects of AOS (Foyer et al., 1991). Superoxide dismutase (SOD), as one of the enzymatic mechanisms, that plays an important role in response to different stimuli. SOD catalyses the dismutation of superoxide into oxygen and hydrogen peroxide (Fridovich, 1975). There are three distinct types of SOD enzymes containing Mn, Fe, or CU plus Zn as prosthetic metals. Several SOD forms (Mn-SOD, Fe-SOD, and Cu/Zn-SOD) are known to occur in different plant cell compartments. Each type of SOD has several forms. Manganese-containing superoxide dismutase (Mn-SOD) localized in the mitochondria and peroxisomes; copper- and zinc-containing superoxide dismutase (CuZn-SOD) occurs within chloroplasts, cytosol and mitochondria (Foster and Edwards, 1980; Sakamoto et al., 1995), while Fe-SOD appears to be located exclusively in chloroplasts (Fig 5.1) (Salin and Bridges, 1980, Niewiadomska et al., 1997). Cu-Zn-SODs forms are very sensitive to cyanide (CN^-) and hydrogen peroxide (H_2O_2), while Fe-SODs are

sensitive to hydrogen peroxide (H_2O_2) but not sensitive to cyanide (CN^-). Manganese forms of SOD (Mn-SODs) are not sensitive to both hydrogen peroxide (H_2O_2) and cyanide (CN^-) (Fridovich, 1975). At physiological pH, superoxide dismutase (SODs) may produce significant amounts of hydrogen peroxide and this is considered as an important feature of SODs (Fridovich, 1989). Despite the fact that excess (H_2O_2) is potentially harmful, the maintenance of hydrogen peroxide (H_2O_2) in plants in low levels is critical because it is a signal molecule that controls gene expression in response to biotic and abiotic stimuli (Lamb and Dixon 1997; Levine et al. 1994). For instance, transient and local accumulations of hydrogen peroxide (H_2O_2) are involved in the hypersensitive response and programmed cell death (Grant and Loake, 2000; Levine et al., 1994).

5.1.3. Hydrogen peroxide (H_2O_2)

In plants, biotic and abiotic stresses are known to raise the concentrations of reactive oxygen species (ROS) such as hydrogen peroxide, super oxide and hydroxyl ions. The increase of ROS leads to an oxidative damage at the cellular level (Zhang et al., 2001). Exogenous hydrogen peroxide (H_2O_2) application induces the plant defence signals in response to pathogen attack (Levine *et al.,* 1994; Alvarez et al., 1998) abiotic (Prasad et al*.,* 1994; Van Camp et al*.,* 1998) and oxidative stresses (Morita *et al.,* 1999). Hydrogen peroxide plays a dual role in plants. In plants, hydrogen peroxide at low concentration act as a messenger molecule involved in acclimatory signalling and triggering tolerance against various biotic abiotic stresses (Karpiniski et al., 1999; Dat et al., 2000). High concentrations of hydrogen peroxide lead to programmed cell death (Dat et al., 2003). High extracellular hydrogen peroxide (representative of apoplastic (H_2O_2) under stress conditions) stimulated a PM hyperpolarization-activated Ca^{2+} conductance in elongation zone epidermal protoplasts. The molecular mechanisms of H_2O_2 signal transduction in plants still mysterious. More studies are needed to answer this question; how H_2O_2 can trigger two extreme responses? (Kovtun et al., 2000; Neill et al., 2002).

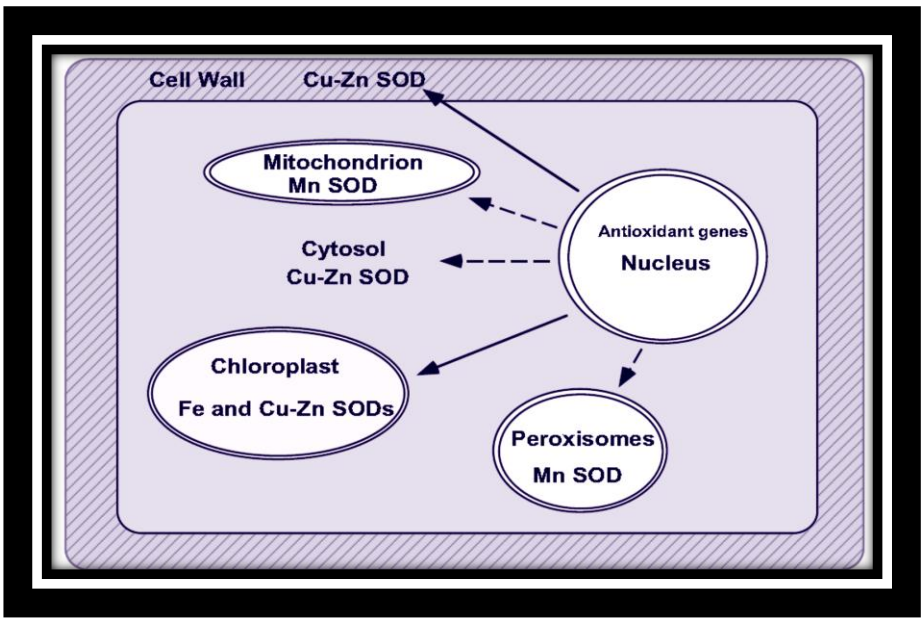

Fig 5.1. Location of SOD thorough the plant cell (Elstner, 1991).

5.1.4. Salt stress response in rice

Rice (*Oryza sativa*) is considered as one of the major food crops worldwide, but stress conditions such as salinity often cause severe yield loss.

Salinity is considered as an important toxicity encountered by rice but the crop is relatively tolerant to salinity (Greenland, 1990). Various metabolic changes have been observed in exposed plants to ionic stress. For instance, activation of Na^+/H^+ exchange processes followed by passing K^+ across the cell membrane (Watad *et al.,* 1986) and then Na^+ can be pumped into tonoplasts (Binzel *et al.,* 1988; Garbarino and DuPont, 1989). In addition, an increase in the ratio of glycoproteins to phospholipids occurred to facilitate the entry of more solutes into cells (Hirayama and Mihara, 1987). Generally, increase the concentrations of osmoprotectants including sugars, organic acids and proteins in response to salt stress (Binzel *et al.,* 1987; La Rosa *et al.,* 1987). Other studies have revealed changes at the cellular levels of a small number of proteins, such as osmotin in dicots (Singh *et al.,* 1985, 1987). In rice, rab21 as an osmotically regulated gene has been identified (Munday and Chua, 1988). Another gene *sal* T, encodes a protein associates with patterns of Na^+ accumulation and its mRNA accumulates in rice seedlings sheaths and roots of rice seedlings during salt stress (Claes *et al.,* 1990). Further to these changes, salt stress can induce oxidative stress. Plants under salinity stress generate reactive oxygen species. Plants have evolved protective and defence

mechanisms to reduce oxidative damage. Under salt stress, the balance between AOS production and the activity of AOS scavenging enzyme is upset and leads to oxidative stress (Hernandez et al., 1995; 1999). The effects of salinity in plants have been well examined on important metabolic processes for instance ion uptake and transport, respiration, photosynthesis and the detoxification processes of AOS (Active Oxygene Species) (Marschner, 1995; Dionisio-Sese and Tobita, 1998; Jayasundara et al., 1998; Meneguzzo et al., 1998). In rice, salt-tolerant varieties have higher SOD activity and lower lipid peroxidation compared to the salt-sensitive varieties (Dionisio- Sese and Tobita, 1998). For instance, isolated mitochondria from leaves of salt-sensitive peas exhibit increased activities of both Mn-superoxide dismutase and Cu, Zn-superoxide dismutase in response to salt stress. By contrast, isolated mitochondria from leaves of salt-tolerant plants showed an increase in Mn-superoxide dismutase activity in response to salt stress (Hernandez *et al.,* 1993). In treated plants with NaCl, an increase of superoxide generation occurred in sub mitochondrial particles of NaCl sensitive compared with the NaCl-tolerant plants (Hernandez et al., 1993). Transgenic plants, with a reduced or an increased expression of antioxidant enzymes have been used to support the evidence on the involvement of antioxidant enzymes in salt tolerance. Transgenic plants with reduced CAT activity are hypersensitive to salt and other oxidative stresses (Willekens et al., 1997). Overexpression of cytosolic APX increases the resistance level of plants in response to salt stress (Torsethaugen *et al.,* 1997). Overexpression of Fe-SOD enhanced the tolerance in response to oxidative stress (Van Camp et al*.,* 1996).

The overall aim of this study was to investigate the differences of SOD activity and isoforms in both susceptible (TN1) and moderately resistant (IR64) rice cultivars in response BPH feeding. The second aim was to investigate the difference of SOD activity and isoforms in susceptible rice cultivar TN1 in response to abiotic stresses (hydrogen peroxide and NaCl). SOD assay was carried out according to (Dhindsa et al. (1980). A gel assay for SOD was conducted following the procedure of Lee and Lee, (2000).

5.2 Material and methods

5.2.1. Plant Materials and Growth Conditions

Two rice varieties were used in the present study, TN1 (Taichung Native 1) and IR64 which are susceptible and moderately resistant to the Brown planthopper, respectively. These varieties were kindly supplied by IRRI (International Rice Research Institute), Philippines. Plants were maintained at 28°C during days and 21°C during nights with a photoperiod 16h day: 8h night, 70% RH.

5.2.2. Insects

Insects were cultured according to the procedures outlined in section 3.2.2.

5.2.3. Experimental design

5.2.3.1. Hydrogen peroxide treatment

Rice plants at 4^{th}-5^{th} leaf stage were used for this study .TN1 and IR64 seedlings were sprayed or systemically treatedwith 10mM of hydrogen peroxide for 24 hours. Controlswere sprayed and systemically applied with water. Infested plants (after removal of BPH) and their respective non-infested control plants were immediately flash frozen in liquid nitrogen 24 hours post treatment. Three individual plants were used as biological replicates for each time point and each variety.

5.2.3.2. Salt treatment

Rice plants at 4^{th}-5^{th} leaf stage were used for this study .TN1 and IR64 seedlings were systemically applied with 160mM of NaCl for 24 hours. Controls were systemically applied with water as a control treatment. Infested plants (after removal of BPH) and their respective non-infested control plants were immediately flash frozen in liquid nitrogen 24 hours post treatment. Three individual plants were used as biological replicates for each time point and each variety.

5.2.3.3. BPH infestation

Rice cultivars TN1 and IR64 at the 4^{th} -5^{th} leaf stage were each infested with 10 BPH at 3^{rd} to 4^{th} nymphal instar. Infested plants (after removal of BPH) and their

respective non-infested control plants were immediately flash frozen in liquid nitrogen at the following time points post infestation: 0, 3, 6, 12, 24. Three individual plants were used as biological replicates for each time point and each variety.

5.2.4. Preparation of Enzyme Extracts

Leaves and roots (1 g) frozen with liquid N2 were ground to a fine powder in a mortar. Soluble proteins were extracted by suspending the powder in 4 ml of 100 mM potassium phosphate buffer (pH 7.8), containing 0.1 mM EDTA, 1% PVP-40, and 0.5% Triton X-100. The homogenate was filtered through four layers of cheesecloth and centrifuged at 18,000 g at 4° C for 20 min. The supernatant was then used for determination of antioxidant enzyme activities following the method of Lee and Lee, (2000). Protein content was measured according to Lowry et al. (1951).

5.2.5. Enzyme assay

SOD activity assay was based on the method of Dhindsa et al. (1980) which is based on the inhibition in the photochemical activity reduction of nitroblue tetrazolium (NBT) measured spectrophotometrically at 560 nm. About 3 ml of reaction mixture, containing 50mM potassiumphosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 0.1 µM EDTA, 4 µM riboflavin and the required amount of enzyme extract. The reaction was started by adding riboflavin and placing the tubes under two 15 W fluorescent lamps for 15 min. A complete reaction mixture without enzyme, which gave the maximal colour, served as control. A non-irradiated complete reaction mixture served as a blank. Three tubes were used for each sample as replicates and the assay repeated twice. Log of the samples devided by blank to produce SOD activity as shown in (Fig 5.2). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm, which was measured according to the method of Giannopolitis and Ries (1977).

5.2.6. PAGE Analysis of Enzyme Activities

5.2.6.1. Samples and native gel preparation

An in gel SOD activity assay was run following the procedure of Lee and Lee, (2000). Bromophenol blue and glycerol were added to plant extracts containing equal amounts of protein to a final concentration of 12.5% and subjected to discontinuous PAGE under non-denaturing, non-reducing conditions, except that SDS was omitted and the gels were supported by 10% glycerol. The separating gel prepared by mixing the following reagents (6.5 ml of Sigma Aldrich 30% acrylamide/bisacrylamide solutions, 3.75 ml of 4X Tris HCl^- pH8.8, 6.25 ml of deionised H_2O_2, 0.05 ml of 10% w/v ammonium persulphate (APS) and 0.01 ml of Tetramethylethylenediamine (TEMED). The stacking gel was prepared by mixing the following reagents (0.65ml of 30% acrylamide, 1.25ml of 4X Tris Cl^- pH6.8, 3.05 ml of deionised H_2O_2, and 0.025ml of 10% APS and 0.005ml of TEMED). Electrophoresis was performed at 4℃ for 6 h with a constant current of 30 mA.

5.2.6.2. Native gel staining

SOD activity was detected following the modified procedure of Beauchamp and Fridovich, (1971). After completion of electrophoresis the gel was incubated in two changes of 50 mM potassium phosphate buffer (pH 7.8) for a total of 10 minutes, then in 1 mg/ml NBT solution for 25 min, and finally 50 mM potassium phosphate buffer (pH 7.8) containing 0.01 mg/ml riboflavin and 3.25 mg/ml tetramethyl ethylene diamine (TEMED) for 10 minutes at 24 °C with gentle agitation in darkness. Presence of SOD was visualised by light exposure for 10–20 min at room temperature.

5.3. Results

5.3.1. Superoxide dismutase (SOD) activity in susceptible TN1 and moderate resistant IR64 rice cultivars in response to BPH feeding

The results show that the specific SOD activity was significantly higher ($p<0.01$) in the moderate resistant IR64 compared to susceptible TN1 in response to BPH feeding. At zero time point (control) SOD activity in TN1 was 0.106 unit/mg^{-1} compared to 0.119 unit/mg^{-1} activity in moderately resistant IR64. Three hours post BPH feeding, the SOD activity for moderate resistant IR64 increased significantly to 0.19 unit/mg^{-1} ($p<0.01$) compared to 0.13 unit/mg^{-1} activity in susceptible TN1. Thereafter, SOD activity in IR64 was significantly higher 0.17 unit/mg-1 compared to 0.099 unit/mg^{1-} in TN1 six hours post BPH feeding. Twelve hours post BPH feeding; SOD activity for IR64 increased significantly 0.19 unit/mg^{1-} compared to 0.077 unit/mg^{1-} in TN1 twelve hours post BPH feeding. Interestingly, The SOD activity in IR64 increased significantly ($p<0.01$) to the highest level 0.21 unit/mg^{1-} compared to 0.70 unit/mg^{1-} in TN1 twenty four hours post BPH feeding (Fig 5.2).

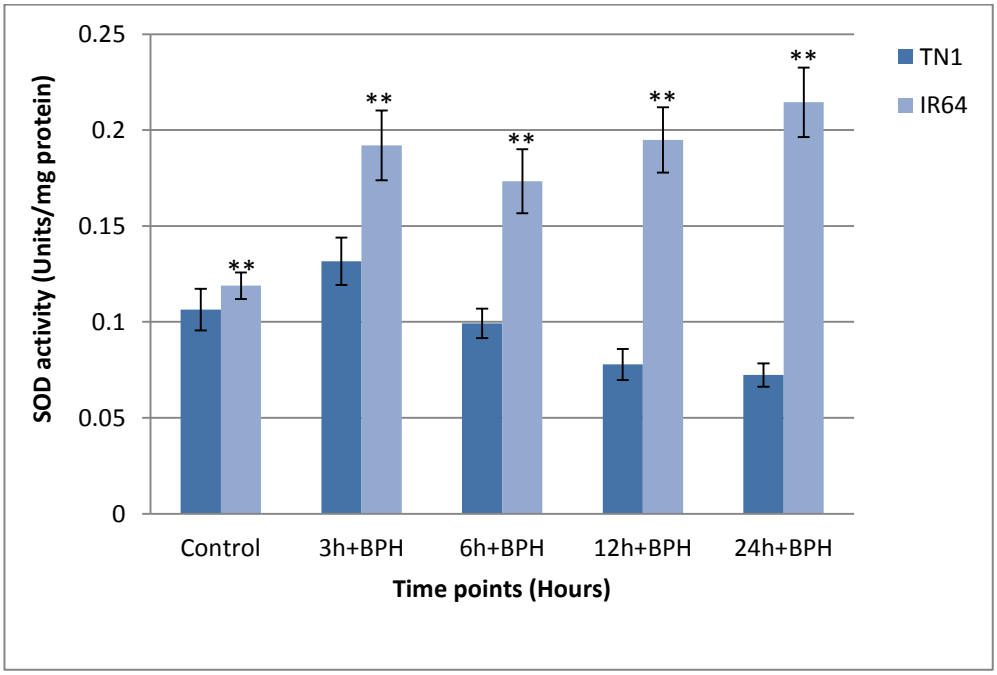

Fig 5.2. Effects of BPH infestation on the total activity of superoxide dismutase (units/mg protein) in rice cultivars TN1 and IR64. Results represent the total activity of superoxide dismutase (SOD) in rice cultivars TN1 and IR64 at time points 0, 3, 6, 12 and 24h post BPH infestation. Data are means ±SE (*n* =6). Significant differences in total activity of SOD were indicated with, * $p<0.05$, **$p< 0.01$, *** $p<0.001$. T test was used to generate the *p* values.

5.3.2. Superoxide dismutase (SOD) isoforms in susceptible TN1 and moderate resistant IR64 rice cultivars in response to BPH feeding

5.3.2.1. Susceptible rice cultivar TN1

SOD activity isoforms showed connected decrease with the total activity in response to BPH feeding. The number of detected isoforms in TN1 was seven in response to BPH feeding. The isoform SOD-2, SOD-3 and SOD-7 showed increase in the SOD activity, 3h, 6h and 12 hours post BPH feeding plus the control; however SOD-1, SOD-4, SOD-5 and SOD-6 activities were decreased at all time points. Forty eight hours post BPH feeding; SOD activity was very low in all detected isoforms (Fig 5.3).

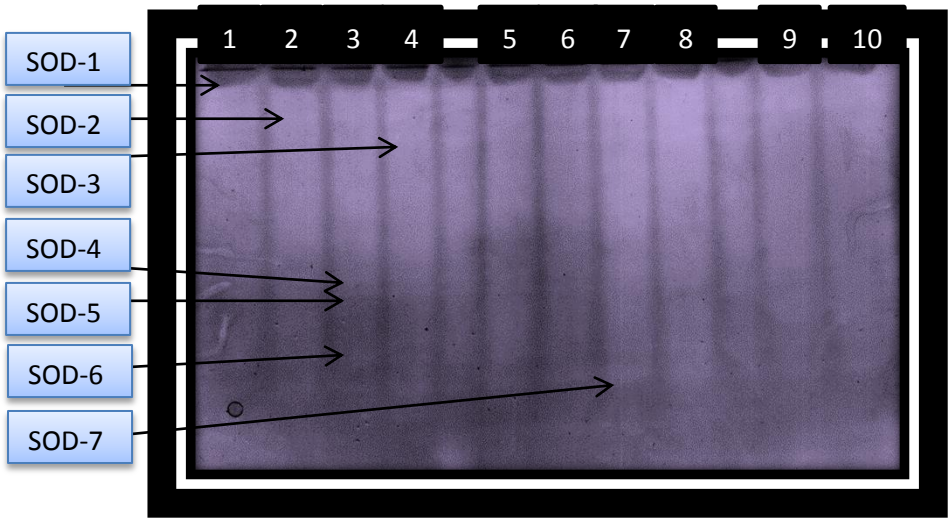

Fig 5.3. The responses of SOD activity and isoforms in the BPH-infested rice cultivar TN1. Rice seedlings at the 3rd-5th leaf stage were infested with BPH at the following time points 0, 3, 6, 12 and 24h. SOD isoforms were detected on native polyacrylamide gels. Crude protein was extracted from infested rice seedlings at different time points. Equal amounts of protein (200 µg) were loaded on each lane in the gel. Lanes 1and 2, control; lanes 3 and 4 , 3h post BPH feeding; lanes 5 and 6, 6h post BPH feeding; lanes 7 and 8, 12 h post BPH feeding; lanes 9 and 10, 24h post BPH feeding. The gel image was adjusted for clarity.

5.3.2.2. Moderate resistant rice cultivar IR64

SOD isoforms activity showed an increase in line with the total activity in response to BPH feeding. Ten SOD isoforms were detected in the moderately resistant IR64 in response to BPH feeding. SOD isoforms SOD-3, SOD-5, SOD-6, SOD-7, SOD-8, SOD-9 and SOD-10 showed an increase in the activity 3h,

6h, 12, and 24 hours post BPH feeding compared to low activity in the control (no-BPH). However, SOD-1, SOD-2 and SOD-4 isoforms showed high activity 3h post BPH feeding and control treatment. The activity of SOD-1, SOD-2 and SOD-4 isoforms was decreased at time points 6h, 12h and 24 hours post BPH feeding (Fig 5.4).

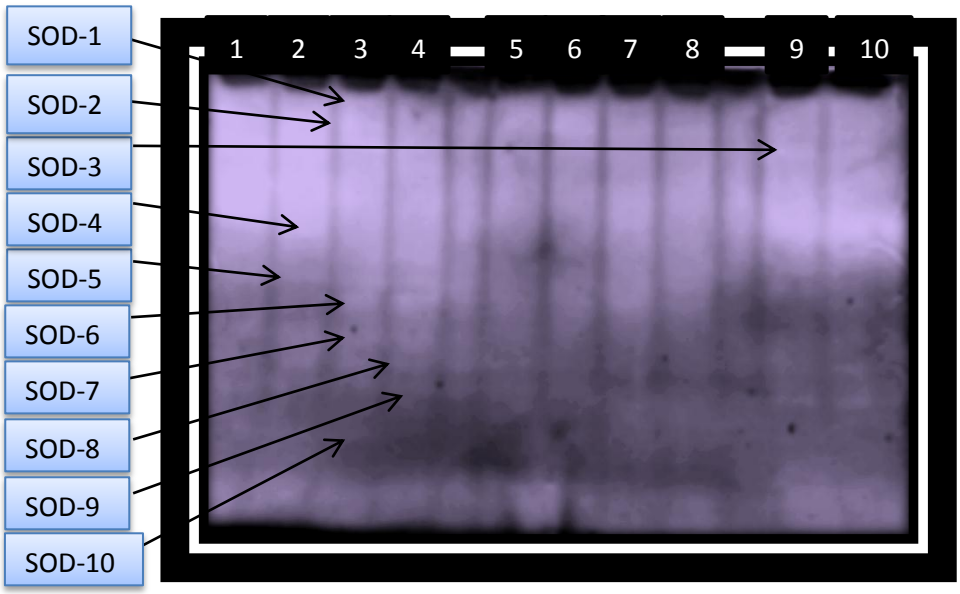

Fig 5.4. The responses of SOD activity and isoforms in the BPH-infested rice cultivar IR64. Rice seedlings at the 3rd-5th leaf stage were infested with BPH at the following time points 0, 3, 6, 12 and 24h. SOD isoforms were detected on native polyacrylamide gels. Crude protein was extracted from infested rice seedlings at different time points. Equal amounts of protein (200 µg) were loaded on each lane in the gel. Lanes 1and 2, control; lanes 3 and 4, 3h post BPH feeding; lanes 5 and 6, 6h post BPH feeding; lanes 7 and 8, 12 h post BPH feeding; lanes 9 and 10, 24h post BPH feeding. The gel image was adjusted for clarity.

5.3.3. Effects of salinity and hydrogen peroxide treatment on the total activity of SOD in rice cultivar TN1 (shoots and roots)

5.3.3.1. TN1 leaves

The results show that SOD activity in rice leaves was near the same level in response to response to salt stress and hydrogen peroxide treatment compared to the control. All SOD activity was recorded 24h post treatment. The total SOD activity in the control (non-treated plants) was 0.108unit/mg^{1-} after 24h. However, total SOD activity increased significantly 0.21 unit/mg^{1-} ($p<0.05$) in stressed TN1 seedling with 160mM for compared to 0.108 unit/mg^{1-} activity in

the control. Twenty four hours post treatment with 10mM hydrogen peroxide, the SOD activity was significantly ($p<0.05$)high 0.203 unit/mg^{1-} compared to the control (Fig 5.5).

5.3.3.2. TN1 Roots

SOD activity was investigated 24h post treatment with salt, hydrogen peroxide and control (non-treated plants). The total activity of SOD was significantly high ($p<0.01$) 0.165 unit/mg^{1-} 24h post salt stress compared to 0.085 unit/mg^{1-} in the control. Twenty four hours post hydrogen peroxide treatment, the total activity of SOD was significantly high ($p<0.05$) compared to 0.085 unit/mg^{1-} in the control (Fig 5.5).

5.3.3.3. TN1 leaves and roots

The total SOD activity in both control shoots and roots had no significant difference. However, the total SOD activity in salt stressed TN1 shoots was significantly high ($p<0.05$) with 0.21 unit/mg^{1-} compared to 0.165 unit/mg^{1-} in stressed roots. Twenty four hours post treatment with hydrogen peroxide, total activity in shoots was significantly (0.01) high 0.203 unit/mg^{1-} compared to 0.123 unit/mg^{1-} in the roots (Fig 5.5).

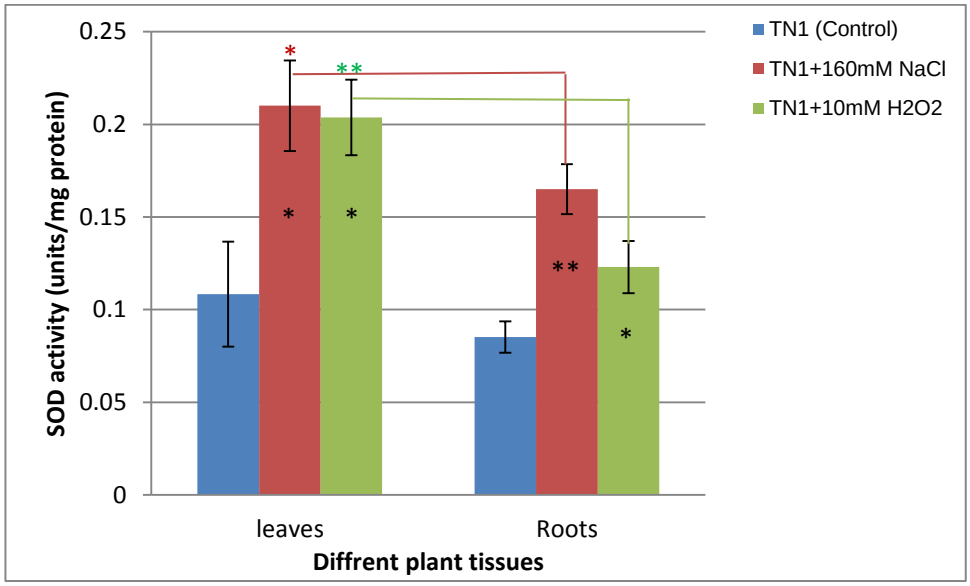

Fig 5.5. Effects of salinity and hydrogen peroxide treatment on the total activity of SOD (units/mg protein) in rice cultivar TN1 (shoots and roots). Results represent the total activity of superoxide dismutase (SOD) in rice cultivar TN1 at time point 24h post treatment with 160mM NaCl, 10mM H_2O_2 and control plants (treated with water). Data are means ±SE (*n* =6).

Significant differences in total activity of SOD were indicated with, * p<0.05, ** p< 0.01, ***, p<0.001. Two ways ANOVA were conducted to generate the *p* values.

5.3.4. Effects of salinity and hydrogen peroxide treatment on SOD isoforms in rice cultivar TN1 (shoots)

SOD activity isoforms showed connected increase with the total activity in response to salt stress and hydrogen peroxide treatment. The number of detected isoforms in TN1 shoots was seven in response to salt stress and hydrogen peroxide treatment compared to 4 isoforms in the control. The SOD isoforms (SOD-1, 2, 3, 4, 5, 6, and 7) showed an increase in the activity in response to salt stress compared to the control (Fig 5.6). Twenty four hours post hydrogen peroxide treatment, the isoforms (SOD-1, 2, 3, 4, 5, 6, and 7) showed an increase in the activity compared to control. However, Salt stressed shoots showed an increase in the isoforms activity compared to treated plants with hydrogen peroxide (Fig 5.6).

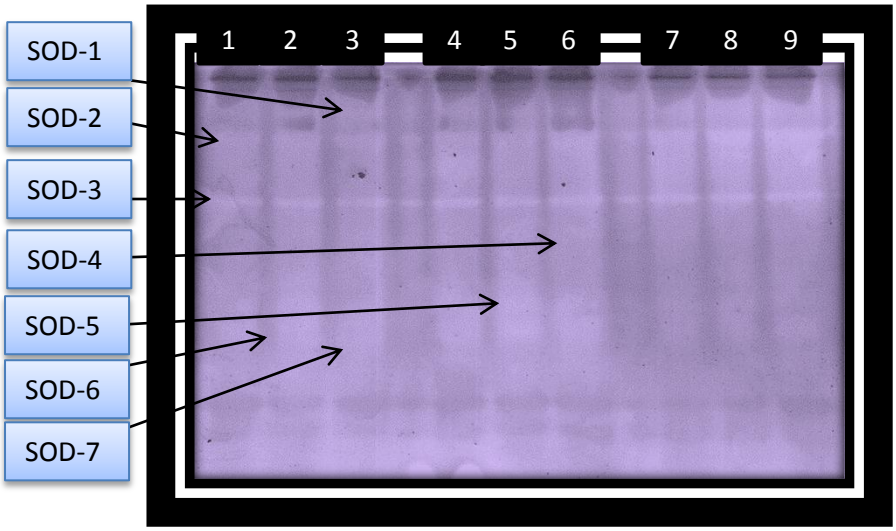

Fig 5.6. The responses of SOD activities and isoforms in the NaCl and hydrogen peroxide -treated rice cultivar TN1 shoots. TN1 seedlings at the 3rd -5th leaf stage were treated with 160 mM NaCl for 24h, 10mM hydrogen peroxide for 24h and water (control) for 24 h. SOD isoforms were detected on native polyacrylamide gels. Crude protein was extracted from infested rice seedlings at different time points. Equal amounts of protein (200 µg) were loaded on each lane in the gel. Lanes 1, 2 and 3, control after 24h; lanes 4, 5 and 6, TN1 treated with 160mM NaCl for 24h ; Lanes 7, 8 and 9, TN1 treated with 10mM hydrogen peroxide for 24h. The gel image was adjusted for clarity.

5.3.5. Effects of salinity and hydrogen peroxide treatment on SOD isoforms in rice cultivar TN1 (roots)

SOD activity isoforms showed connected increase with the total activity in response to salt stress and hydrogen peroxide treatment. The number of detected isoforms in TN1 roots was seven in response to salt stress and hydrogen peroxide treatment compared to 4 isoforms in the control. The SOD isoforms (SOD-1, 2, 3, 4 and 5) showed an increase in the activity in response to salt stress compared to the control (Fig 5.7). Twenty four hours post hydrogen peroxide treatment; the isoforms (SOD-1, 2, 3 and 4) showed an increase in the activity compared to control. However, Salt stressed roots showed an increase in the isoforms activity compared to both treated plants with hydrogen peroxide and control (5.7).

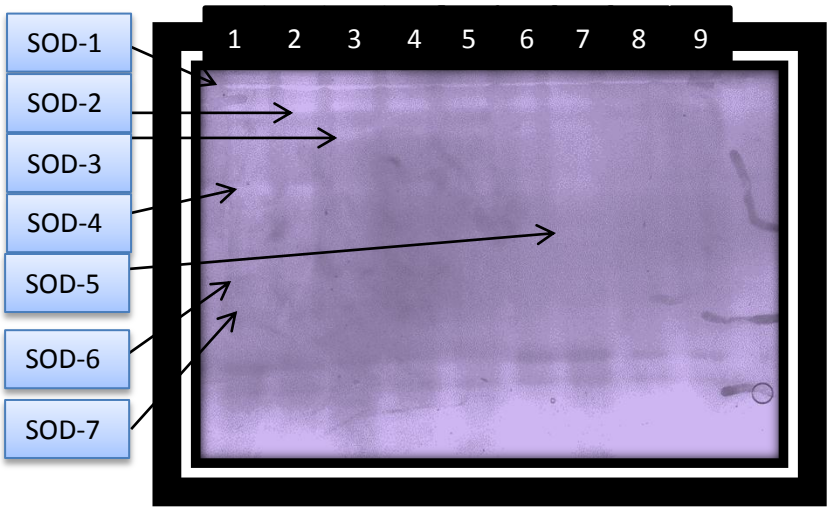

Fig 5.7. The responses of SOD activities and isoforms in the NaCl and hydrogen peroxide -treated rice cultivar TN1 roots. TN1 seedlings at the 3rd -5th leaf stage were treated with 160 mM NaCl for 24h, 10mM hydrogen peroxide for 24h and water (control) for 24 h. SOD isoforms were detected on native polyacrylamide gels. Crude protein was extracted from infested rice seedlings at different time points. Equal amounts of protein (200 µg) were loaded on each lane in the gel. Equal amounts of protein (200 µg) were loaded on each lane in the gel. Lanes 1, 2 and 3, TN1 roots 24h post treatment with water; lanes 4, 5 and 6, TN1 roots 24h post treatment with 160mM NaCl; Lanes 7, 8 and 9, TN1 roots stressed with 10mM hydrogen peroxide for 24h. The gel image was adjusted for clarity.

5.4. Discussion

Reactive oxygen species (ROSs) for instance the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH*) are regularly generated from normal cellular metabolic processes as well as respiratory electron flow and photosynthesis (Halliwell and Gutteridge, 1989). Although ROS are generated in the course of normal aerobic metabolism, the biological effects of ROS on these intracellular processesare reliant on their concentration and high levels of these species are existent during oxidative stress.

In the susceptible rice cultivar TN1 exposed to BPH, the SOD activity was significantly lower than moderately resistant IR64. ROS function as a secondary messenger in response to insect (BPH) feeding. Signal propagation is complemented by the accumulation of reactive oxygen species (ROS) in the extracellular spaces between cells and by rapid expression of ROS-responsive transcripts. The signal can be blocked by the suppression of ROS accumulation at locations that are distant from the initiation site. Rapid systemic signalling is not only independent of ethylene, JA, or SA signalling, but also can be triggered by wounding, and various abiotic stresses such as salinity, light and heat (Miller et al., 2009). Systemic signals play an important role in the plant defence in response to biotic and abiotic stresses (Jung et al., 2009; Karpinski et al., 1999). Low levels of SOD activity in TN1 in response to BPH feeding increased the free oxygen radicals such as O_2, H_2O_2 and OH^- which are dangerous to plant cells. Therfore, SOD activity plays an important role in clearing excess free radicals. Low level of SOD activity and high level of free radicals lead to plant death.

Results from the present study showed that the total SOD activity and isoforms increased significantly in rice cultivar IR64 compared to TN1 in response to BPH feeding. ROS induced in response to insect feeding (BPH) as an early response second messenger. Therforeas a result of BPH feeding in IR64, ROS function as downstream secondary messengers in response to wounding (Orozco-Cárdenas et al., 2001; Sagi et al., 2004), and also function as early local wound or pathogen response mediators (Maffei et al., 2007). ROS accumulation is required along the path of rapid systemic signalling. The dependence of the rapid systemic signal on RBOHD could suggest that

superoxide generated by RBOHD or its reactive derivatives, or both, as mediators of cell-to-cell communication over long distances in plants. ROS accumulation along a systemic signal front is therefore essential for long-distance signalling in plants in response to different environmental stimuli. Superoxide generated by RBOHD is quickly dismutated to H_2O_2 either spontaneously or through an apoplastic-localized superoxide dismutase (Bowler et al., 1994). So that, ROS (H_2O_2) is the mediator of rapid systemic signal RBOHD responsible for initiation, as well as propagation of rapid systemic signal. As such, activation of RBOHD along the pathway of systemic signal is important for signal propagation. Signal propagation is complemented by the accumulation of reactive oxygen species (ROS) in the extracellular spaces between cells and by rapid expression of ROS-responsive transcripts.

SOD is the important free radical scavenger in the plants. SOD can clear the excessive free oxygen radicals such as O_2, H_2O_2 and OH^- which are dangerous to plant cells so that SOD activity was high in IR64 compared to TN1. SOD has been categorized to three types, Cu/Zn-SOD, Mn-SOD and Fe-SOD (Grace et al., 1990) according to different metal ions bound to it. Mn-SOD was considered as the only essential form for the survival of aerobic life (Carlioz and Touati, 1986). All the three types of SOD are complementary to each other. Interestingly, if the expression of one of the SOD isoforms is suppressed, the expression of the other isoforms can be increased in response to stresses which may lead to the development of stress resistance (Kim et al., 1996).

The activities of rice antioxidant SOD enzyme were increased in the root and shoot under NaCl stress and hydrogen peroxide treatment. But the increase was more significant and consistent in the root in response to salt stress. The activity of SOD increased significantly in the root within 24h of the NaCl treatment, indicating rapid responses of antioxidant enzymes to salt stress in TN1 roots.

Increased total SOD activity in stressed TN1 with NaCl in both shoots and roots caused accumulation of ROS (SOD) in the plant cells and resulted in cell toxicity followed by cell death. Treating TN1 with H_2O_2 could lead to an improvement in the plant's resistance if the applied dose is in the safe range. Rice cultivar TN1 treatment with low dose of hydrogen peroxide induced the second messengers Ca^{2+} and ROS followed by an improvement in the plant resistant in response to different stimuli. On the other hand, high dose of

hydrogen peroxide increase ROS in the plant cell and affect the signal transduction of ROS which leads to cell death. Exogenous hydrogen peroxide (H_2O_2) application induces the plant defence signals in response to pathogen attack abiotic and oxidative stresses. Hydrogen peroxide plays a dual role in plants. Low concentration of H_2O_2 acts as a messenger molecule involved in acclimatory signalling and triggering tolerance against various biotic and abiotic stresses. High concentrations of hydrogen peroxide lead to programmed cell death. In plants, the expression of Mn-SOD gene enhanced the ressistance in response different abiotic stimuli. The genetic engineer of Mn-SOD gene from tobacco into *Medicago truncatula* and over-expression in the mitochondria resulted in an increase of total enzyme activity in the transgenic plants 2 times compared to the control (McKersie et al., 1999). Over expression of Cu/Zn-SOD isoform enhanced the tolerance of potato plants in reponse to drought (Lu et al., 2010).

Chapter 6. Oxi1 mutant playsan important role in *Arabidopsis* resistance against aphid (*Myzus persicae*)

Abstract

The interactions between plants and insect herbivores comprise a complex, co-evolved natural system. Plants raise an array of chemical and physical barriers to inhibit insect feeding and insects strive to avoid these defences. *Arabidopsis* mutants provide a powerful tool for studying plant-insect interactions as the phenotype of individual gene deletions can be studied with relative ease. Oxidative signal inducible (Oxi1) is a serine/ threonine kinase required for oxidative burst-mediated signalling in *Arabidopsis* roots and full activation of MAPKs cascades. Camta3 is a calmodulin-binding transcription activator (also called SR1) is mediated by biotic and abiotic stress. Mutants of Camta3 (Camta3-1 and Camta3-2) have been shown to accumulate high levels of reactive oxygen species (ROS) during development. The expression of β-1,3-glucanase, important in the response to aphid feeding, are also investigated through the use of Gns1, Gns2 and Gns3 mutants.

Oxi1 mutants showed resistance to aphids and delay the developmental rate in both adults and nymphs compared to the Col-0 wild type. β-1,3-glucanase genes Gns1, Gns2, Gns3 and Gns5 were not expressed in Oxi1 mutant. However, Gns2 was expressed to high levels in Col-0 in response to aphid feeding. Also, Gns2 was up-regulated in both Oxi1 null mutant in WS2 and WS2 background. Callose synthase GSL5 was down-regulated in both Oxi1 null mutant in WS2 and WS2 background. Camta3-1 and Camta3-2 died quickly and showed susceptibility in response to aphid feeding compared to Col-0 wild type. β-1,3-glucanase mutants Gns1, Gns2 and Gns3 were resistant to aphid feeding and aphid development in both adults and nymphs.

Callose synthase is important for plant resistance especially callose synthase GSL5. Expression of β-1,3-glucanase genes, especially Gns2, play an important role in plant susceptibility in response to aphid feeding. Oxi1 mutants showed resistance in response to aphid feeding. Potentially through the induction of callose deposition via MAPKs resulting in inducing ROS as an early response and signal transduction improves the resistance level of the plant.

6.1. Introduction

The previous chapters focused in the BPH-rice interaction. Rice plants as standared model for monocots because of the whole sequenced genome and BPH as sucking insects as one of the majore problems in rice crop production. The main conclusion of the previous work, callose synthase gene GSL5 is playing an important rol in plant resistance and β-1,3-glucanase genes Gns2 and Gns5 are playing important roles in plant susceptibility. Exogenous application of hydrogen peroxide could mimic the Oxi1 mutants in Arabidopsis and improved the resistance level in susceptible rice cultivar TN1 to become moderately resistance. Exogenous application of hydrogen peroxide in the susceptible rice cultivar TN1 trigerred calcium signalling pathway which in turn stimulates callose synthase genes followed by an increase callose production in response to BPH feeding. However, β-1,3-glucanase genes Gns1, Gns2 and Gns5 were downregulated in response to BPH attack. Furthermore, antioxidant superoxide dismutase activity was increased in response to hydrogen peroxide treatment and BPH feeding. Additionally, TN1 treatment with hydrogen peroxide improved ROS signal transduction which in turn improved the resistance in susceptible rice cultivar TN1. Rice (Oryza sativa), a model system for grasses, has also shown collinearity with other monocots (Tarchini et al., 2000). *Arabidopsis* and rice are expected to have great value as models for dicot and monocot genomic studies, respectively (Gale and Devos 1998).

Arabidopsis thaliana is a small flowering plant that is widely used as a model organism in plant biology. *Arabidopsis* is a member of the mustard (Brassicaceae) family. *Arabidopsis* is not of major agronomic significance, but it offers important advantages for basic research in genetics and molecular biology. Over the past twenty six years, *Arabidopsis thaliana* has been utilized as a model plant to study plant growth, development and adaptation to the environment. *Arabidopsis* has also provided valuable information on plant-insect interactions, including those involving insects in the orders Coleoptera, Diptera, Hemiptera, Lepidoptera and Thysanoptera (Koornneef and Meinke, 2010). *Arabidopsis* mutants are very strong tool for researchers because of their known pathways. The Brown planthopper (BPH)-Rice and Aphid-*Arabidopsis* provide good models for studying the induced responses in plants to phloem-feeding insects. The availability of new molecular tools and progress of genome

sequences of several phloem-feeding insects will enable exploring Arabidopsis—phloem-feeding insect interactions from the perspective of both the plant and the insect. These tools will allow determining how alterations in activity of Arabidopsis genes and mechanisms involved in defense and susceptibility impact gene expression in the insect, and thus provide clues on how insect physiology is impacted on these Arabidopsis mutant and transgenic plants.

6.1.1. Plant-insect interaction

Generally, plant feeding insects can be classified as either generalist or specialist herbivores (Bernays and Chapman, 1994). Whereas specialist herbivores have developed a tolerance for a particular species or group of plants and often recognize plant chemical defences as attractive signals. The interaction between plants and insect herbivores is not passive, but involves the active transcriptions of genes and processing of proteinaceous and chemical compounds to mount a defence response towards the insect attack. Often these response lead to the production of feeding deterrents such as protease inhibitors (Gatehouse, 2002; Broadway and Colvin, 1992) and the release of volatiles that attract predators of the insect herbivores (Mattiacci et al., 1995). Both methyl jasmonate (McConn et al., 1997) and ethylene (Kahl et al., 2000; Stotz et al., 2000) have been implicated as important signalling molecules that mediate induced insect defences in plants.

6.1.2. *Aphid (Myzus persicae)*

Aphids (Homoptera: Aphididae: Aphidinae) and some of the other homoptera target and feed from phloem sieve elements, functional units in the sieve tubes, using their stylet mouthparts. The majority of insects with piercing mouthparts can suck phloem sap from a single sieve element for hours or even days. Aphids keep phloem sieve elements available and sieve plates pores open by inhibiting those phloem proteins (p-proteins) that are responsible for callose formation and deposition (Tjallingii and Hogen Esch, 1993; Prado and Tjallingii, 1994). Electrical penetration graphs (EPG) have been used to monitor plant penetration by aphids and other insects with sucking mouth parts (Tjallingii, 1988). This technique can record signal waveforms and help elucidate different

insect feeding activities, such as mechanical stylet work, saliva secretion, and sap ingestion. During the pathway and phloem phases, EPG shows four periods of saliva secretion with at least two types of saliva. One period of gelling salivation forms a layer of solid saliva that covers the stylets inside the plant tissue. The other three periods are termed non-gelling or watery salivation. Soluble or watery salivation occurs intercellularly in the plant tissue during plant penetration and phloem feeding (Fig 6.1). It is assumed that watery saliva contains adjuvants that are used by the aphid to prevent or suppress the defence responsive p-proteins in the wounded sieve elements thus maintaining the sieve element as suitable feeding site (Knoblauch and Van Bel, 1998; Eckardt, 2001; Will and Van Bel., 2006).

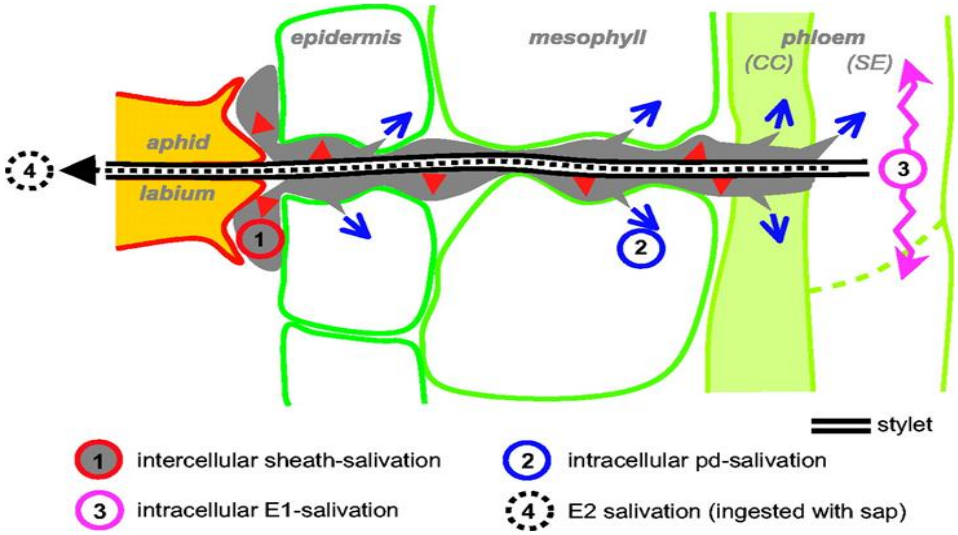

Fig 6.1. Model showing all salivation periods detected by the EPG (Tjallingii, 1995). E1 (Salivation into sieve elments), E2 (2nd salivation), SE (sieve elements), CC (companion cells) and pd (potential drop).

6.1.3. Callose Structure and Callose Synthase genes in *Arabidopsis*

Callose is a linear homopolymer β-1, 3-glucan with partial β-1,6-branches, it is a major polysaccharide component of cell walls in a variety of higher pants. During biosynthesis of callose, UDP-glucose is used as a substrate. Molecular and biochemical studies in many plant cultivars have shown that callose is synthesized by callose synthase enzymes (Verma and Hong, 2001; Brownfield

et al., 2008). Twelve genes encoding putative callose synthase have been identified in the model plant *Arabidopsis thaliana* (Richmond and Sommerville, 2000, Verma and Hong, 2001). Consequently, two different nomenclatures have been approved for the *Arabidopsis* genes. Verma's group uses the CalS (Callose synthase) system to name the twelve genes: AtCalS1-AtCalS12 based on their relative similarity to AtCalS1 (Hong et al., 2001). The Somerville group used different naming system to the twelve *Arabidopsis* genes as *GSL* (Glucan synthase-like) genes, and has designated them as *Arabidopsis thaliana* GSL1 (AtGSL1*)* to *Arabidopsis thaliana (*AtGSL12*) (*Jacobs et al., 2003*).* The GSL nomenclature system has been used because of the wide usage by callose synthase research community *(*Jacobs et al., 2003; Thiele et al., 2008*)*. The GSL family has been classified into four main subfamilies according to the phylogenetic analysis of the AtGSL family. The first subfamily contains AtGSL1, AtGSL5, AtGSL8 and AtGSL10, the second subfamily contains AtGSL2, AtGSL3, AtGSL6 and AtGSL12, the third subfamily contains AtGSL7 and AtGSL11, and the last subfamily includes AtGSL4. According to the previous characterization of callose functions, GSL genes and members of subfamilies show partial roles during pollen development or fertilization. A single GSL gene can also have different functions; for example, callose GSL5 is normally induced in response to wound- and pathogen attack in leaf tissue; and it also plays an important role in exine formation and pollen wall patterning (Jacobs et al., 2003; Enns et al., 2005). Callose synthase genes GSL8 and GSL10 are individually required for asymmetric microspores and for the entry of microspores into mitosis (Toller et al., 2008; Huang et al., 2009). Callose synthase GSL8 is necessary cytokinesis and cell patterning (Xiong-Yan Chen et al. 2009). Mutation of callose synthase gsl8 has been reported as a male gametophytic lethal, with abnormal gsl8 pollen failing to enter pollen mitosis (I) (Toller et al., 2008). According to the gene structure modelling, the majority of *GSL* genes have 40–50 exons; with the exception of GSL1 and *GSL5* which have two and three exons, respectively. The functional enzyme product of the AtGSL gene typically contains around 2,000 amino acids, which is larger than most plant genes (Verma and Hong, 2001; Enns et al., 2005). In *Arabidopsis*, all GSL proteins contain multiple transmembrane domains clustered at the N- and C-terminal regions, leaving a large hydrophilic central loop that faces the cytoplasm. The hydrophilic central loop contains the putative catalytic domain

which has been further subdivided into two domains: the UDP-glucose binding domain and the glycosyl transferase domain (Chen et al., 2009). The characterization of these domains relied on the presence of multiple aspartic acid triplets (DDD) and a QXXRW motif that is conserved in the CeSA superfamily (Verma and Hong, 2001; Thiele et al., 2008; Dong et al., 2005). Most of the knowledge that has been gained about callose in plants during the last decade has relied on the analysis of a number of mutations that affect callose synthesis. All mutations were made by knocking down the individual callose synthase genes of *Arabidopsis* plants (Jacobs et al., 2003; Dong et al., 2005).

6.1.4. Callose synthase plays a major role in plant defence

Callose plays an important role in plant defence (Fig 6.2) in response to biotic and abiotic stresses, plus a major role in a wide variety of processes during plant development. The synthesis and deposition of callose in plants is stimulated by cytosolic Ca^{2+}. Callose is the final product of callose synthases and it is normally degraded by β-1,3-glucanases. Although the importance of callose in plants, the molecular mechanism of its synthesis hasbeen determinedrecently (Verma, 2001).

Callose is widespread in higher plants, and it occurs at particular stages of growth and differentiation in the cell walls or cell wall-associated structures (Stone et al., 1992). Callose in the cell wall is normally synthesized at certain developmental stages like the cell plate (Verma, 2001; Samuels et al., 1995) and in pollen tube walls (Dumas and Knox, 1983) and in response to wounding, pathogen infection and insect attack (Aidemark et al., 2009). Callose deposition also occurs at cell plates during cytokinesis (Dumas and Knox, 1983; Radford et all., 1998). Callose deposition also occurred at the plasmodesmata (PD) (Radford et al., 1998; Northcote et al., 1989) and at sieve plates (McNairn et al., 1967) to regulate intercellular transport of molecules by controlling the size exclusion limit of plasmodesmata as a response to developmental signals or environmental signals, e.g., wounding and pathogen attack (Kauss, 1996; Köhle et al., 1985). Callose deposition strengthens the cell wall at the location of the attack (Aist, 1976; bell, 1981), but callose can also be found at plasmodesmata

in non-infected cells bordering the infected site to prevent the spread of a fungal infection in resistant cultivars (Trillas et al.,2000). Callose deposition can be induced by treating plants with aluminium (Bhuja et al., 2004; Schreiner et al., 1994) to seal plasmodesmata (sivaguru et al., 2000; Levy et al., 2007). Callose deposition can also be synthesized by abscisic acid, and other physiological stresses (Fig 6.3) (Stone and Clarke, 1992). Recently two independent laboratories reported that the callose synthase 7 enzyme (Csl7 or GSL7) is responsible for callose deposition in the sieve plates. Mutants of callose synthase 7 (GSL7) were unable produce callose in sieve pores in response to different stresses (Bo and Zonglie, 2011).

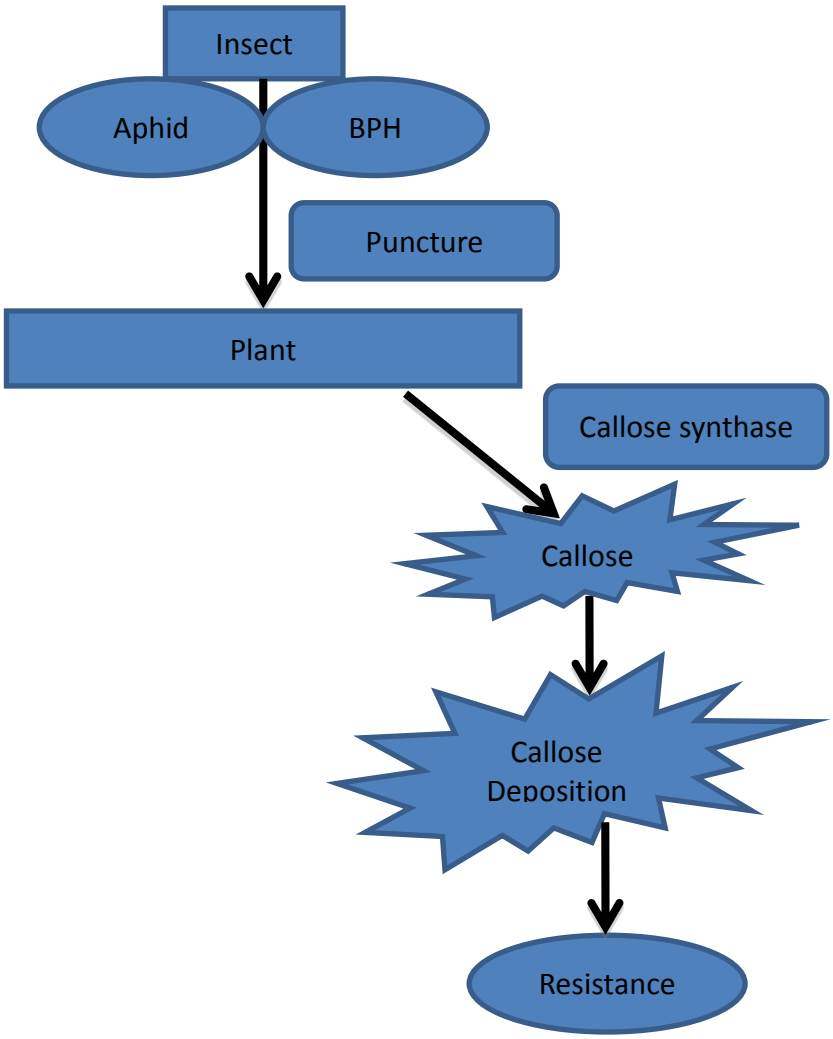

Fig 6.2. Schematic diagram showing the role of callose synthase in plant resistance.

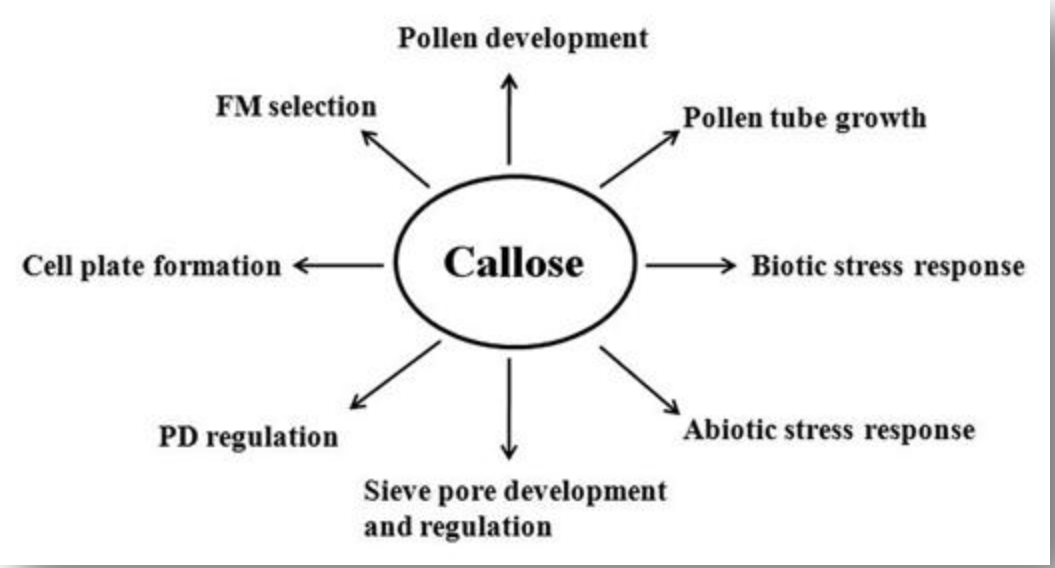

Figure 6.3. Callose is involved in multiple aspects of plant growth and development and response to biotic and abiotic stress (Chen and Kim, 2009). FM (functional megaspore); PD (plasmodesmata).

6.1.5. β-1, 3- glucanase genes

β-1,3-glucanases are a class of hydrolytic enzymes that hydrolyse the 1,3-β-D-glucosidic linkages in β-1,3-glucans (Fig 6.4) (Bachman and McClay 1996). β-1,3-glucanase is one of the commonly known pathogenesis-related proteins. Pathogenesis-related (PR) proteins are a group of heterogeneous proteins encoded by genes that are rapidly induced by pathogenic infections but also by salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). They are widely used as molecular markers for resistance response to pathogens and systemic acquired response (SAR). The PR proteins include all pathogen-induced proteins and their homologs, and are routinely classified into 17 families based on their biochemical and molecular biological properties, from PR-1 to PR-17. β 1,3-glucanase belongs to PR2 group of pathogen-related (PR) proteins, they are induced in response to pathogen infection (van Loon et al. 2006). β-1,3-glucanase are induced by pathogen infection and demonstrate an antifungal capacity by hydrolysing the fungal β-1,3-glucans, a major cell wall structural component of both fungal and also plant species (Leubner-Metzger and Meins, 1999). In *Arabidopsis* the expression of PR1 and β-1,3-glucanase 2 (Gns2)

genes increases in response to Aphid *(Myzus persicae)* feeding and these two genes are also over-expressed as a result of exposure to the signalling molecule salicylic acid (SA), causing the transcription level of PR1 and Gns2 was 10 and 23 fold respectively. The transcription level of PR1 and Gns2 genes have been measured by using Northern blot (Moran and Thompson, 2001). Another interesting role of β-1, 3-glucanase related to PR function is their involvement in cold response (Griffith and Yaish, 2004; Yaish et al., 2006) and β-1,3-glucanase also play critical roles in normal developmental plant processes. The *Arabidopsis* β‑1,3‑glucanase family consists of 50 different genes and 44 genes from this large family have been grouped into 13 expression clusters represented as A–M based on microarray data (Doxey et al., 2007). Groups A–C of β‑1,3‑glucanases are specific to leaves and (D) roots. Proteins in the A-D groups are pathogenic related (PR) genes whose transcription is up regulated in response to pathogen infection. Groups H and K contain genes expressed specific to flower organs, and they are involved in reproductive processes like microspore maturation and pollen tube growth. *Arabidopsis thialina* β-1,3-glucanase plasmodesmata (AtBG‑pap*)* belongs to group (M), which contains 13 genes whose expression products are abundant in a wide range of plant tissues with a high relative expression in the shoot‑apex. Members of M group were suggested to be involved in cell wall morphogenesis or cell division. Group M members are not PR genes and they show insignificant response to most stresses and hormones, and a slight negative response to biotic stresses.

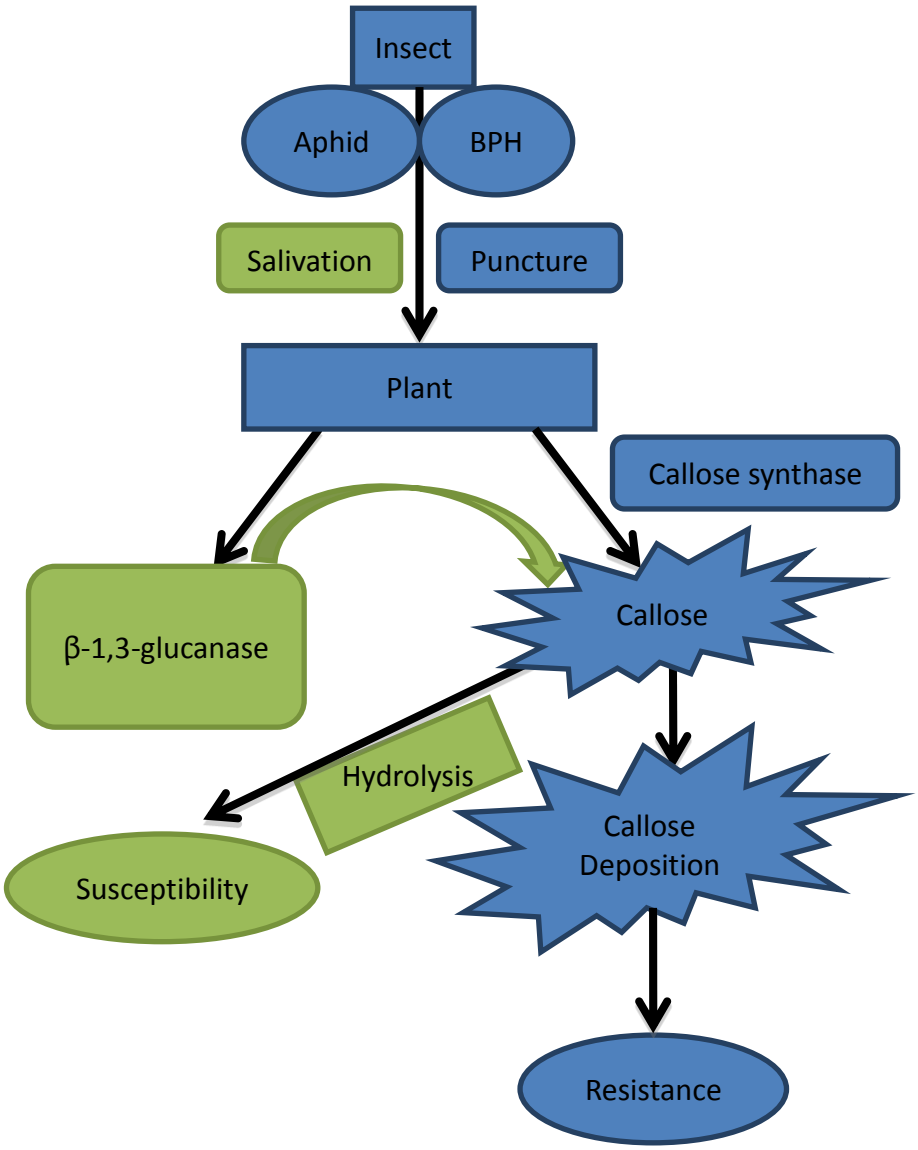

Fig 6.4. Schematic diagram showing the role of beta-1,3-glucanase in plant susceptibility.

6.1.6. Oxidative Signal Inducible (Oxi1)

Oxidative signal inducible 1 (Oxi1) is a serine/threonine kinase required during oxidative burst-mediated signalling in *Arabidopsis* roots. Oxi1 serine/threonine kinase is a member of protein kinase family (AGC) (Alessi, 2001; Bogre et al., 2003). The expression of Oxi1 (serine/threonine kinase) has been demonstrated to increase following the exogenous application of H_2O_2, thus demonstrating the role of H_2O_2 as a signalling molecule in this response cascade. In *Arabidopsis*, AGC2-2 (At4g13000) shares more than 60% of sequence identity to Oxi1 (Bogre et al., 2003) and therefore must be considered as its homolog. Oxi1 itself is required for the partial activation of mitogen-

activating protein kinases 3 and 6 (MPK3 and MPK6) (Fig 6.5) in this critical cascade (Rentel et al., 2004).

It has been shown that in *Arabidopsis* Oxi1 plays a major role in plant immunity against the bacterial pathogen *Pseudomonas syringae.* During periods of plant stress brought on through plant–pathogen interactions NADPH-produced ROS have been shown to initiate expression of Oxi1. The tight control of, and expression levels of Oxi1 appear very important in mediating suitable defence responses, interestingly both down-regulation and overexpression of Oxi1 results in enhanced susceptibility to biotrophic pathogens. Oxi1 MAPKs have emerged as key components linking Reactive Oxygen Species (ROS) accumulation to disease resistance in response to virulent *Hyaloperonospora parasitica* attack (Anthony et al., 2006; Rentel et al., 2004). The Oxi1 null mutant showed an increase in plant susceptibility compared to wild-type *Arabidopsis* following infection with *Hyaloperonospora parasitica* Emco5. Still however relatively little is known regarding the signalling events triggered by oxidative stress that induce the defence system in plants in response to both biotic and abiotic stress.

Pei et al. (2000) showed that treatment with H_2O_2 caused a cytosolic elevation of Ca^{2+} through activation of hyperpolarization- activated Ca^{2+}-permeable channels in the guard cells of *Arabidopsis*. These channels play an important role in abscisic acid signal transduction and mediate Ca^{2+} influx across the plasma membrane in response to H_2O_2 treatment. The induction of calcium pathway signalling in response to H_2O_2 also induces the expression of glutathion-s-transferase, one of the key genes in response to accumulation of ROS. Glutathion-s-transferase is important in the response to oxidatively produced compounds to reduced glutathione, which facilitates their metabolism, sequestration, or removal (Maike and Knight, 2004). The cytosolic Ca^{2+} concentration controls stomata closure, it is also associated with the initiation of signal transduction pathways for a number of genes in response to different stimuli. Furthermore an elevation of cytosolic Ca^{2+} regulates accumulation of salicylic acid through an interaction with calmodulin binding transcription factor protein (Camta) that mediated local and acquired resistance in response to pathogens (Du et al., 2009).

6.1.7. Mitogen-activated protein kinases and cytoskeleton

Mitogen-activated protein kinases (MAPKs) are considered as one of the best and well characterized families of signalling molecules in higher plants (Jonak et al., 2002; Šamaj et al., 2004a) MAPKs contribute in the regulation of a wide range of critical cellular processes including cell division, survival, polarization, stress responses and metabolism. In *Arabidopsis*, there are at least 20 MAPK, 10 MAPKK and 60 MAPKKK genes (MAPK group, 2002). In eukaryotic cells, MAPKs cascades are seen as universal mediators of diverse extracellular and intracellular signals. MAPKs belong to the serine/threonine class of protein kinases and they respond at the cellular level of the host lead to cell division, development, stress responses, survival, and differentiation (Garrington and Johnson, 1999). MAPK signalling pathways are generated from dynamic protein complexes involving three cascades of MAPK kinases (Fig 6.5). In general these cascades involve three functionally linked protein kinases, a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAPK. In plants, MAPKs contribute in transmitting abiotic and biotic stress signals, in the regulator of cell division and the other developmental processes regulated by hormones and biologically active compounds. MAPKs play a major role in the plant response to a divers range of pathogen (Meskiene and Hirt, 2000; Jonak et al., 2002). Direct interaction between scaffold proteins and phosphatases regulate all biological outputs from the MAPKs signalling pathway. Scaffold proteins give specificity to MAPK signalling pathways and ensure precise control of the subcellular assembly, targeting and recruitment of MAPK components into multi protein complexes and MAPKs modules to various membrane compartments (for example, the plasma membrane or signalling endosomes). Phosphatases are required for the resetting of signalling pathways by dephosphorylation of and therefore inactivation of MAPKs (Meskiene et al., 1998) in certain circumstances they can also tether MAPKs in the cytoplasm or within the nucleus (Mattison et al., 1999) leading to signal termination (Volmat et al., 2001). The cytoskeleton provides a structural basis for polarity establishment and maintenance in plant cells (Mathur and Hülskamp 2002; Wasteneys and Galway, 2003). Cytoskeleton and MAPK signalling pathways and the crosstalk between them is important for controlling crucial cellular activities. In general, there is great similarity in MAPK cascades between plants,

yeast and mammalian cells indicating the ubiquitous nature of this type of signalling mechanism (Fig 6.5).

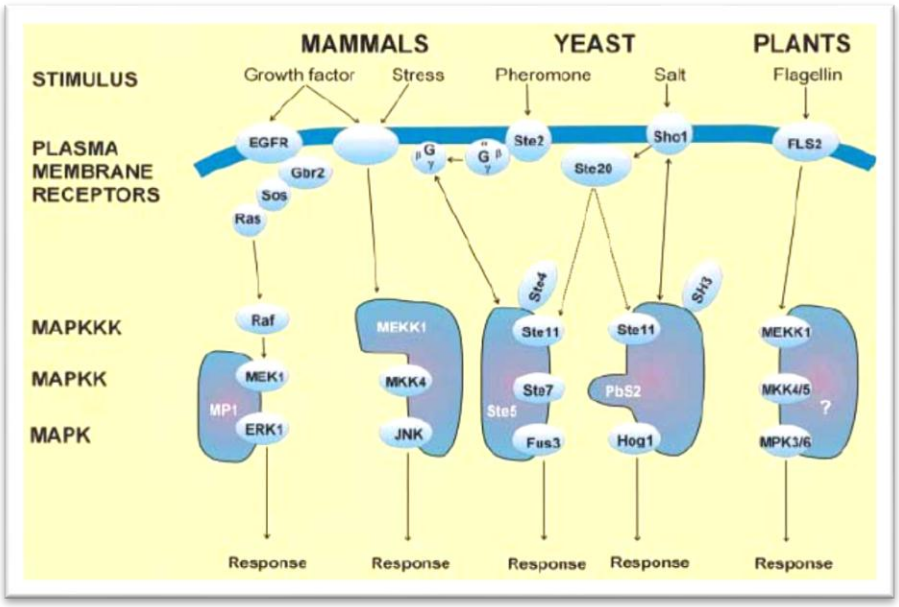

Fig 6.5. Structure of different MAPK signalling pathways in plant, yeast and mammals (Šamaj et al., 2002c, 2003b).

6.1.8. Camta3-1 and 3-2 mutants

Camta-3 is a calmodulin-binding transcription activator and also called SR1 is mediated by biotic and abiotic stress. The two mutants (camta3-1, camta3-2) used in this study are homozygous T-DNA insertion mutants. CAMTA's transcriptional factor activity is induced via Ca^{2+}/CAM (Fig 6.24). The signalling component Ca^{2+} has an important role in plant growth and development. Camta3 mutants show an enhanced level of resistance against bacterial and fungal pathogens and it is believed that this is related to the induction of PR1 genes in addition to high accumulation of Reactive Oxygen Species (ROS) especially H_2O_2 (Bouche et al., 2003; Balague et al., 2003; Ma and Berkowitz, 2007).

The present study demonstrates the involvement of Oxi1, Gns1, Gns2, Gns3, Camta in the interaction between a model plant-aphid system at both the phenotypic level effect on the insect herbivore and also the transcriptional levels of key indicator genes (callose synthase β-1,3-glucanase genes). Specifically aphid survival bioassays are reported using a number of *Arabidopsis* mutants

(Oxi1, Gns1, Gns2, Gns3, Camta3-1 and Camta3-2) in comparison to their wild type backgrounds. Thereafter, RT-qPCR was performed to investigate the transcript level of selected callose synthase β-1,3-glucanase genes.

6.2. Material and methods

6.2.1. Plant Materials and Growth Conditions

Arabidopsis thaliana plants were grown in John Innes compost number 2 in a controlled environment chamber under a 16/8 h light/dark cycle at 23°C, 55% relative humidity. Col-0 wild type, Oxi1, Camta3-1, Camta3-2, Wisconsin Wild-type and the Oxi1 null mutant in Wisconsin background were kindly supplied by as a gift from Prof. Marc Knight (Durham University). β-glucanases 1, 2, 3 and 5 mutants were obtained from the *Arabidopsis* Biological Resource Centre (Nottingham Arabidopsis Stock Centre).

6.2.2. Insect bioassays

The aphid *Myzus persicae* was reared on Arabidopsis Col-0 before starting the bioassay and experiment. *Arabidopsis thaliana* plants were grown as stated in (6.2.1). All bioassay were carried out on plants that were between 25-30 days old (i.e. rosette stage 5-10 leaves). The varieties tested were the Oxi1 mutant in and its background Col-0 wild type; Oxi1 null mutant in WS2 and its background (Wisconsin) WS2. Aphid *Myzus persicae were* reared on Arabidopsis Col-0 and WS2 before starting the bioassay and experiment. The bioassay were performed by adding two aphid adults in the upper side of the *Arabidopsis* leaf and when the adults started producing nymphs , all insects were taken away and two nymphs were left on the leaf to start the bioassay. Zero time point (day 1) for the bioassay started when the two nymphs became adults. The bioassay for all mutants and backgrounds was conducted concurrently. The number of both aphid adults and nymphs were recorded every two days. Bioassay were performed in Oxi1 mutant and its background Col-0, Oxi1 null mutant in WS2 and its background WS2 and β-1,3-glucanase Gns1, Gns2 and Gns3 mutants and its background Col-0.

6.2.3. Experimental design

Arabidopsis plants (i.e. rosette stages 5-10 leaves) were used for all studies. Oxi1 mutant, Col-0 background, Oxi1 mutant in Wisconsin two mutants and Wisconsin background were used in all studies. Each *Arabidopsis* Plant was infested with 10 aphids at different time points (3, 6, 12, 24 and 48 hours).Three

time points (zero, 3 and 48 hours) were used as control for this study. Three *Arabidopsis* plants were used as an individual biological replicates.

6.2.4. RNA extraction

Total RNA was isolated from approximately 200 mg of frozen leaf tissue using Trizol Reagent (Invitrogen), according to the manufacturer's protocols. The concentration and purity of the RNA samples was determined using a Nanodrop (ND-1000 Spectrophotometer; Nanodrop Technologies). All samples had an absorbance ratio (absorbance at A_{260}/A_{280} nm) of between 1.9 and 2.2. Following quantification, all RNA samples were normalized to 100ng μl^{-1}.

6.2.5. Primer design

To ensure maximum specificity and efficiency during QPCR amplification under a standard set of reaction conditions, Allele ID 7.7 software was used to design QRT-PCR primers (Table 6.1). Initially four reference genes were used in this study but only EF (elongation factor) gave consistent expression levels. This was selected as the internal reference gene.

Table 6.1. Specific primers for QRT-PCR.

Gene	Accession number	Specific Primers for Real-Time PCR		Expected Size bp
		Forward Primer (5`-3`)	Reverse Primer (5`-3`)	
EF-1α	AT5G60390	TGAGCACGCTCTTCTTGCTTTCA	GGTGGTGGCATCCATCTTGTTACA	**147bp**
UBQ10	AT4G05320	GGCCTTGTATAATCCCTGATGAATAAG	AAAGAGATAACAGGAACGGAAACATAGT	**120bp**
TIP41	AT4G34270	GTGAAAACTGTTGGAGAGAAGCAA	TCAACTGGATACCCTTTCGCA	**127bp**
helicase	AT1G58050	CCATTCTACTTTTTGGCGGCT	TCAATGGTAACTGATCCACTCTGATG	**140bp**
AGns1	AT3G57270	GAGATGTTATGGTGGTAATGGA	GCTGAAGTAAGTGTAGAGGTT	**89bp**
AGns2	AT3G57260	ACCAATGTTGATGATTCTTCTC	CCGTAGCATACTCCGATT	**81bp**
AGns3	AT3G57240	GATAATGCGAGAACTTAT	ATACTTAGGCTGTAGATT	**177bp**
AGns5	AT5G20340	ACAACAATAGTGACTTCGTAA	AGGAGACCGTAGTTCAAG	**85bp**
AGSL1	AT1G05570	ATTGATGAACATATTGAGAAGGA	GATTAGCCGAACGAACTG	**90bp**
AGSL5	AT2G13680	TCTGTTGCTTGTTCCTTAT	CCAATGCTATCGGTATCTT	**92bp**

6.2.6. Quantitative real time PCR (QRT-PCR)

One step brilliant ll sybr green master mix was used for QRT-PCR. Gradient PCR was used to identify the annealing temperature. A 25-µl reaction volume PCR was employed using 1µl RNA at 100 ng μl^{-1}, 12.5µl of one step Master Mix (Agilent), 2 pmol each gene-specific primer, and UltraPure DNase/RNase-free distilled water (Qiagen) to 25 µl. Amplification of RNA employed the following conditions: 30 minutes incubation at (50°C -60°C) to allow the reverse transcription, initial 10 min of denaturation at 94°C; followed by 39 cycles of denaturation 94°C for 30 s, annealing at (50°C -60°C) for 30 s, and extension at 72°C for 45 s; followed by a final extension for 5 min at 72°C (Table 6.2).

Table 6.2. Protocol setup for Real Time PCR.

Steps	Protocol Setup for Real-Time PCR
0	Temperature Control: Sample Calculation Lid Mode: Constant 100.0C; Shutoff < 30.0C
1	Incubate at 50-60 C for 00:30:00 minutes
2	Incubate at 94.0 C for 00:10:00 minutes
3	Incubate at 94.0 C for 00:00:30 seconds
4	Incubate at 50-60 C for 00:00:30 seconds
5	Incubate at 72.0 C for 00:00:45 seconds
6	Plate Read
7	Go to line 3 for 39 more times
8	Incubate at 72.0 C for 00:05:00 seconds
9	Melting Curve from 45.0 C to 90.0 C read every 0.5 C hold 00:00:05
0	END

6.2.7. QRT-PCR calculations

Efficiency has been calculated from the slopes of the calibration curve according to the equation: $E = 10^{[-1/slope]}$.

Relative expression have been calculated according to Pfaffl equations (Pfaffl, 2001)

1- Relative expression = $2^{[\Delta CT\ control\ -\ \Delta CT\ Target\ gene]}$

2- Relative expression = $2^{\Delta\Delta CT}$

Relative quantification determines the changes in steady-state mRNA levels of a gene across multiple samples and expresses it relative to the levels of an internal control RNA. This reference gene was Elongation factor (EF-1α) gene which was amplified in a separate tube. Therefore, relative quantification does

not require standards with known concentrations. Relative quantification is based on the expression levels of a target gene versus a reference gene and in many experiments is adequate for investigating physiological changes in gene expression levels. Two equations were used to calculate the expression of a target gene in relation to EF-1α reference gene. Calculations were based on the comparison of the distinct cycle determined by various methods, e.g., crossing points and threshold values (Ct) at a constant level of fluorescenc. Threshold cycle of the triplicate reactions were determined by using the Opticon Monitor software.

6.2.8. Statistical methods

Repeated measures ANOVA were conducted to generate the p value for the bioassay. Two way ANOVA with replication was used to test the p value of QRT-PCR results followed by Tukey test.*$p<0.05$, **$p<0.01$ and ***$p<0.001$.

6.3. Results

6.3.1. Aphid (*Myzus persicae*) bioassay in Camta3-1 mutant and its background Col-0 wild type

The results of the aphid bioassay show that the mean number of nymphs on the wild type plants, Col-0, increased rapidly during the first 17 days reaching a total of 120 nymphs (mean per plant) during this period. Once this maximum population density had been reached the numbers decreased until no surviving nymphs were present after 37 days. However in the corresponding Camta 3-1 mutant bioassay, the mean number of nymphs per plant was significantly lower ($p<0.001$) than with the controls, additionally development of the nymphs was slower than with the controls with the peak population density occurring 2 days later, also overall longevity was reduced to 31 days (Fig 6.6).

Similarly the adult aphids on Col-0 showed a more rapid development than those on the Camta3-1 mutant lines reaching a maximum population density after 19 days with 114 adults compared to 21 days with 72 adults respectively. Population density was highly significantly different throughout the assay, but both populations showed the same trends for increase and decrease. However those aphids on Camta3-1 died sooner than those on the wild type (Fig 6.6).

Table 6.3. Aphid (*Myzus persicae*) bioassay in Camta3-1 mutant and its background Col-0.

Days	The mean number of nymphs/plant				The mean number of adults/plant			
	Col-0	P value	Camta3-1	P values	Col-0	P values	Camta3-1	P values
1	0.00	0.000	0.00	[a]-	2.00	-	2.00	-
3	0.00	0.000	0.00	-	2.00	-	2.00	-
5	4.80	0.000	5.25	-	2.00	-	2.00	-
7	11.46	0.000	6.83	-	3.80	-	9.50	0.000
9	22.80	0.000	10.50	-	7.66	-	16.66	0.000
11	34.46	0.000	17.91	-	13.93	-	25.33	0.000
13	56.20	0.000	26.66	-	25.06	-	35.16	0.000
15	105.00	0.000	38.16	-	65.73	-	42.50	-
17	120.20	0.000	55.33	-	106.73	0.000	51.75	-
19	77.80	-	86.91	0.000	114.46	0.000	63.16	-
21	54.46	-	63.91	0.000	85.86	0.000	72.50	-
23	40.86	0.000	35.16	-	72.80	0.000	47.58	-
25	20.33	-	19.83	-	54.46	0.000	30.66	-
27	14.66	0.000	9.41	-	26.06	0.000	15.08	-
29	10.26	0.000	2.66	-	20.33	0.000	6.08	-
31	6.20	0.000	0.00	-	14.20	0.000	0.00	-
33	1.53	-	-	-	4.86	-	-	-
35	0.06	-	-	-	1.53	-	-	-
37	0.00	-	-	-	0.00	-	-	-

[a] no significant differences

Table 6.4. Statestical analysis of aphid bioassay in Camta3-1 mutant and Col-0.

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Sample	14101.38	1	14101.38	3115.446	6.3E-177	3.868012
Columns	335490.5	15	22366.03	4941.374	0	1.694872
Interaction	35817.25	15	2387.816	527.5452	1E-230	1.694872
Within	1593.25	352	4.526278			
Total	387002.4	383				

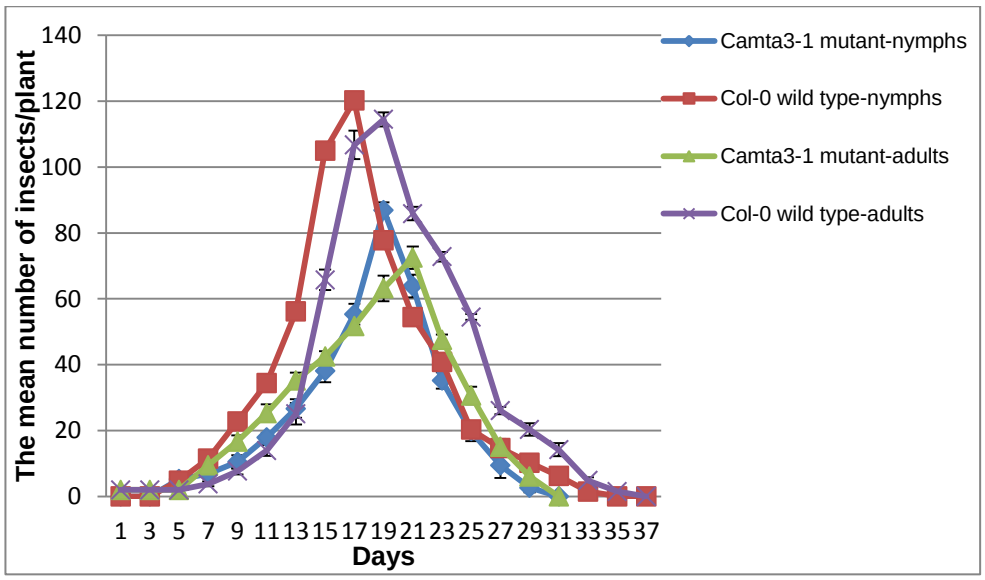

Fig 6.6. Aphid (*Myzus persicae*) bioassay in Camta3-1 mutant and Columbia (Col-0) wild type. Aphid *Myzus persicae were* reared on *Arabidopsis* Col-0 before starting the bioassay and experiment. The bioassay were performed by adding two aphid adults in the upper side of the *Arabidopsis* leaf and when the adults produced nymphs , all insects were taken away and two nymphs were kept in the leaf to start the bioassay. The number of both adults and nymphs were counted every two days. The data represents the mean number of nymphs and adults in Camta3-1 *Arabidopsis* mutant and its background Col-0. All the values are means of 15 plants ±SE (*n* =15). Two ways repeated measures anova test was conducted to generate the *p* values. Significant differences in the number adults was indicated with * p<0.05, ** p< 0.01, *** p<0.001.

6.3.2. Aphid (*Myzus persicae*) bioassay in Camta3-2 mutant and its background Col-0 wild type

Figure 6.7 shows the resulting population densities of aphids on wild type, Col-0, and Camta3-2 mutant *Arabidopsis*. The results show that the mean number of nymphs in Col-0 increased over time and reached the highest population density at day 17 with 120 nymphs per plant. Subsequently the number of nymphs decreased gradually until no viable aphids were present after 37 days. Results from the Camta3-2 mutant show that the time taken to reach the maximum population density and the number of aphids present was significantly lower (p<0.001). However, the population survived for an additional two days compared with those on Col-0 (Fig 6.7).

The trends seen with the nymph populations were also mirrored by the adults. Most interesting is the shift in the time to maximum population density from 19 days to 21 days for Col-0 and Camta3-2 respectively. Also the overall mean

number of adults was significantly less on the Camta3-2 mutant lines, 91 compare to 114 on the controls ($p<0.001$) (Fig 6.7).

Table 6.5. Aphid (*Myzus persicae*) bioassay in Camta3-2 mutant and its background Col-0.

Days	The mean number of nymphs/plant				The mean number of adults/plant			
	Col-0	P value	Camta3-2	P values	Col-0	P values	Camta3-2	P values
1	0.00	[a]-	0.00	-	2.00	-	2.00	-
3	0.00	-	0.00	-	2.00	-	2.00	-
5	4.80	-	3.46	-	2.00	-	2.00	-
7	11.46	0.000	4.80	-	3.80	-	4.00	-
9	22.80	0.000	14.13	-	7.66	-	8.00	-
11	34.46	0.000	24.73	-	13.93	0.000	12.60	-
13	56.20	0.000	33.73	-	25.06	0.000	17.53	-
15	105.00	0.000	44.53	-	65.73	0.000	30.33	-
17	120.20	0.000	53.40	-	106.73	0.000	42.93	-
19	77.80	0.000	70.86	-	114.46	0.000	62.93	-
21	54.46	-	75.33	0.000	85.86	0.000	78.66	-
23	40.86	-	66.73	0.000	72.80	-	91.13	0.000
25	20.33	-	53.93	0.000	54.46	-	72.13	0.000
27	14.66	-	40.40	0.000	26.06	-	55.00	0.000
29	10.26	-	23.66	0.000	20.33	-	36.40	0.000
31	6.20	-	9.33	-	14.20	-	13.00	-
33	1.53	-	3.00	-	4.86	-	6.73	-
35	0.06	-	1.00	-	1.53	-	3.13	-
37	0.00	-	0.13	-	0.00	-	0.60	-
39	-	-	0.00	-	-		0.00	

[a] no significant differences

Table 6.6. Statestical analysis of aphid bioassay in Camta3-2 mutant and Col-0.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	4723.455	3	1574.485	379.8758	1.1E-167	2.613268
Columns	968249.9	18	53791.66	12978.31	0	1.613556
Interaction	221347.8	54	4099.033	988.9731	0	1.348762
Within	4410	1064	4.144737			
Total	1198731	1139				

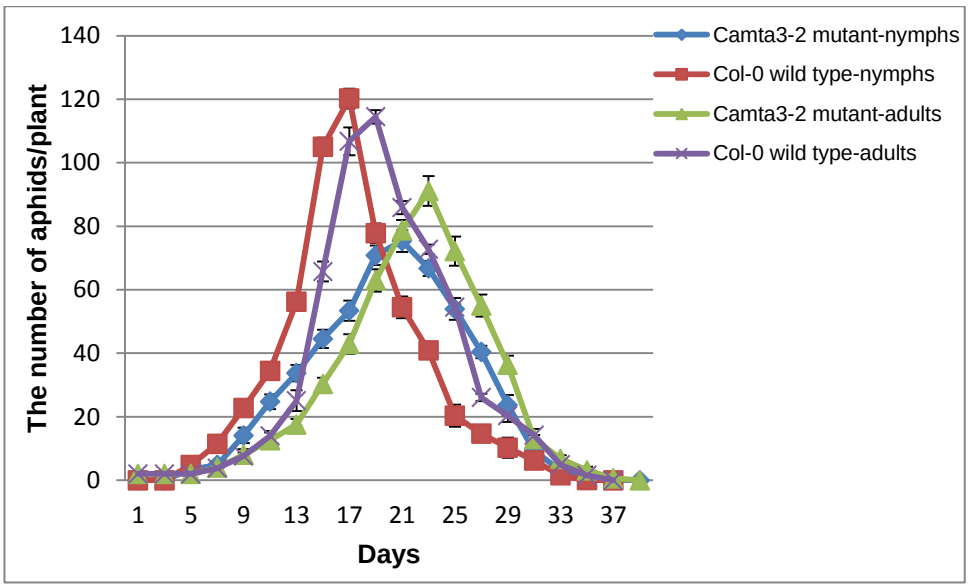

Fig 6.7. Aphid (Myzus persicae) bioassay in Camta3-2 mutant and Columbia (Col-0) wild type. Aphid *Myzus persicae were* reared on *Arabidopsis* Col-0 before starting the bioassay and experiment. The bioassay were performed by adding two aphid adults in the upper side of the *Arabidopsis* leaf and when the adults produced nymphs , all insects were taken away and two nymphs were kept in the leaf to start the bioassay. The number of both adults and nymphs were counted every two days. The data represents the mean number of nymphs in Camta3-2 *Arabidopsis* mutant and its background Col-0. All the values are means of 15 plants ±SE (*n* =15). Two ways repeated measures anova test was conducted to generate the p value. Significant differences in the number adults was indicated with * p<0.05, ** p< 0.01, *** p<0.001.

6.3.3. Aphid (*Myzus persicae*) bioassay in Oxi1 mutants and its background Col-0 wild type

The results show that the number of aphid nymph in Col-0 increased during the bioassay and reached a population maximum at 17 days with 120 nymphs. Subsequently the number of nymphs decreased sharply with no survivors after 37 days. However, in Oxi1 mutant, the number of nymphs was significantly lower and they survived for 53 days ($p<0.001$). Moreover there was a shift in the peak, with a delay of four days (Fig 6.8).

In comparison, the number of adults in Col-0 peaked at day 19 with 114 adult aphids. Thereafter the number decreased gradually with no survivors after 37 days. However, in the Oxi1 *Arabidopsis* mutant, the number of adult aphids was significantly lower after 19 days with 80 adults ($p<0.001$). Thereafter the number of adults decreased gradually with no survivors after 57 days. Furthermore there was also a shift in the peak, with a delay of four days (Fig 6.8).

Table 6.7. Aphid (*Myzus persicae*) bioassay in Oxi1 mutant and its background Col-0.

Days	The mean number of nymphs/plant				The mean number of adults/plant			
	Col-0	P value	Oxi1 mutant	P values	Col-0	P values	Oxi1 mutant	P values
1	0.00	[a]-	0.00	-	2.00	-	2.00	-
3	0.00	-	0.00	-	2.00	-	2.00	-
5	4.80	-	5.66	-	2.00	-	2.00	-
7	11.46	0.000	6.46	-	3.80	-	5.60	-
9	22.80	0.000	11.20	-	7.66	0.000	11.33	-
11	34.46	0.000	16.13	-	13.93	-	14.73	-
13	56.20	0.000	19.40	-	25.06	-	24.53	-
15	105.00	0.000	33.86	-	65.73	0.000	34.46	-
17	120.20	0.000	44.26	-	106.73	0.000	57.20	-
19	77.80	0.000	60.33	-	114.46	0.000	80.60	-
21	54.46	-	96.00	0.000	85.86	-	87.80	-
23	40.86	-	84.66	0.000	72.80	-	93.53	0.000
25	20.33	-	80.33	0.000	54.46	-	82.80	0.000
27	14.66	-	59.06	0.000	26.06	-	64.20	0.000
29	10.26	-	38.06	0.000	20.33	-	45.60	0.000
31	6.20	-	22.93	0.000	14.20	-	33.26	0.000
33	1.53	-	15.93	0.000	4.86	-	16.40	0.000
35	0.06	-	8.00	0.000	1.53	-	12.73	0.000
37	0.00	-	5.20	0.000	0.00	-	9.80	0.000
39	-	-	3.80	-	-	-	8.60	-
41	-	-	2.53	-	-	-	5.86	-
43	-	-	1.06	-	-	-	4.73	-
45	-	-	0.60	-	-	-	3.80	-
47	-	-	0.20	-	-	-	2.13	-
49	-	-	0.13	-	-	-	1.46	-
51	-	-	0.06	-	-	-	1.13	-
53	-	-	0.00	-	-	-	0.73	-
55	-	-	0.00	-	-	-	0.40	-
57	-	-	0.00	-	-	-	0.00	-

[a] no significant differences

Table 6.8. Statistical analysis of aphid bioassay in Oxi1 mutant and Col-0.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	4192.042	3	1397.347	461.4265	5.6E-192	2.613268
Columns	1011102	18	56172.35	18549.01	0	1.613556
Interaction	277732	54	5143.184	1698.362	0	1.348762
Within	3222.133	1064	3.028321			
Total	1296248	1139				

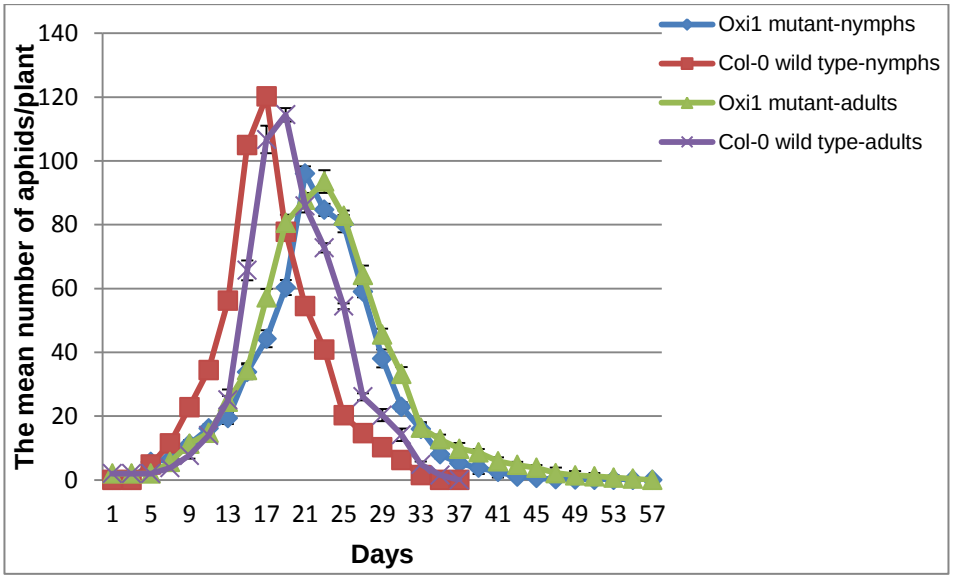

Fig 6.8. Aphid (*Myzus persicae)* bioassay in OXI1 mutant and Columbia (Col-0) wild type. Aphid *Myzus persicae were* reared on *Arabidopsis* Col-0 before starting the bioassay and experiment. The bioassay were performed by adding two aphid adults in the upper side of the *Arabidopsis* leaf and when the adults produced nymphs , all insects were taken away and two nymphs were kept in the leaf to start the bioassay. The number of both adults and nymphs were counted every two days. The data represents the mean number of nymphs per plant in Oxi1 *Arabidopsis* mutant and its background Col-0. All the values are means of 15 plants ±SE (*n* =15). Two ways repeated measures anova test was conducted to generate the p value. Significant differences in the number adults was indicated with * p<0.05, ** p< 0.01, *** p<0.001.

6.3.4. Aphid (*Myzus persicae*) bioassay in β-1,3-glucanase Gns1 mutant and its background Col-0 wild type

The results show that the number of nymphs in Col-0 increased gradually and the highest population density was reached after 17 days with 120 nymphs. Subsequently the number of nymphs decreased gradually until no survivors were present after 37 days. However, in Gns1 mutant, the number of nymphs initially increased more rapidly and peaked at 21 days with 120 nymphs. Thereafter, the number of nymphs decreased gradually with no survivors after 47 days. The maximum population density of the aphids was delayed by four days when on the Gns1 mutant line (Fig 6.9).

Populations of adults in Col-0 peaked at 19 days with 114 adult aphids. Thereafter the number decreased rapidly with no survivors after 37 days. However, in Gns1 *Arabidopsis* mutant, the mean number of adults was

significantly lower after 19 days with 84 adult aphids ($p<0.001$), and the maximum population density (116 adults) was not reached for a further 10 days. Then, the number of adults rapidly decreased with no survivors present after 49 days (Fig 6.9).

Table 6.9. Aphid (Myzus persicae) bioassay in Gns1 mutant and its background Col-0.

Days	The mean number of nymphs/plant				The mean number of adults/plant			
	Col-0	P value	Gns1 mutant	P values	Col-0	P values	Gns1 mutant	P values
1	0.00	[a]-	0.00	-	2.00	-	2.00	-
3	0.00	-	0.00	-	2.00	-	2.00	-
5	4.80	-	5.46	-	2.00	-	2.00	-
7	11.46	-	13.60	-	3.80	-	5.40	0.000
9	22.80	-	29.73	0.000	7.66	-	9.20	0.000
11	34.46	-	42.60	0.000	13.93	-	13.73	-
13	56.20	-	73.73	0.000	25.06	0.000	19.53	-
15	105.00	0.000	94.20	-	65.73	0.000	29.53	-
17	120.20	0.000	112.80	-	106.73	0.000	53.80	-
19	77.80	-	117.60	0.000	114.46	0.000	84.86	-
21	54.46	-	120.86	0.000	85.86	-	97.13	0.000
23	40.86	-	115.20	0.000	72.80	-	104.46	0.000
25	20.33	-	102.66	0.000	54.46	-	107.06	0.000
27	14.66	-	74.93	0.000	26.06	-	112.53	0.000
29	10.26	-	44.26	0.000	20.33	-	116.93	0.000
31	6.20	-	32.33	0.000	14.20	-	91.00	0.000
33	1.53	-	17.00	0.000	4.86	-	66.00	0.000
35	0.06	-	7.86	0.000	1.53	-	35.40	0.000
37	0.00	-	6.66	0.000	0.00	-	27.66	0.000
39	-	-	5.66	-	-	-	22.60	-
41	-	-	3.66	-	-	-	16.86	-
43	-	-	2.66	-	-	-	13.46	-
45	-	-	1.66	-	-	-	10.80	-
47	-	-	0.00	-	-	-	4.00	-
49	-	-	0.00	-	-	-	-	-

[a] no significant differences

Table 6.10. Statistical analysis of Aphid bioassay in Gns1 mutant and Col-0.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	123380.8	3	41126.95	9802.074	0	2.613268
Columns	1306769	18	72598.28	17302.86	0	1.613556
Interaction	518113	54	9594.685	2286.769	0	1.348762
Within	4464.267	1064	4.195739			
Total	1952727	1139				

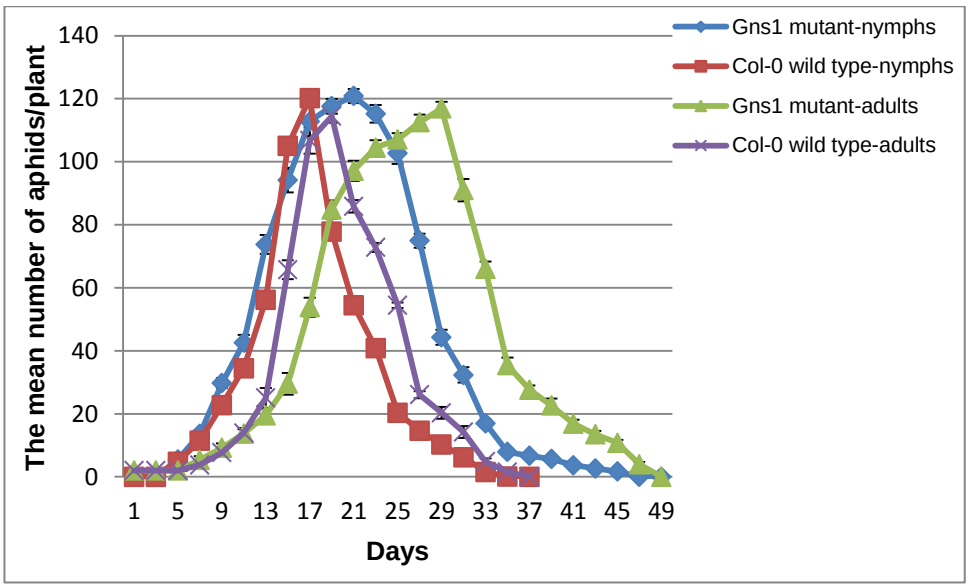

Fig 6.9. Aphid (*Myzus persicae*) bioassay in β-1-3 glucanase (Gns1) mutant and Columbia (Col-0) wild type. Aphid *Myzus persicae were* reared on *Arabidopsis* Col-0 before starting the bioassay and experiment. The bioassay were performed by adding two aphid adults in the upper side of the *Arabidopsis* leaf and when the adults produced nymphs , all insects were taken away and two nymphs were kept in the leaf to start the bioassay. The number of both adults and nymphs were counted every two days. The data represents the mean number of nymphs in Gns1 *Arabidopsis* mutant and its background Col-0. All the values are means of 15 plants ±SE (*n* =15). Two ways repeated measures anova test was conducted to generate the p value. Significant differences in the number adults was indicated with * $p<0.05$, ** $p< 0.01$, *** $p<0.001$.

6.3.5. Aphid (*Myzus persicae*) bioassay in β-1,3-glucanase Gns2 mutant and its background Col-0 wild type

The results show that the number of nymphs in Col-0 increased gradually and reached the highest at 17 days with 120 nymphs. Subsequently the number of nymphs decreased gradually with no survivors after 37 days. However, in Gns2 mutant, the number of nymphs increased gradually and peaked at 29 days with 144 nymphs, a shift in time to maximum population density of 12 days. Thereafter, the number of nymphs decreased gradually with no survivors after 47 days (Fig 6.10).

Similarly the number of adults in Col-0 peaked rapidly at day 19 with 114 adults. Thereafter the number of adults decreased gradually with no survivors after 37 days. However, in Gns2 Arabidopsis mutant, the number of adults was significantly lower after 19 days with 79 adults ($p<0.001$) and the maximum population density was not reached until 12 days later, day 31, where 135

adults were present. Subsequently the number of adults then decreased gradually with no survivors present after 51 days (Fig 6.10).

Table 6.11. Aphid (Myzus persicae) bioassay in Gns2 mutant and its background Col-0.

Days	The mean number of nymphs/plant				The mean number of adults/plant			
	Col-0	P value	Gns2 mutant	P values	Col-0	P values	Gns2 mutant	P values
1	0.00	[a]-	0.00	-	2.00	-	2.00	-
3	0.00	-	0.00	-	2.00	-	2.00	-
5	4.80	-	7.40	-	2.00	-	2.00	-
7	11.46	-	11.20	-	3.80	-	7.46	-
9	22.80	0.000	17.73	-	7.66	-	12.53	0.000
11	34.46	0.000	26.80	-	13.93	-	19.13	0.000
13	56.20	0.000	37.46	-	25.06	-	29.06	0.000
15	105.00	0.000	44.66	-	65.73	0.000	37.53	-
17	120.20	0.000	70.66	-	106.73	0.000	49.86	-
19	77.80	0.000	73.20	-	114.46	0.000	79.60	-
21	54.46	-	75.93	0.000	85.86	-	101.60	0.000
23	40.86	-	78.66	0.000	72.80	-	111.06	0.000
25	20.33	-	85.40	0.000	54.46	-	112.86	0.000
27	14.66	-	127.13	0.000	26.06	-	116.60	0.000
29	10.26	-	144.93	0.000	20.33	-	123.26	0.000
31	6.20	-	98.53	0.000	14.20	-	135.80	0.000
33	1.53	-	57.26	0.000	4.86	-	74.33	0.000
35	0.06	-	28.00	0.000	1.53	-	50.06	0.000
37	0.00	-	9.60	0.000	0.00	-	36.80	0.000
39	-	-	5.46	-	-	-	25.53	-
41	-	-	3.53	-	-	-	19.00	-
43	-	-	2.46	-	-	-	15.33	-
45	-	-	1.86	-	-	-	12.06	-
47	-	-	0.00	-	-	-	4.80	-
49	-	-	0.00	-	-	-	1.73	-
51	-	-	0.00	-	-	-	0.00	-

[a] no significant differences

Table 6.12. Statestical analysis of aphid bioassay in Gns2 mutant and Col-0.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	162978.8	3	54326.27	10786.59	0	2.613268
Columns	1109231	18	61623.96	12235.56	0	1.613556
Interaction	738240.1	54	13671.11	2714.426	0	1.348762
Within	5358.8	1064	5.036466			
Total	2015809	1139				

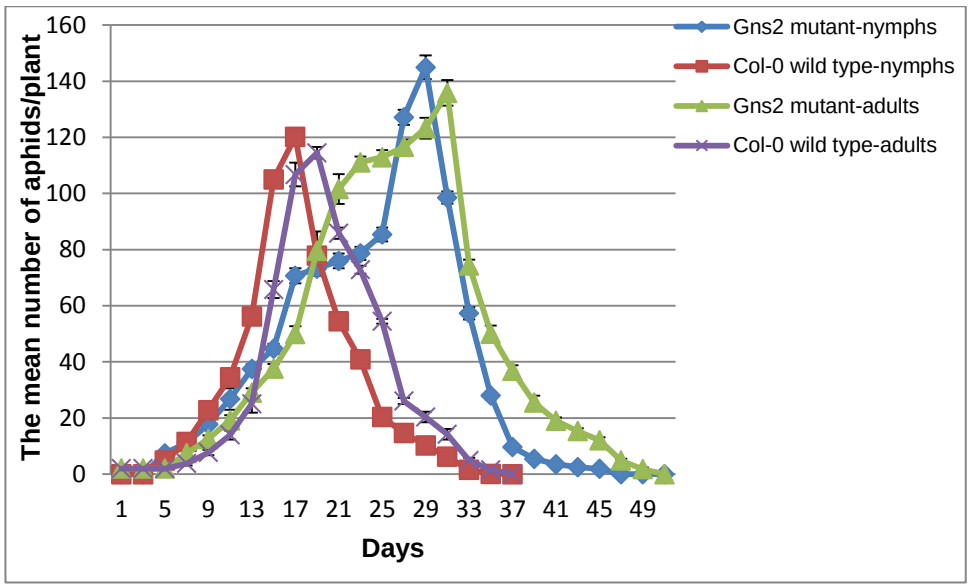

Fig 6.10. Aphid (Myzus persicae) bioassay in β-1-3 glucanase (Gns2) mutant and Colombia (Col-0) wild type. Aphid *Myzus persicae were* reared on *Arabidopsis* Col-0 before starting the bioassay and experiment. The bioassay were performed by adding two aphid adults in the upper side of the *Arabidopsis* leaf and when the adults produced nymphs, all insects were taken away and two nymphs were kept in the leaf to start the bioassay. The number of both adults and nymphs were counted every two days. The data represents the accumulative number of nymph aphids in Gns2 *Arabidopsis* mutant and its background Col-0. All the values are means of 15 plants ±SE (*n* =15). Two ways repeated measures anova test was conducted to generate the p value. Significant differences in the number adults was indicated with * p<0.05, ** p< 0.01, *** p<0.001.

6.3.6. Aphid (*Myzus persicae*) bioassay in β-1,3-glucanase Gns3 mutant and its background Col-0 wild type

The bioassay designed to show the effects of reduced Gns3 expression to aphid population increase and density are shown in figures 6.16 and 6.17. The results show the typical increase of the mean number of nymphs in Col-0 reaching a maximum population density after 17 days with 120 nymphs present per plant. Subsequently the number of nymphs decreased gradually with no survivors after 37 days. For the Gns3 mutant the number of nymphs increased more slowly than with the controls with the population peaking after 23 days. However this, although delayed, population density was highly significantly different to the one observed on the control line. Thereafter the number of nymphs decreased with no survivors after 47 days (Fig 6.11).

When considering the adults the trends of population development and densities were similar to the nymphs. The number of adults in Col-0 peaked at

19 days with 114 adults, and then decreased gradually with no survivors after 37 days. The results show that for the Gns3 *Arabidopsis* mutant, the number of adults was significantly lower after 19 days with 75 adults ($p<0.001$), however the number of adults continued to increased and peaked some 10 days later, at day 29, with 175 adults per plant. The overall longevity of the adult aphids on the Gns3 mutant lines was also greater than when compared to the controls (Fig 6.11).

Table 6.13. Aphid (Myzus persicae) bioassay in Gns3 mutant and its background Col-0.

Days	The mean number of nymphs/plant				The mean number of adults/plant			
	Col-0	P value	Gns3 mutant	P values	Col-0	P values	Gns3 mutant	P values
1	0.00	[a]-	0.00	-	2.00	-	2.00	-
3	0.00	-	0.00	-	2.00	-	2.00	-
5	4.80	-	7.20	0.000	2.00	-	2.00	-
7	11.46	-	14.60	0.000	3.80	-	7.00	0.000
9	22.80	-	24.26	0.000	7.66	-	9.53	0.000
11	34.46	0.000	31.40	-	13.93	-	12.80	-
13	56.20	0.000	43.06	-	25.06	0.000	16.80	-
15	105.00	0.000	63.20	-	65.73	0.000	20.40	-
17	120.20	0.000	73.13	-	106.73	0.000	42.86	-
19	77.80	-	102.33	0.000	114.46	0.000	75.13	-
21	54.46	-	175.33	0.000	85.86	-	135.73	0.000
23	40.86	-	175.60	0.000	72.80	-	157.46	0.000
25	20.33	-	163.53	0.000	54.46	-	163.93	0.000
27	14.66	-	151.06	0.000	26.06	-	170.26	0.000
29	10.26	-	98.20	0.000	20.33	-	175.33	0.000
31	6.20	-	40.26	0.000	14.20	-	81.06	0.000
33	1.53	-	30.93	0.000	4.86	-	54.80	0.000
35	0.06	-	20.86	0.000	1.53	-	34.46	0.000
37	0.00	-	8.93	0.000	0.00	-	29.06	0.000
39	-	-	4.26	-	-	-	24.73	-
41	-	-	3.00	-	-	-	16.93	-
43	-	-	1.93	-	-	-	11.13	-
45	-	-	1.53	-	-	-	4.86	-
47	-	-	0.00	-	-	-	1.66	-
49	-	-	0.00	-	-	-	0.00	-

[a] no significant differences

Table 6.14. statistical analysis of aphid bioassay in Gns3 mutant and Col-0

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	290986.8	3	96995.6	19205.14	0	2.613268
Columns	1819038	18	101057.7	20009.44	0	1.613556
Interaction	1091159	54	20206.65	4000.919	0	1.348762
Within	5373.733	1064	5.050501			
Total	3206558	1139				

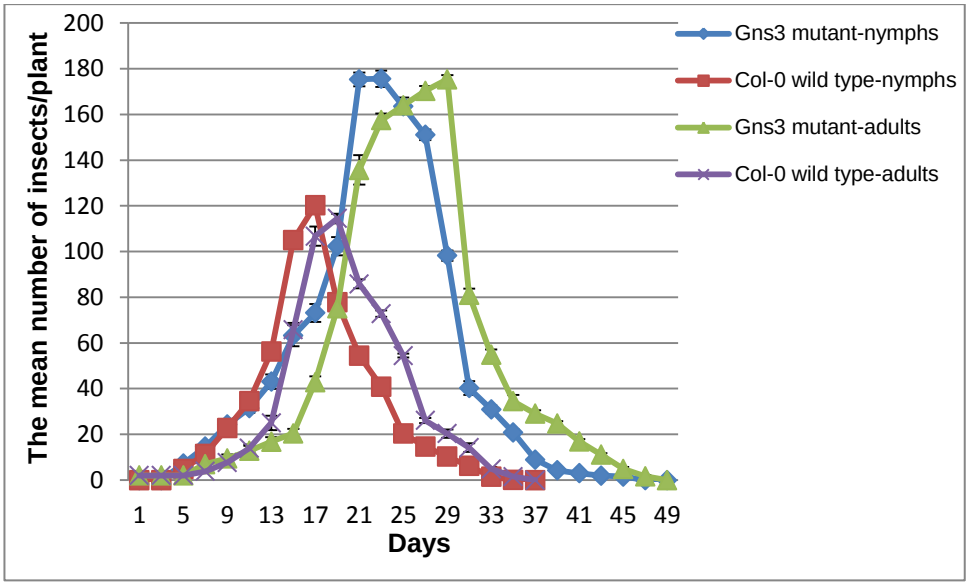

Fig 6.11. Aphid (Myzus persicae) bioassay in β-1,3-glucanase (Gns3) mutant and Colombia (Col-0) wild type. Aphid *Myzus persicae were* reared on *Arabidopsis* Col-0 before starting the bioassay and experiment. The bioassay were performed by adding two aphid adults in the upper side of the *Arabidopsis* leaf and when the adults produced nymphs, all insects were taken away and two nymphs were kept in the leaf to start the bioassay. The number of both adults and nymphs were counted every two days. The data represents the mean number of nymphs in Gns3 *Arabidopsis* mutant and its background Col-0. All the values are means of 15 plants ±SE (*n* =15). Two ways repeated measures anova test was conducted to generate the p value. Significant differences in the number adults was indicated with * p<0.05, ** p< 0.01, *** p<0.001.

6.3.7. Relative expression of Callose synthase genes GSL1 and GSL5 in Oxi1 mutant and its background Columbia Col-0 in response to aphid feeding

The results show that after 3 hours after infestationand at the end of the assay (48 hours) the expression level of GSL1 in Oxi1 mutant was 2.5 fold compared to 1.2 fold in Col-0 for the non-infested plants. Relative expression of GSL1 increased to 14.6 fold in Oxi1 mutant compared to 8.4 fold in Col-0, 3 hours after aphid feeding. After 6 hours of aphid feeding the expression level of GSL1 significantly increased to the highest level 27.2 fold in Col-0 compared to 14.3 fold in Oxi1 mutant. Thereafter, the expression level of GSL1 was 6.6 fold in Col-0 compared to 3.7 fold in Oxi1 mutant 12 hours after aphid feeding. Then Callose synthase GSL1 expressed to 11.4 fold in Col-0 compared to 2.3 fold in

Oxi1 mutant 24 hours after aphid feeding. Suddenly the expression level of GSL1 decreased to 1.2 fold in Col-0 compared to 6.9 fold in oxi1 mutant 48 hours after aphid feeding. Later the expression level of GSL1 in non-infested Oxi1 was 1.2 fold compared to 2.5 fold after 48 hours (Fig 6.12).

Figure 6.13 shows the expression level of callose synthase GSL5 in non-infested plants was 1.4 fold in Oxi1 mutant compared to 1 fold in Col-0 after 3 hours. After 3 hours from aphid feeding, the expression level of GSL5 increased to 2.5 fold in Oxi1 mutant compared to 1.7 fold in Col-0. The expression level of GSL5 increased to 5.3 fold in Oxi1 mutant compared to 1.2 fold in Col-0, 6 hours after aphid feeding. Thereafter, the expression of GSL5 significantly increased to the highest level 6.6 fold compared to 1.8 fold 12 hours after aphid feeding. Then, the expression level of GSL5 decreased to 2.9 fold in Oxi1 mutant compared to 1.9 fold in Col-0, 24 hours after aphid infestation. Suddenly, after 48 hours aphid feeding GSL5 was down-regulated but GSL5 was expressed to 2.3 fold in oxi1 mutant. Later, the expression level of GSL5 was 1.2, 1.3 in Col-0 and Oxi1 mutant respectively, in non-infested plants after 48 hours (Fig 6.13).

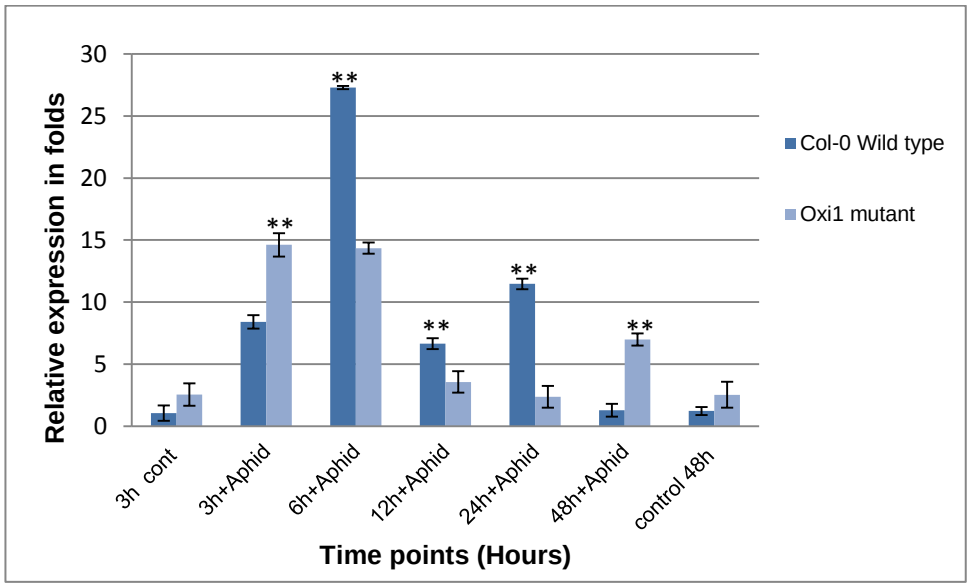

Fig 6.12. Relative expression level of Callose synthase gene (GSL1), in Oxi1 *Arabidopsis* mutant and its background Columbia (Col-0), in response to aphid (*Myzus persicae*) feeding. Results of real-time PCR represent the expression analysis of Callose synthase gene GSL1 in Oxi1 *Arabidopsis* mutant and Col-0 wild type in response to aphid feeding. Total RNA was extracted from *Arabidopsis* leaves (rosette stage 5-10 leaves) of infested plants with aphid at time points (3, 6, 12, 24 and 48 hours) and non-infested *Arabidopsis* plants control at time

points (0, 3 and 48 hours). Expression of genes was quantified relative to the value obtained from 0 (h) samples (aphid-free plants). Each RNA sample was extracted from approximately 200 mg of fresh leaf of Oxi1 and Col-0 *Arabidopsis* plants. *Arabidopsis* Elongation Factor (EF) gene was used as reference control. Two ways ANOVA was used to generate the p values. Significant differences in gene expression were indicated with * p<0.05, ** p< 0.01, *** p<0.001. Data are means ±SE (*n* =9).

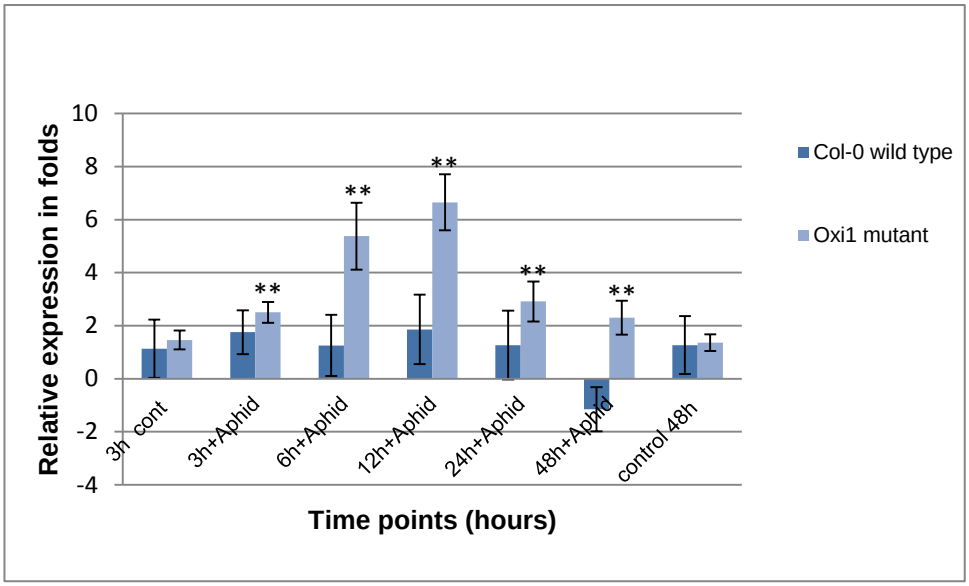

Fig 6.13. Relative expression level of Callose synthase gene GSL5 in *Arabidopsis* Oxi1 mutant and its background Columbia (Col-0) in response to aphid (*Myzus persicae*) feeding. Results of real-time PCR represent the expression analysis of Callose synthase gene GSL5 in Oxi1 *Arabidopsis* mutant and Col-0 wild type in response to aphid feeding. Total RNA was extracted from *Arabidopsis* leaves (rosette stage 5-10 leaves) of infested plants with aphid at time points (3, 6, 12, 24 and 48 hours) and non-infested *Arabidopsis* plants control at time points (0, 3 and 48 hours). Expression of genes was quantified relative to the value obtained from 0 (h) samples (aphid-free plants). Each RNA sample was extracted from approximately 200 mg of fresh leaf of Oxi1 and Col-0 Arabidopsis plants. *Arabidopsis* Elongation Factor (EF) gene was used as reference control. Two ways ANOVA was used to generate the p values. Significant differences in gene expression were indicated with * p<0.05, ** p< 0.01, *** p<0.001. Data are means ±SE (*n* =9).

6.3.8. Relative expression of β-1,3-glucanase genes Gns1, Gns2, Gns3, and Gns5 in Oxi1 mutant and its background Columbia Col-0 in response to aphid feeding

β-1,3-glucanase Gns1, Gns2, Gns3 and Gns5 were not expressed in Oxi1 neither in the control nor post aphid feeding. In Col-0, Gns1, Gns3 and Gns5 genes were expressed such low levels that made it too difficult to quantify gene expression accurately and reliably.

However, the expression level of β-1,3-glucanase gene Gns2 in non-infested Col-0 plants (control) was 1.1, 1.2 fold after 3 and 48 hours respectively. Thereafter the expression level of Gns2 increased 2.4 fold 6 hours post aphid feeding. The expression level of Gns2 increased to a maximum level of 26.4 fold 12 hours post aphid feeding. Subsequently the expression level of Gns2 decreased and returned to the basal level as seen at the beginning of the assay, 2.3 fold and 1.8 fold at 24 and 48 hours respectively post aphid feeding (Fig 6.14).

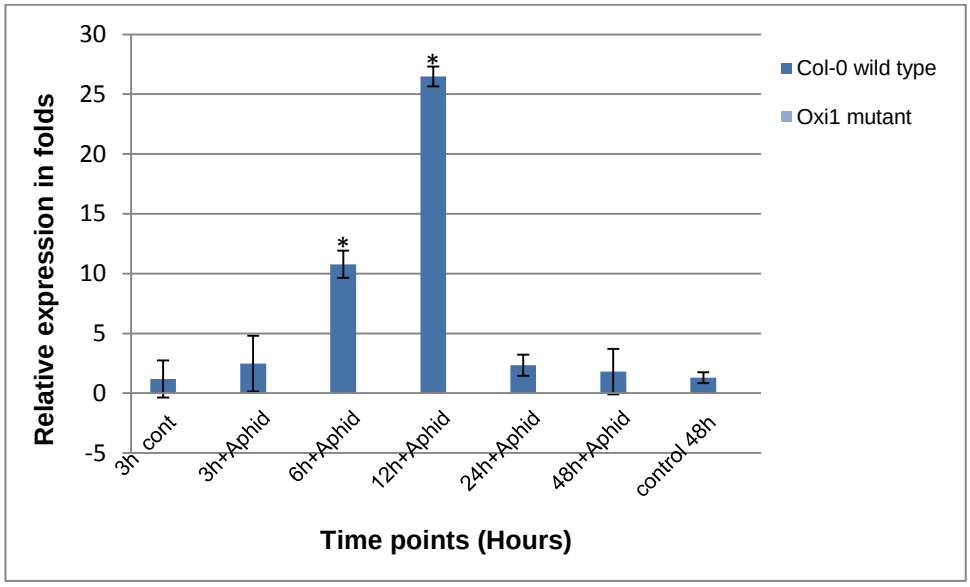

Fig 6.14. Relative expression level of β-1,3-glucanase two gene (Gns2) in *Arabidopsis* Oxi1 mutant and Columbia (Col-0) wild type in response to aphid (*Myzus persicae*) feeding. Results of real-time PCR represent the expression analysis of β-1,3-glucanase gene Gns2 in Oxi1 Arabidopsis mutant and Col-0 wild type in response to aphid feeding. Total RNA was extracted from *Arabidopsis* leaves (rosette stage 5-10 leaves) of infested plants with aphid at time points (3, 6, 12, 24 and 48 hours) and non-infested *Arabidopsis* plants control at time points (0, 3 and 48 hours). Expression of genes was quantified relative to the value obtained from 0 (h) samples (aphid-free plants). Each RNA sample was extracted from approximately 200 mg of fresh leaf of Oxi1 and Col-0 *Arabidopsis* plants. *Arabidopsis* Elongation Factor (EF) gene was used as reference control. Two ways ANOVA was used to generate the p values. Significant differences in gene expression were indicated with * $p<0.05$, ** $p< 0.01$, *** $p<0.001$. Data are means ±SE (*n* =9).

6.3.9. Relative expression of Callose synthase genes GSL1 and GSL5 in Oxi1 null mutant in WS2 (Wisconsin) and its background WS2 in response to aphid feeding

The results show that the expression level of GSL1 was 1 fold in both Oxi1 null mutant in WS2 and its background WS2 (wild type) in non-infested plants (control) after 3 and 48 hours. The expression level of GSL1 increased to 1.7 fold in the null mutant in WS2 compared to 1 fold in WS2 3 hours post aphid feeding. Thereafter, the expression of GSL1 increased to 2.9 fold in Oxi1 null mutant in WS2 but was down-regulated in the wild type 6 hours post aphid feeding. Expression of GSL1 significantly increased to the highest level of 11.2 fold in Oxi1 null mutant in WS2 but was expression remained down-regulated in WS2 background 12 hours after aphid feeding. After that, the expression level of GSL1 in oxi1 null mutant in WS2 was 7.1 fold compared to 1 fold in WS2 background 24 hours post aphid feeding. Later, relative expression of GSL1 was 7.1 fold in Oxi1 null mutant in WS2 but was down-regulated in WS2 background 48 hours post aphid feeding (Fig 6.15).

Figure 6.16 shows the relative expression of GSL5 was 1.2 and 1.6 fold in WS2 and Oxi1 null mutant in WS2 background respectively in non-infested plants after 3 hours. Interestingly, relative expression of GSL5 was down-regulated in both Oxi1 null mutant in WS2 and WS2 background 3, 6, 12, 24, and 48 hours post aphid feeding (Fig 6.16).

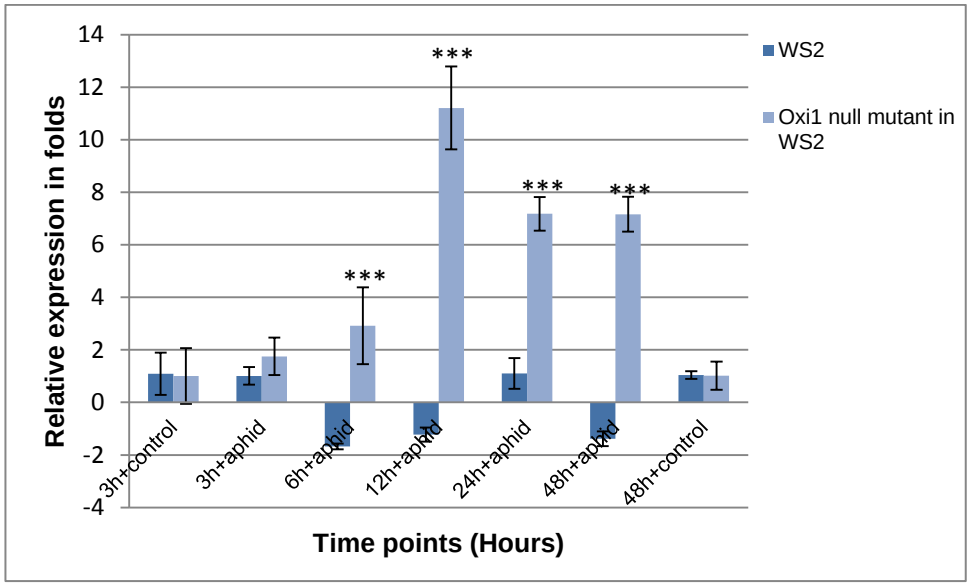

Fig 6.15. Relative expression level of Callose synthase gene (GSL1) in *Arabidopsis* mutant Oxi1 in Wisconsin (Oxi1 in WS2) and its background Wisconsin (WS2) in response to aphid (*Myzus persicae*) feeding. Results of real-time PCR represent the expression analysis of Callose synthase gene GSL1 in Oxi1 null mutant in WS2 and its background WS2 (Wisconsin) in response to aphid feeding. Total RNA was extracted from

Arabidopsis leaves (rosette stage 5-10 leaves) of infested plants with aphid at time points (3, 6, 12, 24 and 48 hours) and non-infested *Arabidopsis* plants control at time points (0, 3 and 48 hours). Expression of genes was quantified relative to the value obtained from 0 (h) samples (aphid-free plants). Each RNA sample was extracted from approximately 200 mg of fresh leaf of Oxi1 and Col-0 Arabidopsis plants. *Arabidopsis* Elongation Factor (EF) gene was used as reference control. Two ways ANOVA was used to generate the p values. Significant differences in gene expression were indicated with * p<0.05, ** p< 0.01, *** p<0.001. Data are means ±SE (*n* =9).

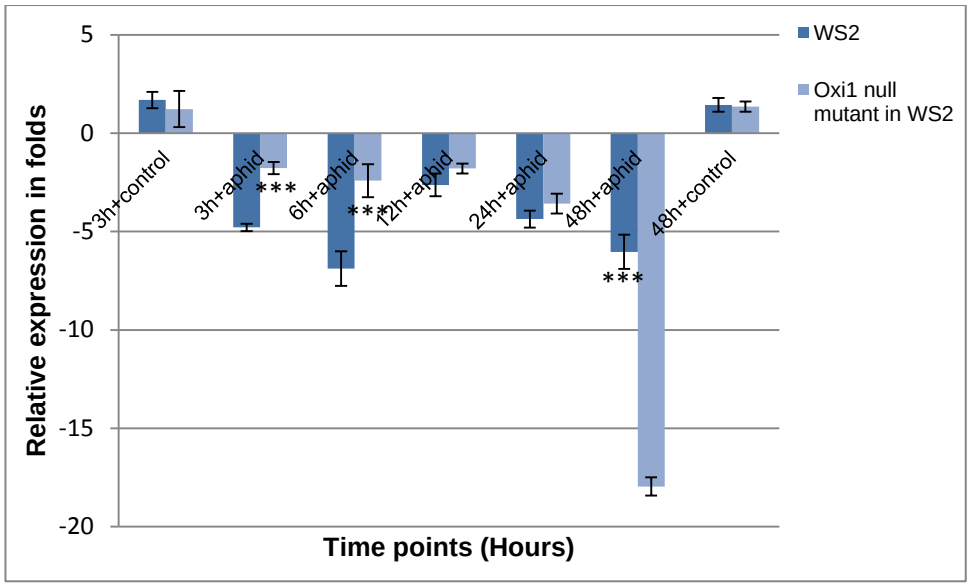

Fig 6.16. Relative expression level of Callose synthase gene (GSL5) in Arabidopsis Oxi1 in Wisconsin (Oxi1 in WS2) and its background Wisconsin (WS2) in response aphid (*Myzus persicae*) feeding. Results of real-time PCR represent the expression analysis of Callose synthase gene GSL5 in Oxi1 null mutant in WS2 and its background WS2 in response to aphid feeding. Total RNA was extracted from *Arabidopsis* leaves (rosette stage 5-10 leaves) of infested plants with aphid at time points (3, 6, 12, 24 and 48 hours) and non-infested *Arabidopsis* plants control at time points (0, 3 and 48 hours). Expression of genes was quantified relative to the value obtained from 0 (h) samples (aphid-free plants). Each RNA sample was extracted from approximately 200 mg of fresh leaf of Oxi1 and Col-0 Arabidopsis plants. *Arabidopsis* Elongation Factor (EF) gene was used as reference control. Two ways ANOVA was used to generate the p values. Significant differences in gene expression were indicated with * p<0.05, ** p< 0.01, *** p<0.001. Data are means ±SE (*n* =9).

6.3.10. Relative expression of β-1,3-glucanase genes Gns1, 2, 3 and 5 in Oxi1 null mutant in WS2 (Wisconsin) and its background WS2 in response to aphid feeding

β-1,3-glucanase genes Gns1, 3 and 5 genes were not expressed in both Oxi1 null mutant in WS2 and WS2 background in response to aphid feeding. Relative

expression of β-1,3-glucanase gene Gns2 was expressed to 1.2 and 1.6 fold in WS2 (wild type) and Oxi1 null mutant in WS2 background in non-infested plants (control) after 3 hours. Three hours after the start of aphid feeding the expression level of Gsn2 in WS2 increased to 3.5 fold compared to 1.8 fold in Oxi1 null mutant in WS2 background. As the aphids spent more time feeding on the plants the relative expression of Gns2 significantly increased to a maximum of 14.2 fold in Oxi1 null mutant in WS2 compared to 1.1 fold in WS2, 6 hours post aphid feeding. After 12 hours, Gns2 was expressed at 3.2 fold in WS2 and at 4.1 in the Oxi1 null mutant in WS2 background. Then, 24 hours post aphid feeding, relative expression of Gns2 was reduced to 7.6 fold in Oxi1 null mutant in WS2 compared to 1.3 fold in WS2. Subsequently after 48 hours, expression of Gns2 was down-regulated in WS2; however it was expressed to 2.9 fold in Oxi1 null mutant in WS2 background, and 48 hours post aphid feeding. Expression of Gns2 in the non-infested control experimental plants was 1.2 and 1.5 fold in WS2 and Oxi1 mutant in the WS2 background respectively (Fig 6.17).

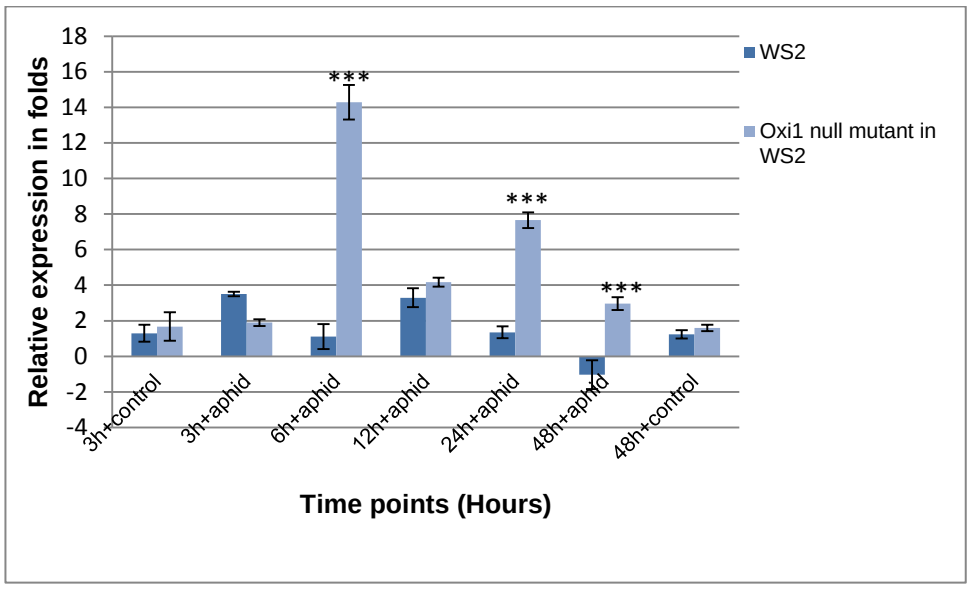

Fig 6.17. Relative expression level of β-1,3-glucanase gene (Gns2) in Arabidopsis OXI1 in Wisconsin(OXI1 in WS) and Wisconsin (WS) wild type at different time points in response to stressed and non-stressed plants with aphid (*Myzus persicae*). Results of real-time PCR represent the expression analysis of β-1,3-glucanase gene Gns2 in Oxi1 null mutant in WS2 and its background WS2 in response to aphid feeding. Total RNA was extracted from *Arabidopsis* leaves (rosette stage 5-10 leaves) of infested plants with aphid at time points (3, 6, 12, 24 and 48 hours) and non-infested *Arabidopsis* plants control at time points (0, 3 and 48 hours). Expression of genes was quantified relative to the value obtained from 0 (h) samples

(aphid-free plants). Each RNA sample was extracted from approximately 200 mg of fresh leaf of Oxi1 and Col-0 *Arabidopsis* plants. Arabidopsis Elongation Factor (EF) gene was used as reference control. Two ways ANOVA was used to generate the p values. Significant differences in gene expression were indicated with * $p<0.05$, ** $p< 0.01$, *** $p<0.001$. Data are means ±SE (n =9).

6.4 Discussion

The plant–aphid interaction is a dynamic system subjected to continual variation and change (Mello and Silva-Filho 2002). In this system, aphids evolve and develop many strategies to overcome plant defence barriers which allow them to feed, grow and reproduce on their host plants. The first activity of aphids is to determine if a plant is suitable for them or not. After selecting a plant, aphids ingest phloem sap from their hosts through narrow piercing-sucking mouthparts (stylets). During probing, aphids' stylets transiently puncture the epidermis, mesophyll, and parenchyma cells to gain access to the phloem, and this mechanical damage may influence plant responses to infestation (Tjallingii and Hogen Esch, 1993). Throughout evolutionary adaptation plants have developed different mechanisms to reduce aphid attack. It has been suggested that two different processes are involved in the elicitation of plant defence (Smith and Boyko, 2007). One process involves the gene-for-gene recognition of aphid-derived elicitors by plant resistance genes, followed by the activation of aphid resistance and defence responses. The second process involves the recognition of aphid-inflicted tissue damage, which leads to changes in plant chemistry, followed by the production of signalling molecules that trigger a general stress response, similar to the basal plant defence to pathogens.

The activation of signalling pathways in response to phloem-feeding aphids alters gene expression, which in turn leads to changes in the molecular composition inside the cell. DNA sequencing analyses have indicated that encoded proteins of these differentially regulated genes function in direct defence, defence signalling, oxidative burst, secondary metabolism, cell maintenance and photosynthesis (Zhu-Salzman et al., 2004).

Reactive oxygen species (ROS), mainly hydrogen peroxide (H_2O_2), are molecules of defence signalling pathways with known involvement in the activation of plant response to aphid attack (De Ilarduya et al., 2003). H_2O_2 is a relatively stable ROS, being only mildly reactive and electrically neutral. H_2O_2 is able to pass through cell membranes and reach cell locations remote from the initial site of formation, thus providing a mechanism for mounting a systemic response. Plant cells produce H_2O_2 in response to various biotic factors (Wojtaszek, 1997), thus H_2O_2 production is a general response of plants to stress conditions and not specific to plants infested by aphids. The H_2O_2

released by the plant in response to infestation by aphids is of significant importance and concerns its involvement in signal transmission, since it is easily transported over considerable distances. H_2O_2 activates defence genes. For example, peroxidases are involved in the stimulation of cell wall reorganization and induce cross-linking of proline-rich plant cell wall proteins. Additionally, a high H_2O_2 level could have a toxic action against aphids, causing damage. Results reported by Kusnierczyk et al. (2008) indicated that the involvement of reactive oxygen species (ROS) and calcium in early signalling in *Arabidopsis thaliana* after infestation by the aphids, *Brevicoryne brassicae*. Hydrogen peroxide activates the protein phosphorylation cascade, which modulates gene expression in response to external stimuli. This cascade involves subsequent phosphorylation events of MAPK, the last of which results in translocation to the nucleus and activation of transcription factors. The expression of five genes coding for MAPKs (MKK1, MKK2, MKK4, MKK9 and MKK11) was positively regulated (Kusnierczyk et al., 2008)

The data presented shows that Oxi1 mutants reduced and delay the accumulation of the aphids, Gns1, Gns2, and Gns3 mutants were able to resistant larger population densities aphid and the plants survived for longer time compared to Col-0, the wild type background. The main reason for that is β-1,3-glucanase Gns1, Gns2, Gns3 and Gns5 were not expressed at different time points in response to aphid feeding. Also, Callose synthase GSL5 was induced in response to aphid feeding. Callose synthase GSL5 is required for wound and papillary callose formation (Jacobs et al., 2003). Oxi1 is a serine/ threonine kinase required for oxidative burst-mediated signalling in *Arabidopsis* roots. Also, it is induced in response to wide range of Reactive Oxygen species especially hydrogen peroxide (Rentel et al., 2004) and it is required for full activation of MAPKs genes especially MAPK3 and MAPK6. MAPKs genes are also required for root development and plant defence against bacterial and fungal pathogens. Full activation of MAPK cascades trigger the calcium pathway (Asal et al., 2002) which in turn, stimulate callose synthase followed by callose production. As a result of callose production *Arabidopsis* plants become resistant in response to aphid. Oxi1 is playing an essential role of signal transduction pathway connecting oxidative burst signals to different downstream responses (Peterson et al., 2009). Induction of Oxi1 expression is the result of

ROS accumulation through the AtrbohD NADPH-oxidase mechanism and it is also improving signal transduction of ROS plus catalase activity (Kwak et al., 2003).

Oxi1 mutant has unique features like, continuous Induction of MAPKs kinase genes, early response ROS accumulation and signal transduction, catalase activity, calcium pathway, and callose synthase. These features allow Oxi1 mutants to tolerate and cope with high population of aphid plus delaying growth rate of both aphid nymphs and adults. However, the Oxi1 null mutant in WS2 and WS2 background showed up-regulation of β-1,3-glucanase Gns2 and down-regulation of Callose synthase GSL5 which may result to plant susceptibility in response to aphid feeding.

On the other hand, the Columbian (Col-0) background Oxi1 did not manage to survive or cope with high populations of aphids. β-1,3-glucanase gene Gns2 was up-regulated to the highest level in Col-0 in response to aphid feeding. Also low expression of GSL5 at different time points and down-regulation 48 hours post aphid feeding had a major effect in plant defence. The possible reason for plant susceptibility is that the insect manage to induce β-1,3-glucanase gene Gns2 in the plant to hydrolyse callose and use the plant nutrient material to reproduce. In Arabidopsis, PR1 and β-1,3-glucanase Gns2 mRNA have been induced in response to Aphid *(Myzus persicae)* feeding and those two induced genes are connected with Salicylic Acid(SA). The transcription level of PR1 and Gns2 was 10 and 23 fold respectively (Moran and Thompson, 2001).

β-1,3-glucanase mutants Gns1, Gns2 and Gns3 have shown resistance to aphid feeding with plants delaying aphid development, being able to sustain larger insect populations and surviving for longer time compared to Col-0 wild type. The possible reason could be knock out or gene silencing for one or all β-1,3-glucanase genes Gns1, Gns2, Gns3 and Gns5 may result in plant resistant. So that, aphid will not be able to induce β-1,3-glucanase genes in the plant to hydrolyse callose and re-attack the plant.

Camta3-1 and Camta3-2 were susceptible to aphid infestation and they died quickly compared to Col-0 background. Camta's transcriptional factor activity is induced via Ca^{+2}/CAM (Fig 6.24) with Ca^{+2} also playing an important role in plant growth and development. Camta3 mutant showed an enhancement of the resistant level against bacterial and fungal pathogen and this is related to the

induction of PR1 genes plus high accumulation of Reactive Oxygen Species (ROS) especially H_2O_2 (Bouche., et al 2003; Balague et al., 2003; Ma and Berkowitz., 2007).

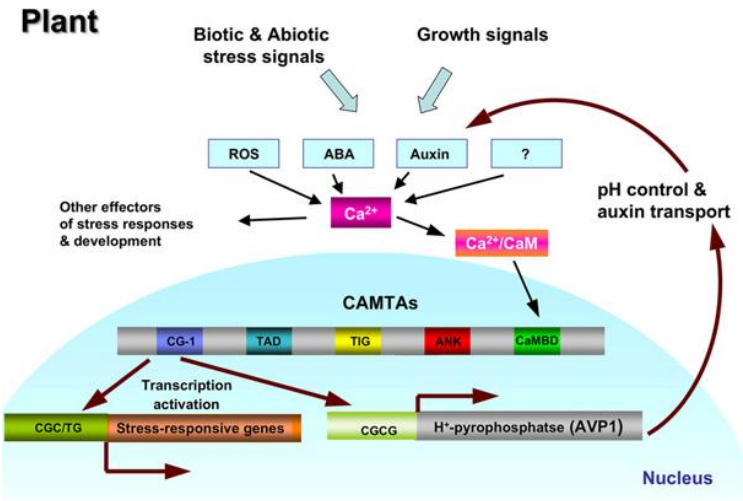

Fig 6.18. A model for Camta -mediated signalling in plants (Finkler et al., 2007). In plants, *Arabidopsis* Camta3 (AtSR1) contains an NLS in the CG-1 domain. However, in rice Camta (OsCBT) two NLS sequences were found, one in the N-terminal CG-1 domain and another in the C-terminal part. Further experimental evidence revealed the occurrence of other functional domains including a transcription activation domain (TAD) in the *Arabidopsis* AtCamta1. Finally, proteins resembling Camtas were originally reported only in multicellular eukaryotes; however bioinformatics analysis of more recent databases revealed CAMTA- like proteins also in some unicellular eukaryotes including the ciliates Paramecium tetraurelia and *Tetrahymena thermophila*.

By contrast, with respect to insects, Camta3-1 and Camta3-2 mutants have shown susceptibility against aphid and they died quickly in comparison to Col-0. Camta3-1 and 3-2 mutants in response to insect as a result to the accumulation of ROS in high level in the mutant plus the induction of ROS in the mutants in response to aphid, decreasing ROS signal transduction may cause cell toxicity which lead to cell death in the end. Another possibility could be the induction of PR genes (e.g., β-1,3- glucanase genes) in Camta3-1, 3-2 mutants cause callose hydrolyses in plants and result in increasing plant susceptibility in response to aphid feeding. Furthermore increasing ROS especially H_2O_2 plus decreasing catalase activity may lead to H_2O_2 accumulation in the plant cell and cause cell death (Finkler et al., 2007).

Chapter 7. Hydrogen peroxide enhances resistance of *Arabidopsis thaliana* to the aphid (*Myzus persicae)*

Abstract

Hydrogen peroxide functions as a key signalling molecule in plants where it plays a dual role**:** at low levels it acts as a messenger molecule involved in mediating signalling pathways, which induces tolerance against various biotic and abiotic stresses, and at high concentrations it coordinates programmed cell death. Wild type *Arabidopsis* plants (Col-0) treated with 10mM H_2O_2 exhibited enhanced resistance in response to aphid infestation with a significant decrease in both nymphs and adults ($p<0.001$) with a delay in the rate of development of 2 days compared to control plants. In contrast the *Arabidopsis* mutant Oxi1 treated with 10mM H_2O_2 exhibited significantly ($p<0.001$) enhanced susceptibility in response to aphid infestation; furthermore all the Oxi1 plants died more quickly compared to control plants. Callose is thought to play a key role in host plant resistance to pests and pathogens; callose synthases are involved in the synthesis of callose whilst β-1, 3-glucanase is responsible for its breakdown. The results showed that in the wild type plants transcript levels for callose synthase 1 (GSL1) were up regulated by 20.7-fold 48h after treatment with 10mM H_2O_2 whilst callose synthase 5 (GSL5) was also significantly upregulated (8.3 fold), but only in plants exposed to the dual stress after 48h (H_2O_2+aphid). In contrast, in the Oxi1 mutant line GSL5 was down regulated in response to the dual stress at both time points (24h and 48h). In wild type *Arabidopsis* plants transcript levels for β-1, 3-glucanase gene 2 (Gns2) were down regulated (26.1-fold) in response to the dual stress, but only after 48h. In the Oxi1 Arabidopsis mutant, Gns2 was upregulated after both 24h and 48h in response to the dual stress; H_2O_2 alone had no significant effects on Gns2 or 5. In conclusion, wild type *Arabidopsis* plants treated with hydrogen peroxide increased resistance to biotic stress, whilst H_2O_2 treatment of the Oxi1 mutants increased susceptibility. Callose synthase 5 (GSL5) is a key gene for plant resistance in response to insect attack, whilst the β-1,3-glucanase gene 2 (Gns2) plays an important role in plant susceptibility in response to aphid infestation.

7.1. Introduction

Arabidopsis mutants Oxi1, Gns1, Gns2, and Gns3 showed resistance to aphid feeding. Callose synthase gene GSL5 expressed to the highest level in Oxi1 mutant compared to the Col-0 background. β-1,3-glucanase genes Gns1, gns2, Gns3 and gns5 were not expressed at different time points in Oxi1 mutant in response to aphid feeding. However, β-1,3-glucanase genes Gns2 was expressed to the highest level in Col-0. *Arabidopsis* mutants Camat3-1, camta3-2 and Oxi1 in WS2 showed susceptibility to aphid feeding. The expression level of callose synthase genes (GSL1 and GSL5) and β-1,3-glucanase genes (Gns1, gns2, Gns3 and Gns5) were completely different in the null mutant Oxi1 in WS2 compared to its background WS2.

Exogenous application of hydrogen peroxide in the *Arabidopsis* background Col-0 could mimic the Oxi1 mutant and improve the resistance level of Col-0 in response to aphid feeding.

7.1.1. Abiotic and biotic stress response crosstalk in plants

Plants are continuously challenged with various biotic and abiotic stresses. To cope with the diverse types of abiotic and biotic stresses plants have evolved different defence mechanisms. Various pathways of complicated cellular signalling are activated to enable organisms to respond to stress (Fraire-Velázquez et al., 2011). In plants, detecting biotic and abiotic stress induces different signalling cascades that activate production of reactive oxygen species (ROS), kinase cascades, ion channels accumulation of hormones such as salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and abscisic acid (ABA) (Fraire-Velázquez et al., 2011) leading to an overall defence response. The expression levels of different defence genes vary at different growth stages of the plant, different insects and different environmental factors.

7.1.2. Calcium-dependent protein kinases (CDPKs) and MAPKs crosstalk in response to abiotic and biotic stress

The transitory changes in cytosolic calcium content detected under biotic or abiotic stress conditions require diverse calcium sensors. Calcium-dependent protein kinases (CDPKs) are the largest and most well defined group of calcium sensors; they have many different substrates. Acarboxy terminal calmodulin-like

domain containing EF-hand calcium-binding sites and N-terminal protein kinase domain sensors are members of CDPKs (Cheng et al., 2002). In *Arabidopsis*, 34 CDPKs have been described encoded by different genes, but only a few substrates for these enzymes have been identified (Uno et al., 2009). MAPKs are a large family of serine/threonine protein kinases. They control different cellular activities, for instance gene expression, mitosis, differentiation, proliferation, and cell survival/apoptosis and respond to extracellular stimuli. MAPKs work downstream of sensors/receptors and transfer the signals from extracellular stimuli into intracellular responses plus amplification of the transmuted signals. MAPKs act as the final product of protein cascade and transduce extracellular stimuli into transcriptional responses in the nucleus (Wurzinger et al., 2011). MAPK signalling pathways are generated from dynamic protein complexes involving three cascades of MAPK kinases. In general these cascades involve three functionally linked protein kinases, a MAPK kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAPK. In MAPK modules, the MAPKKK, serine/threonine kinase, phosphorylates and activates MAPKKs which, in turn, perform T and Y dual phosphorylation of MAPKs. In *Arabidopsis*, there are 20 MPKs, 10 MPKKs, and 80 MPKKKs (Colcombet and Hirt, 2008). In eukaryotes, MAPKs and CDPKs signalling cascades are widely induced in response to biotic and abiotic stresses. In a number of pathosystems, MAPKs and CDPKs signalling cascades have been shown to be stimulated in response to the same stressing factor proposing a crosstalk between both of them (Wurzinger et al., 2011). On the other hand a specific CDPK or MAPK could be stimulated in response to diverse biotic and abiotic stresses. MPK3 and MPK6 play a role in the mitogen-activated protein kinase cascade. In *Arabidopsis,* several studies demonstrate that MPK3 and MAPK6 are activated in response to biotic and abiotic stresses (Gerold et al., 2009). MKK2 plays an important role as a key regulator for cold- and salt-stress response (Teige et al., 2004) and has also been shown to be involved in *Arabidopsis* resistance in response to *Pseudomonas syringae* (Brader et al., 2007). On the other hand CDPKs, CPK6 and CPK3 are involved in the control of ABA regulation of guard cells and stomatal closure (Mori et al., 2006). CDPKs also play major role in adaptation to abiotic stress (Dat et al., 2010). In plants, calcium-dependent enzymes and the MAPKs are involved in defence signalling pathways.

7.1.3. Hydrogen peroxide as an important product of Reactive Oxygen Species (ROS) and regulatory agent in plants

Reactive Oxygen Species (ROS) are early response molecules to different stimuli, and control many different processes in plants. ROS, such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) are regularly produced from normal cellular metabolic process as well as photosynthesis and respiratory electron flow (Halliwell and Gutteridge, 1989). Hydrogen peroxide (H_2O_2) production is a key response molecule responsible for the organisation of numerous processes as well as cell wall rigidification, transcription of defence-related genes and hypersensitive (programmed) cell death (Levine et al., 1994; Neill et al., 2002). Hydrogen peroxide functions as a signalling molecule and is up regulated in response to various stimuli indicating that it is a key factor for tolerance induction in stressed plants (Neill et al., 2002). However hydrogen peroxide is also known to be a toxic cellular metabolite.

7.1.4. Hydrogen peroxide turnover in the plant cell

Hydrogen peroxide is constantly generated from a number of sources during normal metabolism. It is generated and enhanced in response to different stimuli via the Mehler reaction in chloroplasts, electron transport in mitochondria and photorespiration in peroxisomes. Hydrogen peroxide is also enhanced in response to both biotic and abiotic stress via enzymatic sources, for instance plasma membrane localized NADPH oxidases (RBOH), or cell wall peroxidases (Sagi and Fluhr, 2001; Torres et al., 2002; Bolwell at al., 2002). H_2O_2 distributes freely through peroxiporin membrane channels (Henzler and Steudle, 2000). Cellular levels of hydrogen peroxide are estimated by H_2O_2 production rates and metabolism via catalase and the ubiquitous ascorbate-glutathione cycle, which includes ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Noctor and Foyer, 1998) (Fig 7.1).

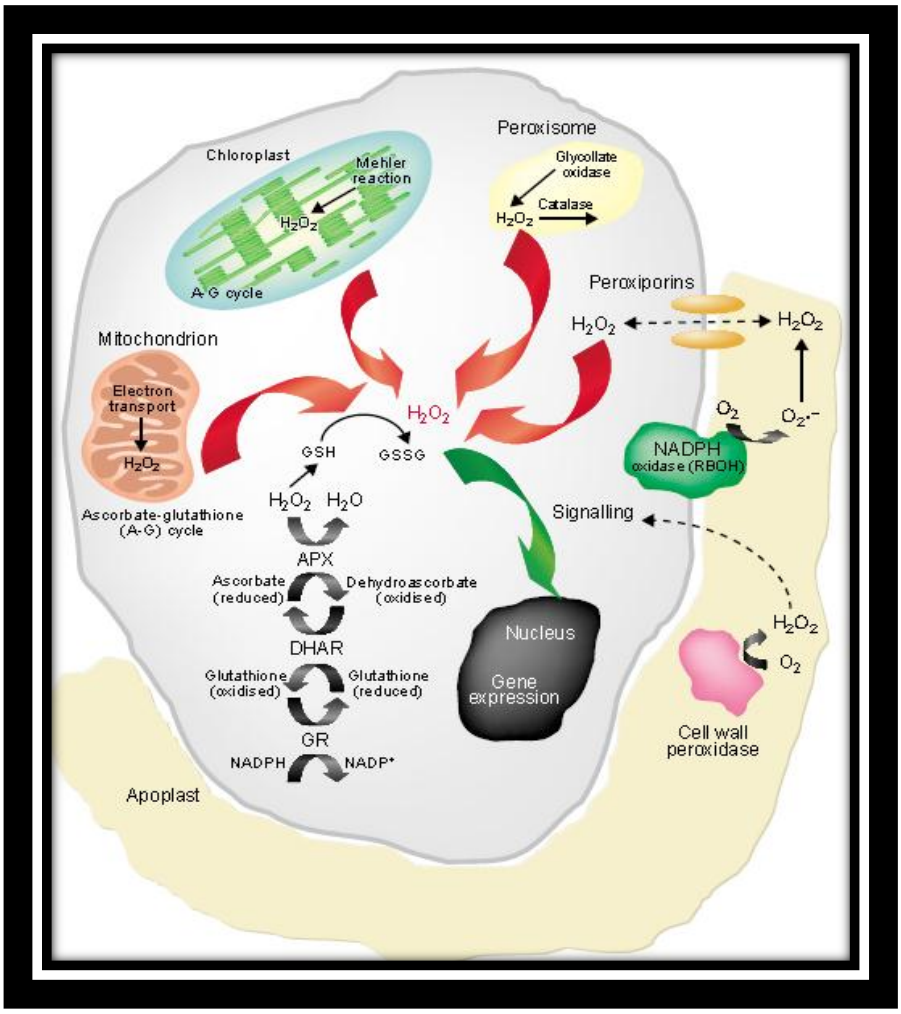

Fig 7.1. Hydrogen peroxide turnover in the plant cell (Noctor and Foyer, 1998). H_2O_2 is generated in normal metabolism via the Mehler reaction in chloroplasts, electron transport in mitochondria and photorespiration in peroxisomes. Peroxisomes may also contain other systems that generate H_2O_2. Abiotic and biotic stresses enhance H_2O_2 generation via these routes and also via enzymatic sources such as plasma-membrane-localised NADPH oxidases (RBOH or cell wall peroxidases. H_2O_2 diffuses freely, perhaps facilitated by movement through peroxiporin membrane channels. Cellular H_2O_2 levels are determined by the rates of H_2O_2 production and metabolism via catalase and the ubiquitous ascorbate-glutathione cycle (A-G cycle, which involves ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). H_2O_2 also reacts with glutathione to convert it from its reduced state (GSH) to its oxidised state (GSSG).

The role of hydrogen peroxide production varies from one plant to another and is also influenced by the environment. For example in *Arabidopsis thaliana,* it was found that hydrogen proxide have risen from 60 µM – 7 mM compared to 1 – 2 mM in maize and rice (Veljovic-Jovanovic, 2001; Karpinski, 1999; Jiang and Zhang, 2001; Lin and Kao, 2001). However, high concentrations of hydrogen peroxide can be controlled by an efficient antioxidant system (Noctor and Foyer, 1998; Corpas et al., 2001). Different stresses can disturb the

balance of hydrogen peroxide in the plant cells and this may cause an increase in H_2O_2 levels followed by induction of numerous of signalling responses. Abiotic stresses such as dehydration, low and high temperatures, and excess irradiation can perturb the hydrogen peroxide balance and cause an increase in its level in the cell. These high levels initiate signalling responses for enzyme activation, gene expression, programmed cell death (PCD) and cellular damage. An oxidative burst with rapid H_2O_2 synthesis is a common response to different stimuli, for instance pathogens, elicitors, wounding, heat, ultra-violet light and ozone (Bolwell, 1999; Rao and Davis, 2001). In *Arabidopsis*, knockout experiments have shown that *AtrbohD* and *AtrbohF* genes (encoding NADPH oxidase) are needed for producing H_2O_2 in response to bacterial and fungal infection; although a number of NADPH enzyme homologues have been identified in plant genomes, more work is still required to confirm its activity. Activity of the NADPH oxidase enzyme is directly activated via calcium Ca^{2+} binding. In plants, there are a large number of NADPH oxidase gene families and they all differ in their biological activity (Torres et al., 2002). Rops (Rho-like small G proteins) play an important role in regulating H_2O_2 production via NADPH oxidase (Baxter et al., 2002). Previous studies have demonstrated that Rops signalling is involved in the generation of H_2O_2 (Yang, 2002). Oxygen deprivation was found to induce Rop signalling that in turn stimulated NADPH oxidase. Xanthine oxidase, amine oxidase and cell wall peroxidase are considered as potential enzymatic sources of H_2O_2 (Corpas et al., 2001; Blee et al., 2001; Bolwell et al., 2002). In *Arabidopsis thaliana*, a peroxidase-mediated oxidative burst has been verified in response to fungal elicitors. For example, *Arabidopsis* plants transformed with an antisense peroxidase construct have shown hypersensitivity in response to both fungal and bacterial infection (Bolwell et al., 2002). Furthermore, a number of studies have revealed the effects of high and low expression of antioxidant enzymes on cell physiology (Mittler et al., 1999). For instance, the reduction of antioxidant activity causes an increase in hydrogen peroxide that stimulates gibberellin (GA)-induced PCD in the barley aleurone (Fath et al., 2001). A high antioxidant status of cells possibly inhibits H_2O_2 transport through the cell. As a result, responses to H_2O_2 are probably limited to micro domains (i.e. 'H_2O_2 hot-spots') within the cell.

7.1.5. Callose deposition in response to stress

Callose plays major roles during plant growth and development. In addition, callose deposition occurs at the plasma membrane and cell wall interface in response to a wide range of wound stresses. Callose deposition has been shown to be induced within minutes in response to mechanical damage, chemical or ultrasonic treatments. Callose deposition is also induced by plasmolysis in response to physiological or biotic stress, temperature extremes and microbial infection (Stone et al., 1992). Mutants for callose synthase (GSL5/PMR4/CalS12) confirmed their role for callose synthesis in the sporophytic tissue in response to wounding and/or pathogen attack, since these mutants failed to synthesize callose on the fungal papillae. Surprisingly, reduction or depletion of callose in *gsl5* mutants makes the plants more resistant to pathogens, not more susceptible. Callose has a negative effect on plant defence in response to pathogen attack as it is thought that it delays the plants' defence system against pathogen attack. It is therefore possible that *gsl5* mutant activates plant defence systems (Jacobs et al., 2003). The other possibility is that the lack of callose in GSL5/PMR4/ CalS12 mutants may enhance salicylic acid (SA) signalling, which results in increased resistance to pathogens (Nishimura et al., 2003). Recently two independent laboratories reported that callose synthase 7 (Csl7 or GSL7) was responsible for callose deposition in the sieve plates. Mutants of callose synthase 7 (GSL7) were unable produce callose in the sieve pores in response to different stresses (Bo and Zonglie, 2011).

The overall aim of the present study was to investigate the role of hydrogen peroxide in inducing callose synthase genes 1 and 5 via triggering the calcium pathway. In this study wild type *Arabidopsis thaliana* seeds (cultivar Col-0) were treated with 10mM hydrogen peroxide and then infested with aphid (*Myzus persicae*). Aphid performance was then measured on the treated and non-treated plants and the transcript levels for gene encoding callose synthase 1 and 5 genes and β-1,3-glucanase 1,2,3 and 5 genes were measured using QRT-PCR.

7.2. Material and methods

7.2.1. Plant Materials and Growth Conditions

Arabidopsis thaliana plants were grown in John Innes compost number 2 in a controlled environment chamber under a 16/8 h light/dark cycle at 21°C, 55% relative humidity. Col-0 wild type and Oxi1 mutant seeds were kindly supplied as a gift from Prof. Marc Knight (Durham University).

7.2.2. Insect bioassays

The aphid *Myzus persicae* was reared on *Arabidopsis* Col-0 before starting the bioassays. *Arabidopsis thaliana* plants were grown as stated in (7.2.1). All bioassays were carried out on plants that were between 25-30 days old (i.e. rosette stage 5-10 leaves). The bioassay were performed by adding two aphid adults in the upper side of the *Arabidopsis* leaf and when the adults started producing nymphs, all insects were taken away and two nymphs were kept in the leaf to start the bioassay. The starting time point was day one (zero time point) with 2 adults. The two varieties tested were the Col-0 wild type and the Oxi1 mutant in the same genetic background. Three plants were used per treatment.

The treatments are summarised in Table 7.1

Table 7.1. Summary of treatments with *Arabidopsis thaliana* wild type Col-0 and the Oxi1 mutant.

Plant	Treatment	Time point
Col-0 wild type	Water as a contact spray	0h
Col-0 wild type	10mM H_2O_2 as contact spray and systemic application	24h
Col-0 wild type	Aphid infestation post H_2O_2 treatment	24h
Col-0 wild type	10mM H_2O_2 as contact spray and systemic application	48h
Col-0 wild type	Aphid infestation post H_2O_2 treatment	48h
Oxi1 mutant	Water as a contact spray	0h
Oxi1 mutant	10mM H_2O_2 as contact spray and systemic application	24h
Oxi1 mutant	Aphid infestation post H_2O_2 treatment	24h
Oxi1 mutant	10mM H_2O_2 as contact spray and systemic application	48h
Oxi1 mutant	Aphid infestation post H_2O_2 treatment	48h

7.2.3. RNA extraction

RNA was exctracted according to the procedures outlined in section 6.2.2.

7.2.4. Primer Design

QRT-PCR primers were designed according to the procedures outlined in section 6.2.4.

7.2.5. Quantitative real time PCR (QRT-PCR)

QRT-PCR was performed according to the procedures outlined in section 6.2.5.

7.2.6. QRT-PCR calculations

QRT-PCR results were calculated according to the procedures outlined in section 6.2.6.

7.2.7. Statistical methods

Repeated measures ANOVA were conducted to generate the p value for the bioassay. Two way ANOVA with replication was used to test the p value of QRT-PCR results followed by Tukey test.*$p<0.05$, **$p<0.01$ and ***$p<0.001$.

7.3. Results

For all bioassays, each plant was originally inoculated with 2 adults; the following day the adults and all nymphs except for two were removed. These two nymphs were allowed to develop to adulthood and the number of progeny produced was recorded (Fig 7.2a; 3a). The number of nymphs developing to adulthood was also recorded (Fig 7.2b; 3b).

7.3.1. Effects of hydrogen peroxide on tolerance of *Arabidopsis* wild type Col-0 to aphid infestation

The results also show that for the wild type plants treated with water the number of nymphs peaked at 17 days with 120 nymphs (Fig 7.2a). Subsequently the numbers of nymphs decreased gradually with none after 37 days; at this stage they had either reached adulthood or had died. However, plants treated with 10mM H_2O_2, caused a significant ($p<0.001$) reduction in the number of nymphs, with the maximum number (77) at 19 days, representing a shift of 2 days (Fig 3b); by 35 days there were no nymphs remaining.

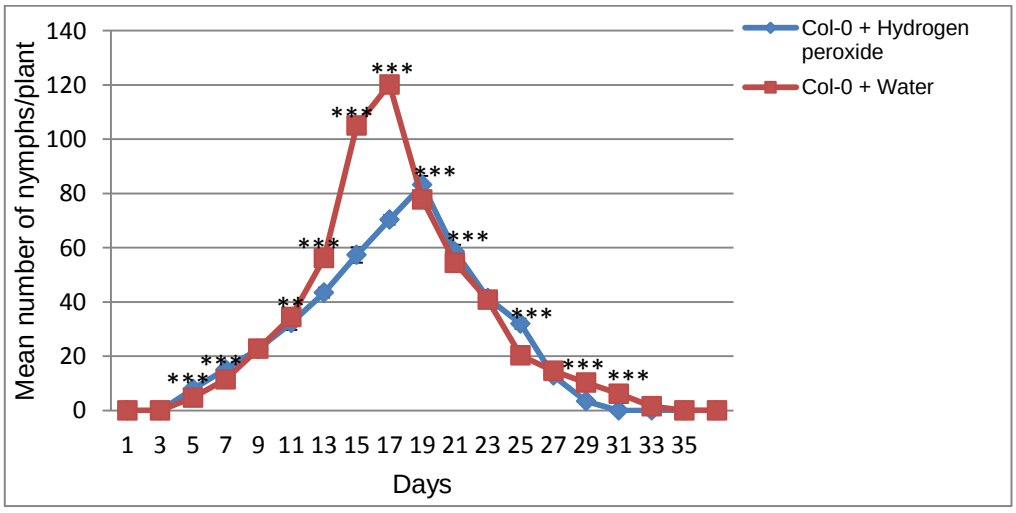

Fig 7.2a. Bioassay of aphid nymphs (*Myzus persicae)* on *Arabidopsis* wild type Col-0 treated with 10mM hydrogen peroxide. Col-0 control plants were treated with equal volumes of distilled water. The data represents mean number of nymphs in treated *Arabidopsis* wild type Col-0 and control plants. Significant differences in the number of nymphs were indicated with * $p<0.05$, ** $p< 0.01$, *** $p<0.001$. Data are means ±SE (n =15). Stuednt's t-test was used to generate the p values.

The number of adult aphids peaked at 19 days with 114 aphids on *Arabidopsis* wild type Col-0 plants treated with water (Fig 7.2b). Thereafter the numbers decreased rapidly with no survivors after 37 days. However, in plants that had been treated with 10mM H_2O_2, the number of adult aphids was significantly ($p< 0.01$) lower, this being reduced to 87, again with a shift in the peak of two days (Fig 7.2b).

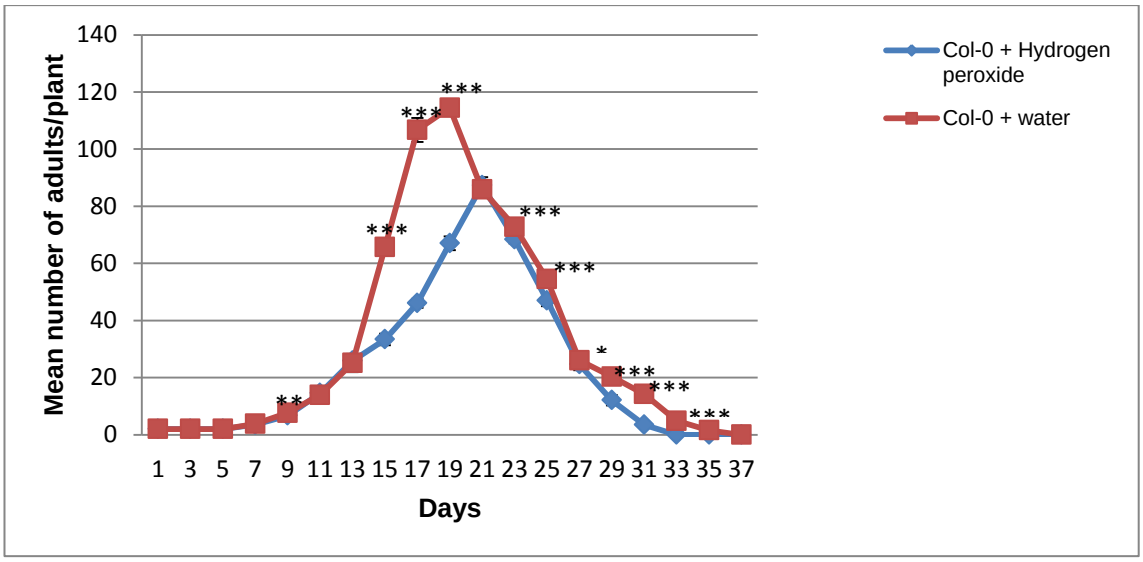

Fig 7.2b. Bioassay of adult aphids (*Myzus persicae)* on *Arabidopsis* wild type Col-0 treated with 10mM hydrogen peroxide. Col-0 control plants were treated with equal volumes of distilled water. The data represents the mean number of adult aphids in treated Col-0 *Arabidopsis* wild type and control plants. Significant differences in the number adults was indicated with * $p<0.05$, ** $p< 0.01$, *** $p<0.001$. Data are means ±SE (*n* =15). Student's t-test was conducted to generate the p values.

7.3.2. Effects of hydrogen peroxide on tolerance of *Arabidopsis* Oxi1 mutant to aphid infestation

The results show that when *Arabidopsis* mutant Oxi1 plants were treated with 10mM H_2O_2 (both when applied as a spray and systemically) the mean number of nymphs peaked at 21 days with 125 nymphs (Fig 7.3a). Subsequently the numbers decreased gradually with none survivors after 45 days. In the plants treated with water, the number of nymphs was significantly ($p<0.001$) lower, but survival was extended to 51 days.

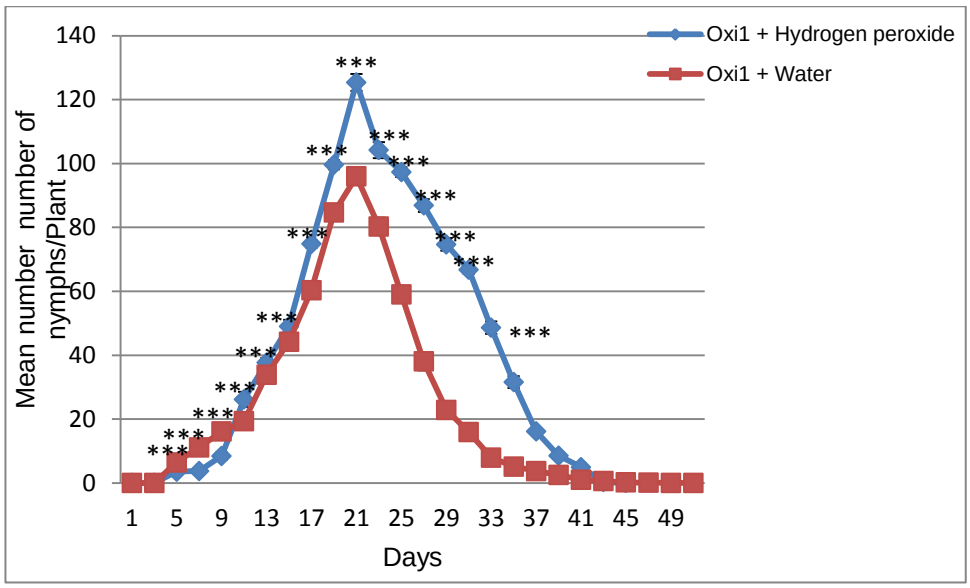

Fig 7.3a. Bioassay of aphid nymphs (*Myzus persicae)* on Oxi1 *Arabidopsis* mutants treated with 10mM hydrogen peroxide. Control Oxi1 plants were treated with equal volumes of distilled water. The data represents the mean number of nymphs in treated and control Oxi1 Arabidopsis mutant plants. Significant differences in the number of nymphs were indicated with * p<0.05, ** p< 0.01, *** p<0.001. Data are means ±SE (*n* =15). Student's t-test was used to generate the *p* values.

The results show that when *Arabidopsis* mutant Oxi1 plants were treated with 10mM H_2O_2 (both when applied as a spray and systemically) the mean number of adult aphids peaked at 23 days with 114 aphids. Thereafter the numbers decreased rapidly with no survivors after 44 days. However, in *Arabidopsis* mutant Oxi1 plants treated with water (control plants), the number of aphids was significantly (p<0. 001) reduced peaking at 93 by day 25 (Fig 7.3b).

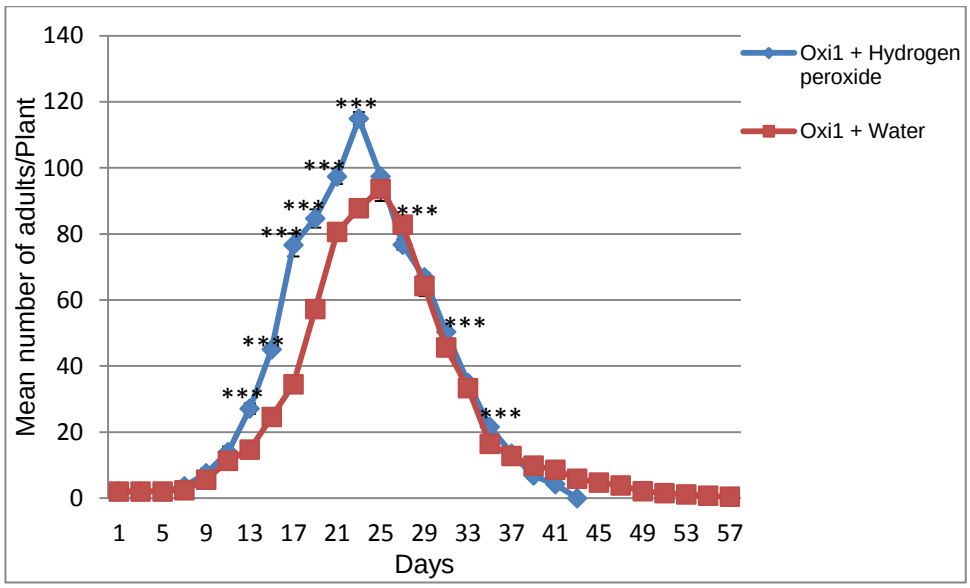

Fig 7.3b. Bioassay of adult aphids (*Myzus persicae)* on Oxi1 *Arabidopsis* mutants treated with 10mM hydrogen peroxide. Control Oxi1 plants were treated with equal volumes of distilled water. The data represents the mean number of adult aphids in treated Oxi1 *Arabidopsis* mutant and control plants. Significant differences in the number adults was indicated with * p<0.05, ** p< 0.01, *** p<0.001. Data are means ±SE (*n* =15). Student's t-test was used to generate the *p* values.

7.3.3. Relative expression of callose synthase genes GSL1 and GSL5 in the wild type *Arabidopsis* Col-0 in response to 10mM H_2O_2 and aphid infestation

The transcript levels for callose synthase gene 1 (GSL1) were shown to increase significantly (p<0.001) in response to the abiotic stresser (H_2O_2) after 48h, compared to the non-stressed control plants (Fig 7.4), representing an increase in transcript levels of 20.7-fold. Plants exposed to the dual stress (i.e. to both H_2O_2 and aphid infestation) also showed a significant increase in transcript levels for GSL1, these being 17.1 and 18.5-fold for 24h and 48h respectively. Interestingly, changes in transcript levels for callose synthase 5 (GSL5) were not significantly different to the controls plants when exposed to H_2O_2 alone or indeed when exposed to the dual stress after 24h. However, after 48h the levels were significantly (p<0.05) increased by 8.3-fold in response to the dual stress.

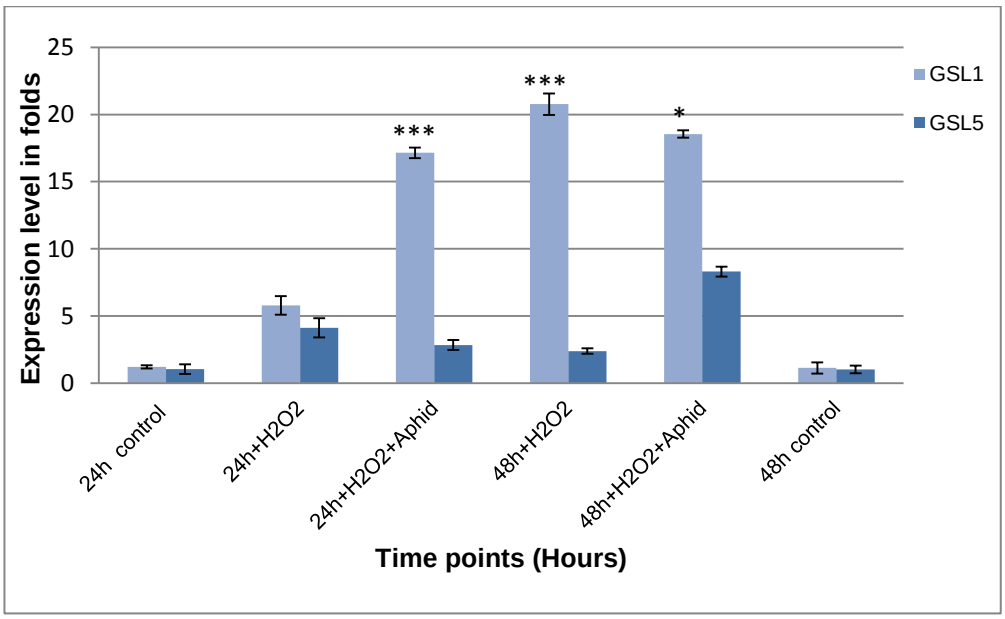

Fig 7.4. Relative expression levels for callose synthase 1 and 5 genes in wild type *Arabidopsis* (Col-0) in response to 10mM H_2O_2 and aphid (*Myzus persicae*) infestation. Results of real-time PCR represent the expression analysis of callose synthase genes GSL1 and GSL5 in *Arabidopsis* wild type (Col-0) in response to 10mM hydrogen peroxide treatment and aphid feeding (Table 7.2). Total RNA was extracted from *Arabidopsis* leaves (rosette stage 5-10 leaves) of plants treated with 10mM H_2O_2 for 24 and 48 hours, and plants treated with 10mM H_2O_2 for 24h and 48h followed by aphid infestation for 24h and 48h and non-treated plants (control) (Table 7.2). Expression of genes was quantified relative to the value obtained from 0 (h) samples (aphid and hydrogen peroxide -free plants). Each RNA sample was extracted from approximately 200 mg of fresh leaf of Col-0 *Arabidopsis* wild type plants. Arabidopsis Elongation Factor (EF) gene was used as reference control. Data are means ±SE (*n* =9). Significant differences in gene expression were indicated with, * $p<0.05$, ** $p< 0.01$, *** $p<0.001$. Two ways ANOVA were used to generate the p values.

7.3.4. Relative expression of β-1,3-glucanase genes Gns1, Gns2, gns3 and Gns5 in the wild type *Arabidopsis* Col-0 in response to 10mM H_2O_2 and aphid infestation

Following aphid and/or H_2O_2 treatment, transcripts were only detected for the β-1,3-glucanase gene 2 (Gns2), with no transcripts detected for Gns1, Gns3, or Gns5, irrespective of the time or treatment. Under conditions of either the abiotic stress i.e. H_2O_2, or biotic stress (aphid infestation), transcripts for Gns2 were down-regulated for both time points. Similarly, these transcripts were also down regulated in plants receiving both stresses compared to the non-stressed plants. These results were highly significant ($p<0.001$) in plants exposed to the dual stress for 48h, were the decrease in expression was 26.1-fold.

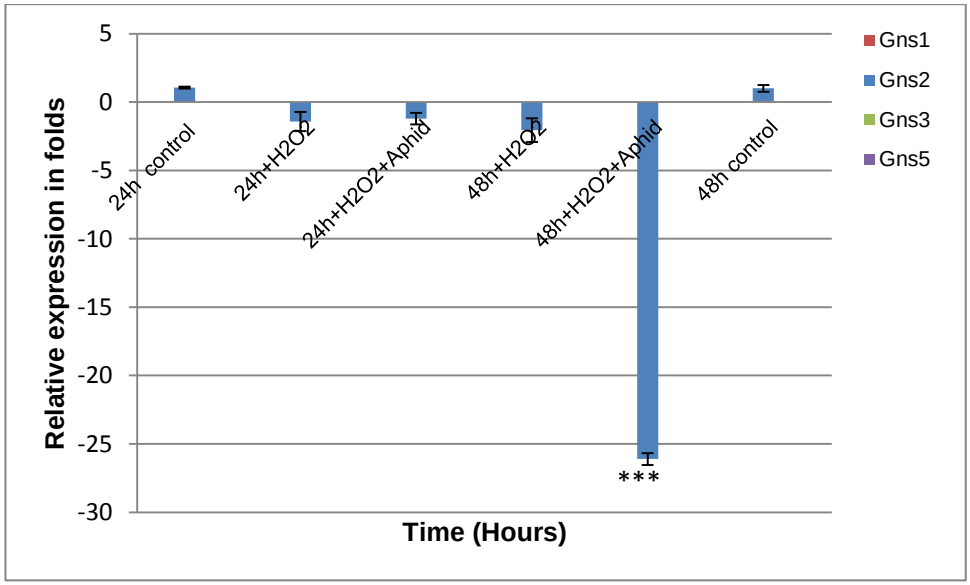

Fig 7.5. Relative expression levels for β-1, 3-glucanase genes (1, 2, 3 and 5) in wild type *Arabidopsis* (Col-0) in response to 10mM H_2O_2 and aphid (*Myzus persicae*) infestation. Results of real-time PCR represent the expression analysis of β-1,3-glucanase genes Gns1, Gns2, Gns3 and Gns5 in wild type (Col-0) in response to 10mM hydrogen peroxide treatment and aphid feeding (Table 7.2). Total RNA was extracted from *Arabidopsis* leaves (5-10 rosette stage) of treated plants with 10mM H_2O_2 for (24 and 48 hours), post treated plants with 10mM H_2O_2 for (24 and 48 hours) followed by aphid infestation for (24 and 48 hours) and non-treated plants (control) (Table 9.2). Expression of genes was quantified relative to the value obtained from 0 (h) samples (aphid and hydrogen peroxide -free plants). Each RNA sample was extracted from approximately 200 mg of fresh leaf of Col-0 *Arabidopsis* wild type plants. *Arabidopsis* Elongation Factor (EF) gene was used as reference control. Data are means ±SE (*n* =9). Significant differences in gene expression were indicated with, * $p<0.05$, ** $p< 0.01$, *** $p<0.001$. Two ways ANOVA was used to generate the p values.

7.3.5. Relative expression of callose synthase genes GSL1 and GSL5 in the *Arabidopsis* mutant Oxi1in response to 10mM H_2O_2 and aphid infestation

The results showed that there were no significant differences between the transcript levels for the two genes callose synthase 1 and 5 (GSL1, GSL5) at either time point (24h or 48h) for control compared to the H_2O_2 treatment (control or 10mM H_2O_2). However, when the plants were exposed to both stresses i.e. 10mM H_2O_2 followed by aphid infestation, the transcript levels for GSL1 were significantly ($p<0.001$) different to those for GSL5. Relative expression levels for GSL1 were up regulated by 1.3 and 1.4-fold at 24h

(24h+10mM H_2O_2+aphid) and 48h (48h+10mM H_2O_2+aphid) respectively, whilst to the transcript levels for GSL 5 were down regulated at both time points (Fig 7.6).

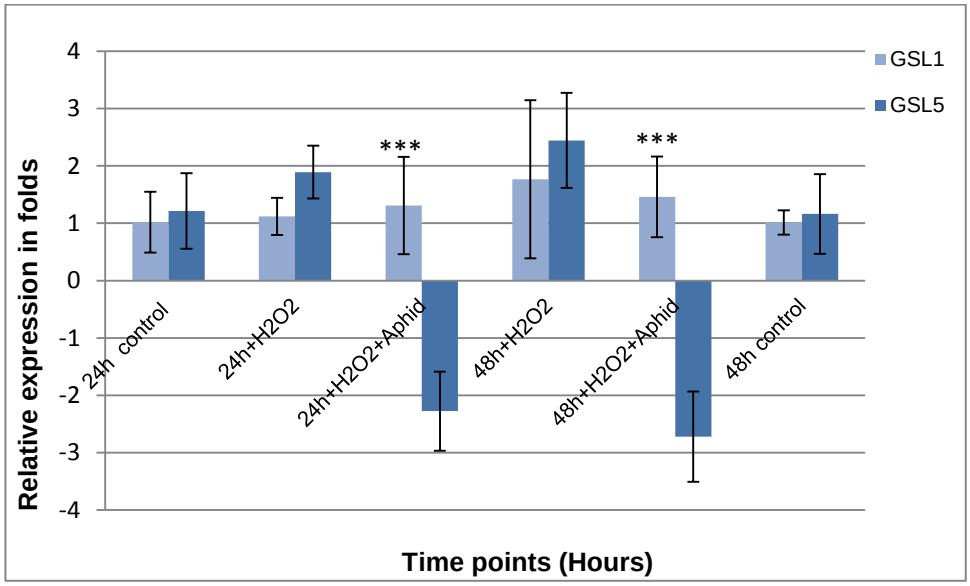

Fig 7.6. Expression levels of callose synthase genes GSL1 and GSL5 in the *Arabidopsis* mutant Oxi1 in response to 10mM H_2O_2 treatment and aphid (*Myzus persicae*) infestation. Results of real-time PCR represent the expression analysis of callose synthase genes GSL1 and GSL5 in the *Arabidopsis* mutant Oxi1 in response to 10mM hydrogen peroxide treatment and aphid feeding (Table 7.2). Total RNA was extracted from *Arabidopsis* leaves (5-10 rosette stage) of treated plants with 10mM H_2O_2 for (24 and 48 hours), post treated plants with 10mM H_2O_2 for (24 and 48 hours) followed by aphid infestation for (24 and 48 hours) and non-treated plants (control) (Table 7.2). Expression of genes was quantified relative to the value obtained from 0 (h) samples (aphid and hydrogen peroxide -free plants). Each RNA sample was extracted from approximately 200 mg of fresh leaf of Oxi1 Arabidopsis mutant plants. Arabidopsis Elongation Factor (EF) gene was used as the reference control. Significant differences in gene expression were indicated with * p<0.05, ** p< 0.01, *** p<0.001. Data are means ±SE (*n* =9). Two ways ANOVA were used to generate the *p* values.

7.3.6. Relative expression of β-1, 3-glucanase genes Gns1, Gns2, Gns3 in the *Arabidopsis* mutant Oxi1 in response to 10mM H_2O_2 and aphid infestation

Following aphid and/or H_2O_2 treatment, transcripts were only detected for the β-1,3-glucanase gene 2 (Gns2), with no transcripts detected for Gns1, Gns3, or Gns5, irrespective of the time or treatment. There was a significant (p<0.001) increase in the transcript levels for β-1,3-glucanase gene 2 of 3.8-fold in plants that were exposed to both stresses (i.e. H_2O_2 and aphid infestation) after 24h,

compared to plants that had received neither stress. This increase in relative expression for Gns2 was further increased to 7.8-fold in plants stressed for 48h (Fig 7.7).

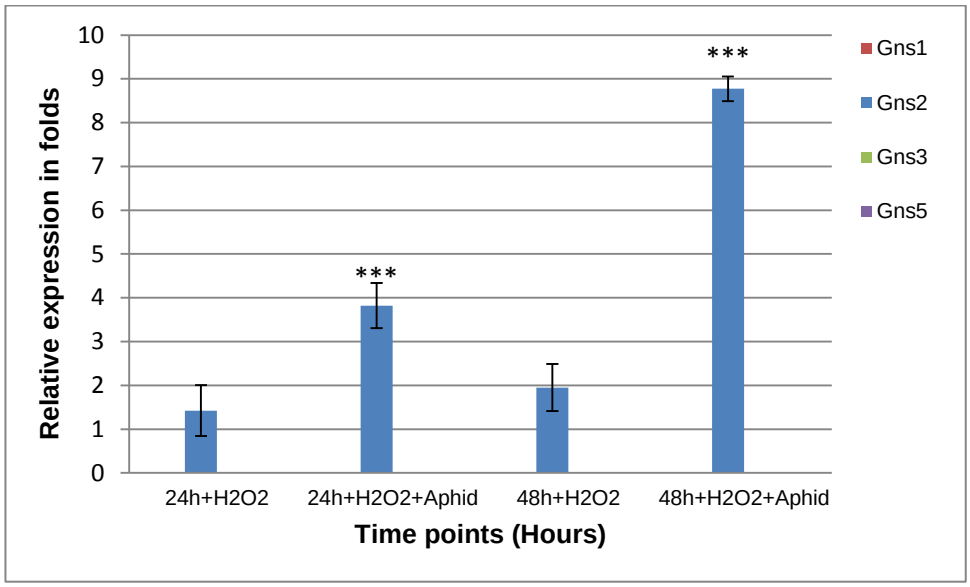

Fig 7.7. Relative expression levels of β-1, 3-glucanase genes Gns1, Gns2, Gns3 and Gns5 in the *Arabidopsis* mutant OxI1 in response to 10mM H_2O_2 and aphid (*Myzus persicae*) infestation. Results of real-time PCR represent the expression analysis of β-1,3-glucanase genes Gns1, Gns2, Gns3 and Gns5 in Oxi1 Arabidopsis mutant in response to 10mM hydrogen peroxide treatment and aphid feeding (Table 7.2). Total RNA was extracted from Arabidopsis leaves (rosette stage 5-10 leaves) of plants treated with 10mM H_2O_2 for 24h and 48h, and plants treated with 10mM H_2O_2 for 24h and 48h followed by aphid. Expression of genes was quantified relative to the value obtained from 0 (h) samples (aphid and hydrogen peroxide -free plants). Each RNA sample was extracted from approximately 200 mg of fresh leaf of Oxi1 *Arabidopsis* mutant plants. *Arabidopsis* Elongation Factor (EF) gene was used as reference control. Data are means ±SE (*n* =9). Significant differences in gene expression were indicated with, * p<0.05, ** p< 0.01, *** p<0.001. Two ways Anova were conducted to generate the *p* values.

7.4 Discussion

7.4.1. Hydrogen peroxide plays a key role in plants

Reactive oxygen species (ROS) are mainly considered as toxic by-products of aerobic organisms. However, plants are also able to use ROS as signalling molecules for regulating plant development, responses to biotic, abiotic stresses and programmed cell death; they act as an early response molecule to different stimuli, and control many different processes in plants. ROS such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) are regularly produced from normal cellular metabolic process as well as photosynthesis and respiratory electron flow (Halliwell and Gutteridge, 1989). Hydrogen peroxide (H_2O_2) is a key signalling molecule involved in numerous processes including cell wall rigidification, transcription of defence-related genes and hypersensitive (programmed) cell death (Levine et al., 1994; Neill et al., 2002). Not only does hydrogen peroxide play an important regulatory role, but it has also been reported to be a toxic cellular metabolite (Gadjev et al., 2008). In addition to the above, hydrogen peroxide functions as a signalling molecule in plants to different stimuli (Gadjev et al., 2008). For example, studies have demonstrated that hydrogen peroxide is up regulated in response to various stimuli indicating that it is the key factor for tolerance induction in stressed plants (Neill et al., 2002). Furthermore, hydrogen peroxide plays a dual role in plants**:** at low levels it acts as a messenger molecule involved in mediating signalling pathways which trigger tolerance against various biotic and abiotic stresses (Dat et al., 2003). However, at high concentrations, it coordinates programmed cell death (Dat et al., 2003).

7.4.2. Oxidative signal inducible (Oxi1) protein kinases are induced by application of hydrogen peroxide

Oxidative signal inducible (Oxi1) protein kinase is a serine/ threonine kinase of the AGC family (AGC2-1) and is required for oxidative burst-mediated signalling in *Arabidopsis* roots. Oxi1 is induced in response to a wide range of Reactive Oxygen species, especially hydrogen peroxide (Rentel et al., 2004); it is also required for full activation of MAPKs genes. MAPKs genes are involved in many developmental processes e.g. root development, but also form a valuable component of the plant's inducible defence system to protect the plant against

bacterial and fungal pathogens. Recently, oxidative signal-inducible 1 (Oxi1) was shown to be necessary for ROS-mediated responses in *Arabidopsis*. The oxi1 mutant was compromised in ROS-dependent processes, such as root hair elongation, and displayed enhanced susceptibility to biotrophic pathogens, such as the fungal pathogen *Hyaloperonospora parasitica* and the bacteria *Pseudomonas syringae* (Petersen et al 2009). The kinase activity of Oxi1 was itself induced by H_2O_2, wounding, cellulases and various elicitor treatments mimicking pathogen attack. Oxi1 plays an essential role in the signal transduction pathway connecting oxidative burst signals to different downstream responses. Induction of Oxi1 leads to accumulation of ROS, and improving signal transduction of ROS. To mimic the Oxi1 mutant, Oxi1 could be induced in response to the exogenous application of hydrogen peroxide. Moreover Oxi1 is essential for the partial activation of MPK3 and MPK6 in response to H_2O_2 and cellulose treatment, imitating pathogen attack (Rentel et al., 2004). MPK3 and MPK6 are involved in the mitogen-activated protein kinase cascade activated following recognition of bacterial flagellin by the receptor-like kinase FLS2 (Asai et al., 2002), which initialises the induction of defence genes such as *WRKY22/29* and *GST* and is effective in defence responses against both bacterial and fungal pathogens (Gomez-Gomez et al., 2001; Asai et al., 2002; Chinchilla et al., 2006).

7.4.3. Callose synthase plays a key role in plant defence

Callose is an important polysaccharide component of plant cells and can account for up to 80% of dry mass in many specialized cell walls, including the callose wall, pollen tubes, and the growing cell plate (Bacic et al., 2009; Verma, 2001). In higher plants, callose synthesis and accumulation are tightly controlled during plant growth and development such as in cell division, cell growth and differentiation. Callose accumulation can also be induced in response to biotic or abiotic stress (Bacic et al., 2009; Verma, 2001; Hong, 2001). Under normal growth conditions callose is present in the sieve plate at low levels. However, it accumulates rapidly and drastically, plugging the sieve pores in response to stress. Genes encoding callose synthases have now been identified in several plant species **(**Aidemark, 2009). An *Arabidopsis* callose synthase, GSL5, is required for wound and papillary callose formation (Jacobs et al., 2003).

Results from the present study showed that post treatment with hydrogen peroxide, in the susceptible Arabidopsis wild type Col-0 to aphid, elevated tolerance to aphid feeding. However, exogenous application of hydrogen peroxide in Oxi1 mutants increased plant susceptibility in response to aphid feeding.

In the *Arabidopsis* wild type Col-0, post application of 10mM hydrogen peroxide followed by aphid infestation, induced Oxi1 MAPKs kinas especially MAPK3 and MAPK6. Oxi1 protein kinase is required for full activation of MAPKs 3 and 6 trigger calcium signalling pathway, which in turn stimulate callose synthase followed by callose deposition in the sieve elements in response to aphid feeding (Fig 7.8). The expression level of callose synthase gene GSL1 increased significantly in response 48 hours post treatment. Also the expression of GSL1 increased in response to dual treatment (hydrogen peroxide+ aphid) but not as high as hydrogen peroxide treatment alone. This result may confirm that induction of GSL1 was as a result of hydrogen peroxide treatment. However, the expression level of GSL5 increased significantly in response to dual treatment with hydrogen peroxide and aphid in comparison to hydrogen peroxide treatment. This result shows that GSL5 is more of an insect wound response gene (Jacobs et al., 2003) rather than an abiotic stress response. In contrast β-1,3-glucanase gene Gns2 were significantly down-regulated in response to dual treatment (hydrogen peroxide + aphid) compared to single treatment with hydrogen peroxide. Down-regulation of Gns2 may refer to induction of Gns2 in the plant by insect to cause callose hydrolysis. This result may confirm that Gns2 is the key gene in plant susceptibility in response to aphid feeding.

In the *Arabidopsis* Oxi1 mutant, exogenous application of hydrogen peroxide followed by aphid infestation, may affect different signalling pathways which results in plant susceptibility. The results show that callose synthase genes GSL1 and GSL5 were expressed in the basal level in response to hydrogen peroxide treatment; however GSL5 was downregulated in response to dual treatment (hydrogen peroxide + aphid). This result may confirm that GSL5 might be key resistance gene in response to aphid feeding. On the other hand β-1,3-glucanase gene Gns2 was up-regulated to the highest level especially in

response to dual treatment (hydrogen peroxide + aphid). The results confirm that exogenous application of hydrogen peroxide to Oxi1 mutant may cause over expression of Oxi1 MAPKs and this might turn off the signalling for MAPKs cascades (Fig 7.8). Over expression or down-regulation of Oxi1 results in plant susceptibility enhancement in response to biotic and abiotic stresses (Rentel et al., 2004).

In conclusion, regulation of Oxi1 expression levels seems important in mediating an appropriate defence response. Oxi1 mutant has unique features like, continuous induction of MAPKs kinase genes, early response ROS accumulation and signal transduction, catalase activity, calcium pathway, and callose synthase. Oxi1 mutant with all distinctive features tolerate and cope with high population of aphid plus delaying growth rate of both aphid nymphs and adults. Exogenous application of hydrogen peroxide led to induction of Oxi1 which is required for partial activation of MAPK3 and 6. Activation of MAPK cascades trigger calcium signalling pathway which is responsible for callose synthase induction followed by callose production. Exogenous application of hydrogen peroxide in Oxi1 mutant led to over expression of Oxi1.

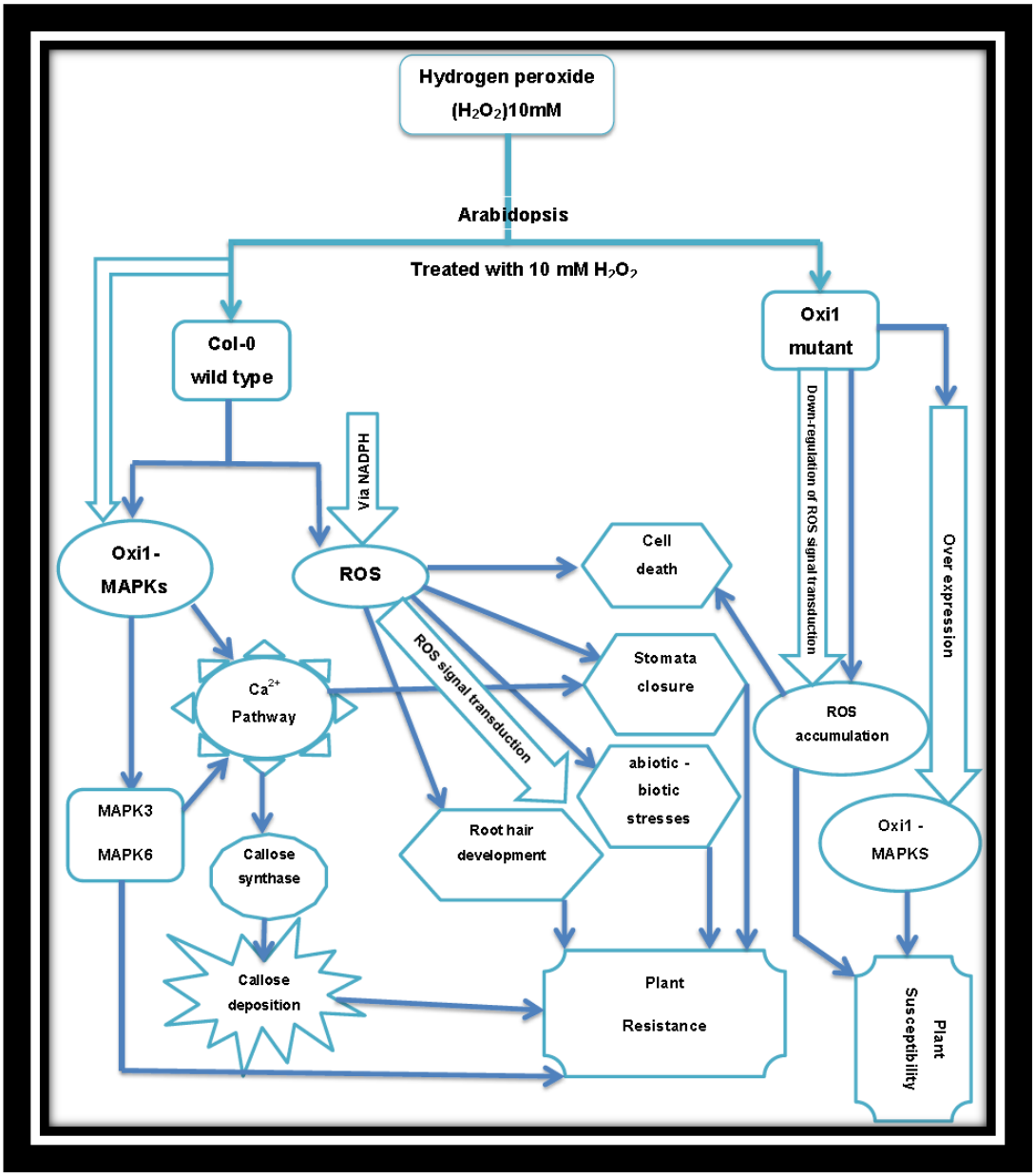

Fig 7.8. Schematic diagram showing predicted signalling pathway in the *Arabidopsis* wild type (Col-0) and Oxi1 mutant in response to hydrogen peroxide treatment. In the *Arabidopsis* wild type Col-0, post application of 10mM hydrogen peroxide followed by aphid infestation, induced Oxi1 MAPKs kinas especially MAPK3 and MAPK6. Oxi1 protein kinase is required for full activation of MAPKs 3 and 6 trigger calcium signalling pathway, which in turn stimulate callose synthase followed by callose deposition in the sieve elements in response to aphid feeding. On the other hand, the *Arabidopsis* Oxi1 mutant, exogenous application of hydrogen peroxide followed by aphid infestation, may affect different signalling pathways which results in plant susceptibility.

Chapter 8. On-going and future work

β-1,3-glucanase (Gns5) gene silencing by using antisense technique in rice cultivar *Oryza sativa* (TN1)

Abstract

Gene silencing is an experimental tool to study gene functions in plants. Recently, gene silencing has become more popular because of its great effect to inhibit the expression of a homologous endogenous gene.

To clone and construct expressing vectors (IRRI 462 and 463) of sense and antisense β-1,3-glucanase gene(Gns5). Total RNA was extracted from susceptible rice cultivar TN1, and then the Gns5 cDNA was amplified by RT-PCR. At the same time the sense and antisense Gns5 gene were formed by binding BamH I and Kpn1 in cis and trans-directions. At the end they were ligated into expressing vectors (pCAMBIA 1300int-ubi-hpRNAi and pCAMBIA1300int-35S-hpRNAi) in cis and Trans directions using DNA recombinant technology. The recombinant vectors were further identified by digestion of BamH I and Kpn1.

The results of sequencing showed that the orientation of the ligations and the reading frame were correct. After digested by BamH I and Kpn1, one fragment exhibiting 290bp for targeted Gns5 (β-1,3-glucanases) sequence and the vector pCAMBIA1300int-ubi-hpRNAi) 11167bp and another fragment 290bp for the ligated Gns5 (β-1,3-glucanases) targeted sequence 9695bp for pCAMBIA1300int-35S-hpRNAi were formed in sense and antisense expressing vectors. Electrophoretic results were completely coincident with theoretical calculation.

β-1,3-glucanase(Gn5) sense and antisense genes were successfully cloned and expressing vectors were successfully constructed.

8.1. Introduction

8.1.1. Rice

Rice is an ancient cereal crop and it has been grown for more than 10,000 years; it is a staple food of two third of the world's population (Isawa and Shamoto, 1996; Giri and Laxmi, 2000). Because of the long history of its cultivation and widespread cultivation, rice is the most genetically diverse among the world's cereal crops. The domesticated rice plants consist of two species – *Oryza sativa* and *Oryza glaberrima*. Of these two species, most of the research has been focused on *O. sativa* because it is the main cultivated rice in the majority of the rice growing regions (Isawa and Shamoto, 1996). *O. sativa* is an important cereal crop, which belongs to the grass family Poaecea in the plant kingdom (Gnanamanickam, 2009). It is diploid, annual and a short day plant, which can self-fertilize (Giri and Laxmi, 2000). It is also one of the essential nutritional crops of mankind.

For many years rice has been the ideal model plant for research into development, genomic and evolution in monocotyledonous species. There are several aspects to the rice that have elevated it to this level, amongst them are the small genome size (~389 Mb) which lead to a rapid annotation of the genome in 2005 by the International Rice Genome Sequencing Project (IRGSP, 2005), the simplicity of *Agrobacterium*-mediated transformation (Hiei et al. 1994), and high levels of genetic synteny with other cereal genomes (i.e. barley, wheat, maize and sorghum) (Bennetzen and Ma, 2003). Prior to the era of modern molecular biology in the 1980's a great wealth of work was carried out through breeding studies to expand the knowledge of this important crop. These studies led to insights into areas such as Mendelian segregation in rice (Van der Stok, 1908), an agreed system of rice chromosome numbering, linkage groups and nomenclature for gene symbolization (Kadam and Ramiah 1943; Nagao and Takahashi 1963).

8.1.2. Rice transformation

During the 1980's considerable research was focussed towards establishing efficient rice transformation protocols. With support of the Rockefeller Foundation, three independent groups discovered that transgenic rice plants

could be regenerated from rice protoplast following electroporation-mediated or PEG-mediated transformation methods (Toriyama et al., 1988; Zhang and WU, 1988, Zhang et al., 1988). However, despite early successes rice genetic engineering remained a challenge because these early gene transfer methods were limited by constraints imposed by the culture systems. As a result, only a few japonicas and an even smaller number of indica varieties could be engineered routinely. Work by Christou et al., (1991) solved this problem through the development of particle bombardment-based methodologies which allowed the creation of transgenic rice plants from many important cultivars, including indicas and elite japonicas. Additionally Chan et al. (1993) and Hiei et al., (1994) developed highly efficient *Agrobacterium*-mediated transformation system for japonica varieties using the mature seed-derived callus as the explant material, the latter of which became the most common transformation method for rice plants. During 2006, *Agrobacterium*-mediated transformation was further improved through reducing the steps necessary for the transformation procedure (Toki et al., 2006). Despite the improvements for japonica transformation indica rice remained hard to transform however some modification by Lin and Zhang (2005) and by Hiei and Komari (2006) led to better transformation efficiency of this variety. More recently in 2008, new protocols for *Agrobacterium*-mediated transformation have been established for both japonica and indica varieties (Hiei and Komari, 2008). These protocols allow for the generation of transgenic lines within 10 weeks by exploiting the extremely high transformation efficiency of the immature embryo, a single immature embryo may produce 5—13 independent transformants. The only disadvantage of this technique is that the collection of immature embryos is hard and limited by the season. These successive developments of rice transformation have facilitated the introduction of several transgenes into the rice genome for both crop improvement and studies of functional genomics (Tyagi and Mohanty, 2000; Chen et al., 2009).

8.1.3. Tissue-specific/inducible expression

Constitutive CaMV 35S and maize Ubiquitin promoters are the two most common promoters used in rice transgenic research. There are certain problems associated with expressing transgenes in all plant tissues and organs at all growth stages using a strong constitutive promoter, for instance,

increasing the metabolic burden of transgenic plants, and contributing to the public's concerns about the food safety due to accumulation of transgenic protein products in the edible parts of engineered plants. Moreover, constitutive expression of some beneficial genes, such as abiotic stress-resistance related transcription factor genes in transgenic plants may potentially lead to abnormal plant growth and development. Thus, tissue-specific/inducible expression is crucial for transgenic breeding, which is usually implemented by making use of tissue-specific/ inducible promoters.

8.1.4. Gene silencing

Gene silencing using RNA interference (RNAi) is the specific downregulation of gene expression by double-stranded RNA (dsRNA). The specificity is sequence-based and depends on the sequence of one strand of the dsRNA corresponding to part or all of a specific gene transcript (Price and Gatehouse, 2008). RNAi is a post-transcriptional control mechanism involving degradation of a target mRNA. This degradation is mediated through the production of small interfering RNAs (siRNAs) from the dsRNA, which is cleaved by dsRNA-specific endonucleases referred to as dicers. In plants and nematodes, RNAi can have systemic effects on gene expression, so that gene knockout spreads throughout the organism and persists during development. The basis of this effect is thought to lie in the presence of an RNA-dependent RNA polymerase (RdRP) that is able to interact with the RISC complex (RNA-induced silencing complex) and generate new dsRNA based on the partially degraded target template by using the hybridised siRNA strands as primers. The synthesized dsRNA is then acted on by the dicer enzymes to generate new siRNAs (secondary siRNAs), thus acting as an amplification step. In this way, once a dsRNA is introduced into a cell, its effect can persist during development; in addition, the dsRNAs can be exported to neighbouring cells and thus spread the gene knockout effect through the organism. Guo and Kemphues (1998) demonstrated that sense RNA deactivated the target gene to equivalent levels when compared to using antisense RNA. Furthermore, Fire et al., (1998) concluded that double stranded RNA (dsRNA) was better still at reducing levels of target genes expression than when using either sense or antisense RNA alone (Fire et al., 1998). Later studies on a range of organisms, such as *Caenorhabditis elegans, Mus musculus*, and *Drosophila* demonstrated the universal presence of this

phenomenon, and the underlying principles of the conserved mechanism (Flavell, 1994; Hunter, 2000; Svoboda et al., 2000; Tabara et al., 1998). A significant drawback of these initial experiments was the laborious procedure of forming the specialised construct to guarantee the formation of a hairpin structure especially when applying the technology in the plant kingdom (Chuang and Meyerowitz, 2000; Schweizer et al., 2000). To simplify the time-consuming construction of the RNAi structure, specialised direct PCR cloning–based vectors, pHANNIBAL and TOPO-based pHELLSGATE (Wesley et al., 2001), were developed. Both vectors contain the cauliflower mosaic virus 35S promoter, which limits them to dicotyledonous species. So far, no commercial RNAi vector has been available for monocots, which makes it much more difficult to carry out studies on functions of genes from major crops such as wheat, rice, maize, and barley. Iyer et al. (2000) developed a monocot specific PCR-based RNAi vector, pTCK303. Using this vector system in an *Agrobacterium*-mediated transformation event they were able to demonstrate silencing of the callose synthase gene (OsGAS1) in rice. Molecular evidence supports the conclusion that the novel RNAi vector pTCK303 not only simplified the procedure of RNAi construction but also efficiently silenced the target gene in rice. The efficacy gene silencing relies on the stability and predictability of the integration event. Gene silencing is the major difficulty to the genetic engineering of crops. In dicotyledonous plants, transgene silencing has been studied extensively. However, in monocots, random gene silencing is not completely understood, but is known to occur at both transcriptional and post-transcriptional levels (Iyer *et al*., 2000). Recent publications show that there are several examples where loss of gene function results in enhanced plant performance, such as an increase in the yield or tolerance in response biotic and abiotic stress (Song et al., 2007; Leshem et al., 2006). For example sd1 gene in rice, which encodes GA20 oxidase and GA20ox-2, is involved in gibberellin biosynthesis. Deactivation of sd1 affects the plant development and results in a semi-dwarf phenotype. Furthermore pathogen-related genes have been targeted successfully to reach enhanced virus and insect resistance (Niu et al., 2006; Qu and Ye, 2007; Mao et al., 2007).

The work presented demonstrates the construction of a RNAi vector system using vector IR462 and IR463 (Figures 9.1 and 9.2) to reduce the expression of

β-glucanase Gns5 in susceptible rice varieties following infestations of BPH. β-1,3-glucanase Gns5 plays important functions in the breakdown of callose, as well as in defending against pathogen attack. Expression of Gns5 gene increases following BPH attack and are likely to play important roles in callose decomposition, which ultimately facilitates ingestion of phloem sap by BPHs from susceptible rice plants. Therefore, the absence of expression (or limited expression) of these genes allows the sieve tube occlusions to be maintained as found in the resistant plants, thus conferring resistance to the susceptible variety.

8.2 Material and Methods

8.2.1. Targeted sequence and primer design

Rice genome annotation website (http://rice.plantbiology.msu.edu/) was used to search for the unique targeted sequence in Gns5 (β-1,3-glucanases) gene and compared to all other glucanase genes. Primers were designed by using the following website (http://frodo.wi.mit.edu/primer3/). The restriction site *KpnI* has been added to 5` end for the forward primer and *BamHI* has been added to 5` end for the reverse primer to aid cloning into the multi-purpose cloning site of IR462 and IR 463. Webcutter website (http://users.unimi.it/~camelot/tools/cut2.) was used to check if any other restriction enzymes could cut within the targeted sequence.

8.2.1.1. Gns5 (β-1,3-glucanases) targeted sequence

tccactcccaaacaaaaaggagtctaatatatcaaatcaaacacagtggtgcaatctatcggcgtgtgctacg
gcgtgatcgggaacaacctgccgtcgccgagcgacgtcgtgcagctctacaagtccaacggcatcgactcc
atgcgcatctacttcccaagaagcgacatcctccaggccctcagcggctcaagcatcgccctcaccatggac
gtcggcaacgatcagctcggctccctcgcctccgacccctccgccgccgccgccttcgtccagaacaacatc
c

8.2.2. Plant sampling

Rice shoots were removed from the susceptible variety *Orayza sativa* TN1, kept in liquid nitrogen and stored in a -80°C freezer. Frozen leaves were ground to a fine powder in a mortar with a pestle in the presence of liquid nitrogen in a cold room. RNA was isolated from 100 mg of leaf tissue using TRI Reagent® and the resulting purified RNA quantified using a NanoDrop spectrophotometer. For RT-PCR, cDNA were synthesized using Super-script™ II (Invitrogen). Primers to amplify the targeted region of Gns5 from the rice cDNA were designed (Table 8.1). The resulting products were cloned into pSC-A for sequence validation.

Table 8.1. PCR primers and amplification profile for Gns5 (β-1,3-glucanases).

Primers	Product size	PCR protocol (touch down)
Forward ***KpnI*** atatggtacctccactcccaaacaaaaagg Reverse *BamHI* atatggatcctggatgttgttctggacgaa	290 bp	95°C 5 min; 35 cycles 94°C 30s 60°C 30s (decrease 0.5°C every cycle) 72°C 30s 72°C 5 min

Table 8.2. Double Digestion with *BamHI*, *KpnI* Recommended by Fermentas. Buffer *BamHI* +*BamHI* + 2-fold excess of *KpnI* Incubate at 37°C

Enzyme	Incubation temp.	Recommended buffer	Units for overnight incubation	Thermal inactivation	Restriction enzyme activity, %
					BamHI
					1X
BamHI	37°C	*BamHI*	0.5	80°C	100
KpnI	37°C	*BamHI*	0.4	80°C	100

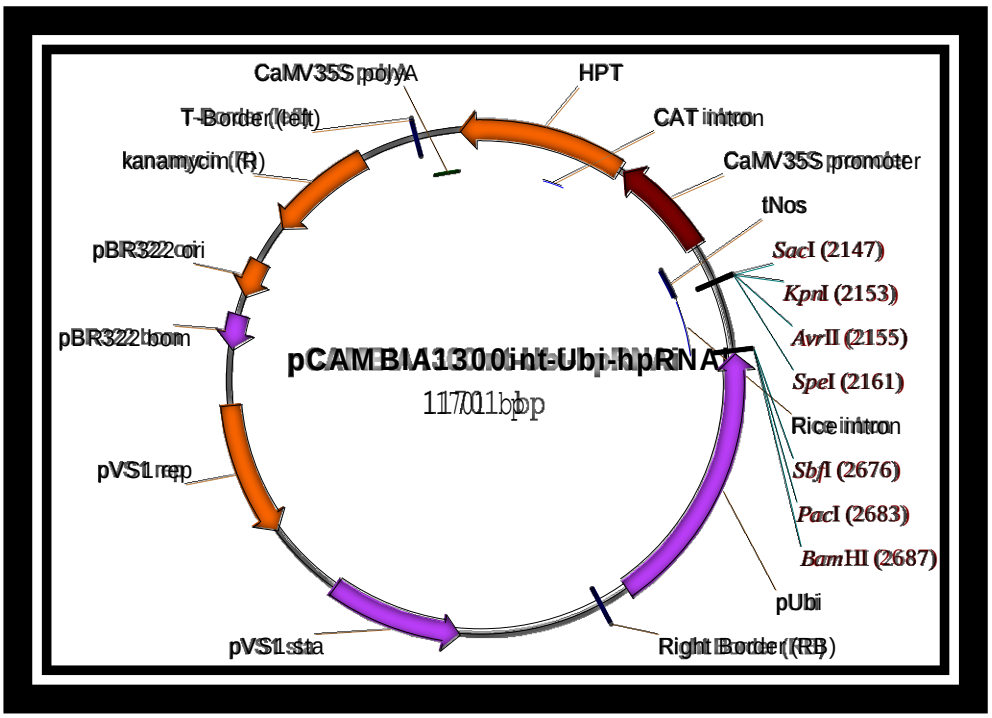

Fig 8.1. IR462 (pCAMBIA1300int-Ubi-hpRNAI) expression vector map.

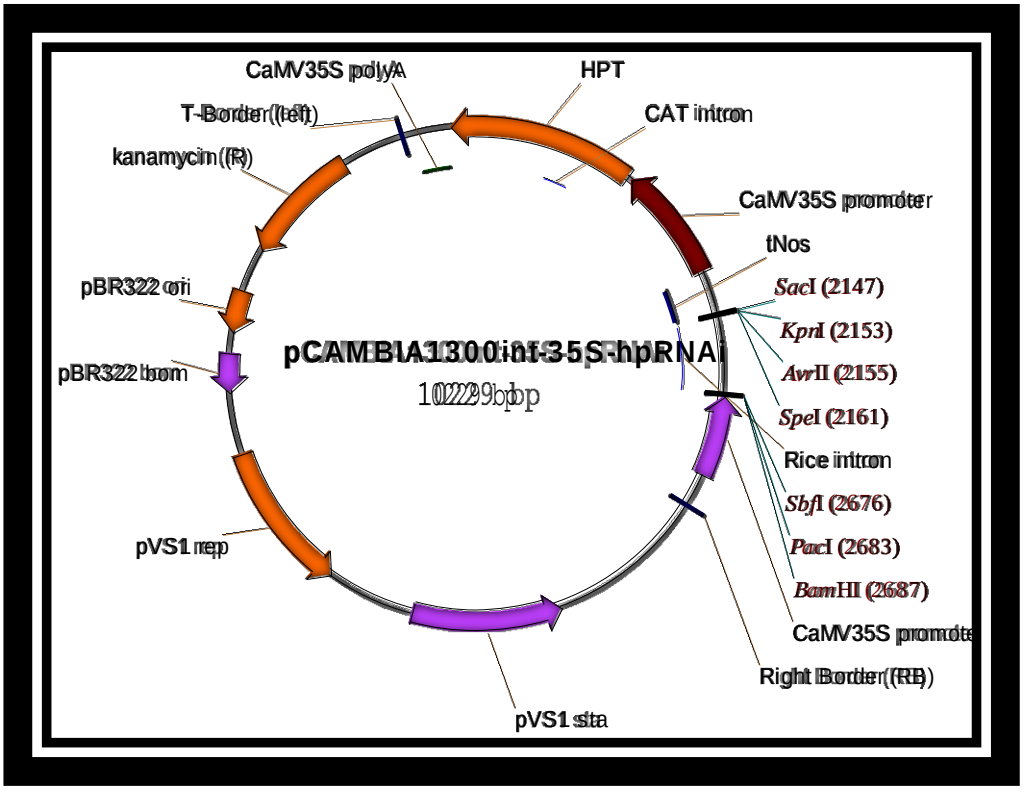

Fig 8.2. IR463 (pCAMBIA1300int-35S-hpRNAI) expression vector map.

8.3 Results

8.3.1 Construction of transformation vector

Following RNA extraction a fragment of the Gns5 was amplified using PCR. The primers used for amplifying the 290 bp fragment were engineered specifically to introduce 2 unique restriction sites to either end of the amplified region. Figure 3 shows the resulting agarose gel after electrophoresis showing the presence of amplified DNA in lane 1 and lane 2 (duplicates). Following subsequent TA-based cloning and sequence verification the Gns5 fragment was released by digesting with *KpnI* and *BamHI*, to enable it to be ligated into the two vectors. Electrophoretic separation of the digestates is shown in (Figure 8.4). Following digestion the 290 bp Gns5 fragment is released (Figure 8.4, lanes 5 and 6) from the pSC-A cloning vector, digestion of the IR462 and IR463 vectors released the 534 bp rice intron stuffer fragment revealing the linear antisense vector (Figure 8.4, lanes 1,2 and 3,4 respectively). Ligation of the Gns5 fragment into IR462 and IR463 completed the construction of the antisense vectors. Correct integration of the fragments into the respected vectors was confirmed both by PCR and restriction fragment analysis. Although difficult to see, due to the concentration of the DNA used, figure 8.5 shows confirmation of the correctly constructed antisense Gns5 vectors. Lane 4 shows the release of the Gns5 fragment from a single recombinant clone of the IR462 vector following digestion with *KpnI* and *BamHI*, lanes 2 and 3 show positive PCR amplification of the Gns5 fragment from the same recombinant vector. Lanes 6 and 7 show release of the Gns5 fragment from 2 independent recombinant IR463 clones and lanes 8, 9 and 10, 11 shows the corresponding (duplicate) PCR amplification of the fragment from the recombinant vectors.

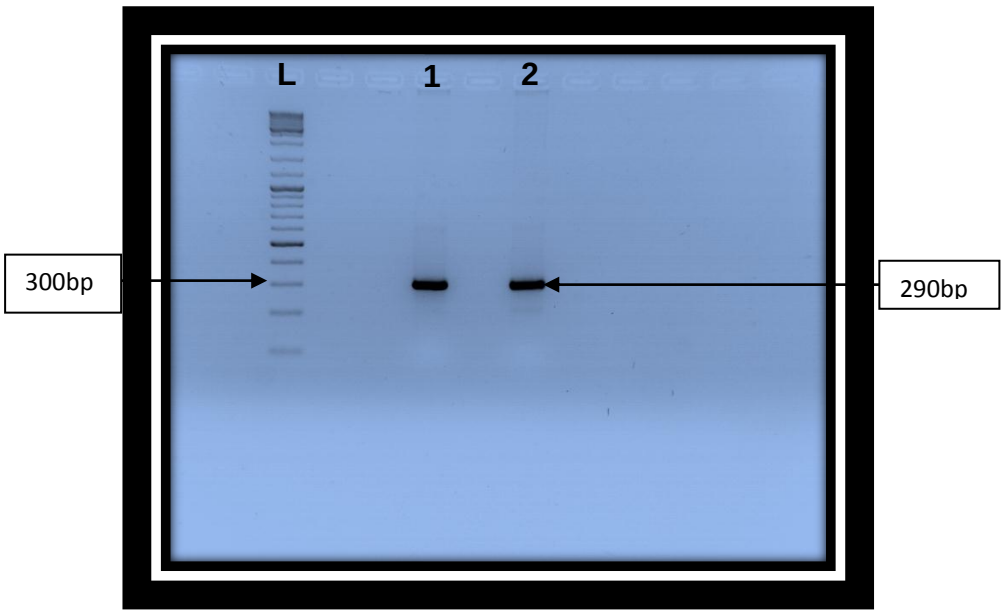

Fig 8.3. PCR product for cDNA synthesis using KpnI forward and BamHI reverse primers. L - Gene Ruler ladder; 1 and 2 PCR products (duplicates, 290 bp) for targeted sequence in rice plant Gns5 (β-1,3-glucanases).

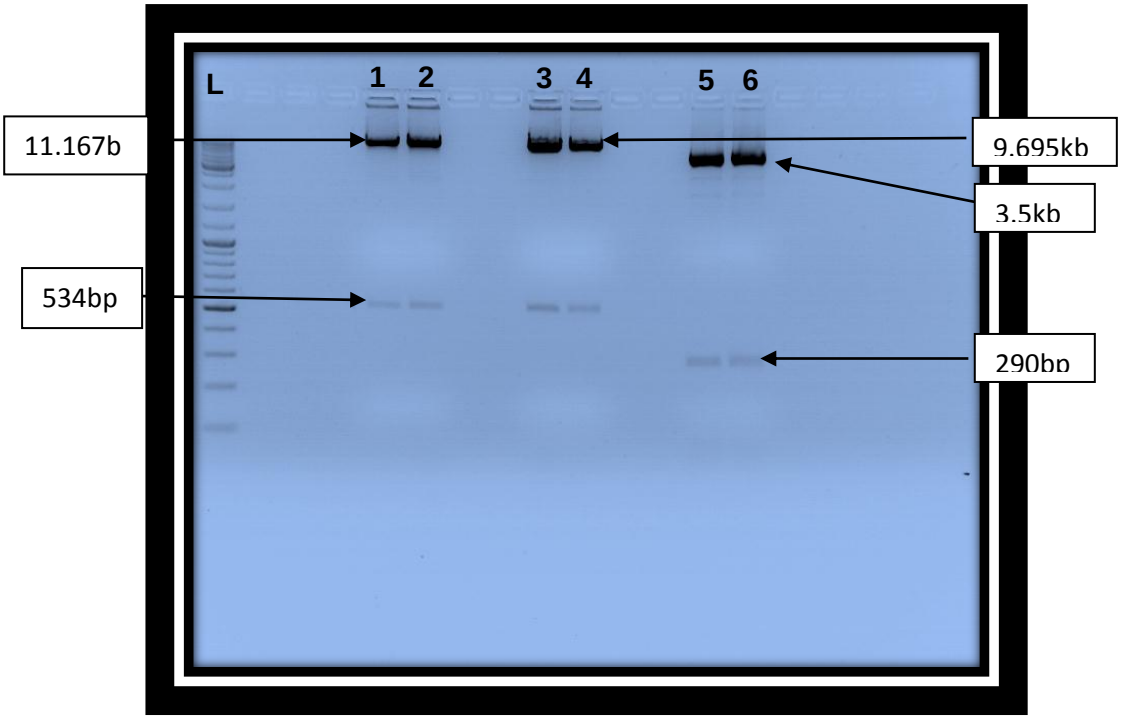

Fig 8.4. Double digestion for expression vectors and cloned Gns5 (β-1,3-glucanases) 290bp using *KpnI* and *BamHI* restriction enzymes. L - Gene Ruler ladder; 1 and 2, IR462 (expression vector + rice intron (534bp)); 3 and 4, IR463 (expression vector + rice intron (534bp)); 5 and 6, pSC-A+ Gns5 (290bp) and pSC-A+ Gns5 (290bp).

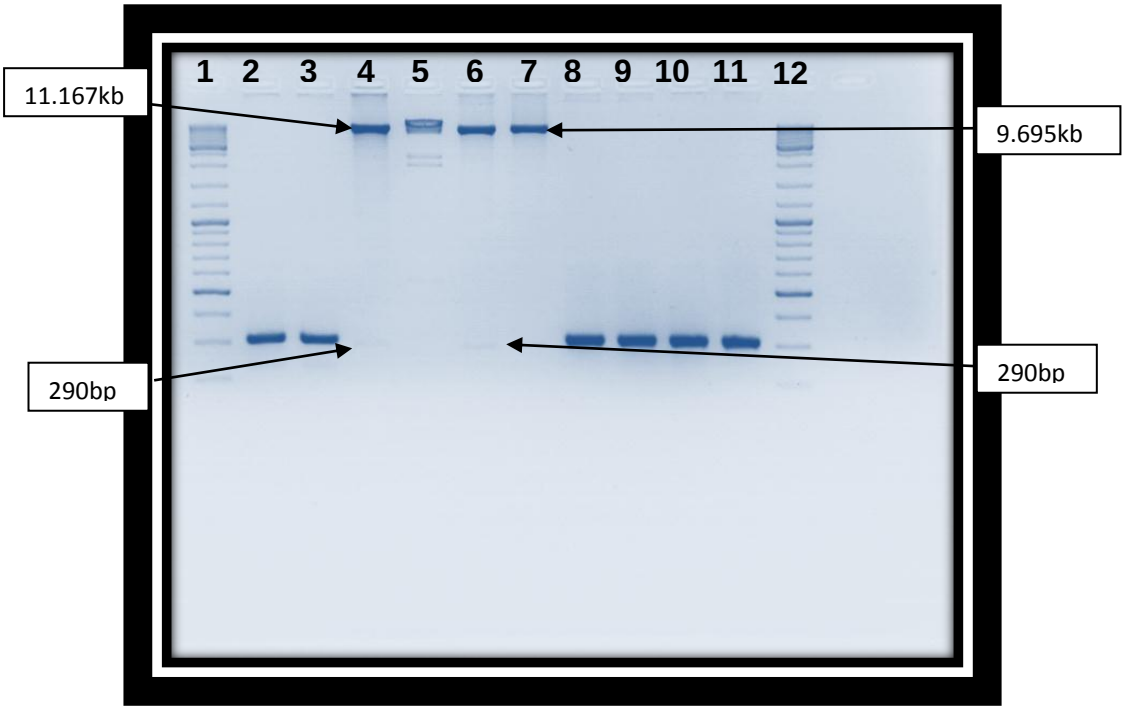

Fig 8.5. Electrophoresis for PCR product of Gns5 (β-1,3-glucanases) using *KpnI* and *BamHI* forward and reverse primers and double digestion for the new construct (IR462 +Gn5 (290bp) and (463+Gns5 (290bp). Lanes 1 and 12 (Gene Ruler Mix); 2, 3 PCR product for ligated Gns5 (290bp) with IR462; 4 double digestion using *KpnI* and *BamHI* for the new construct IR462+Gn5 (290bp); 5(Lambda ladder); 6, 7 double digestion using *KpnI and BamHI* for the new construct IR463+Gns5 (290bp); 8, 9, 10, 11 3 PCR product for ligated Gns5 (290bp) with IR463.

8.4 Discussion

PCR amplification and restriction fragment analysis demonstrated that the 2 antisense vectors had been constructed successfully. Currently these recombinant vectors are with collaborators in the Biotechnology Research Institute, Chinese Academy of Agricultural Sciences for transformation into the susceptible rice variety TN1 to test the original hypothesis that expression of antisense Gns5 will confer resistance to BPH attack by removing the ability of BPH-induced over expression of this specific β-1,3-glucanase.

Two vectors were chosen to be tested simultaneously to investigate the performance of both the maize Ubiqutin (IR462) and the CamV35S (IR463) promoters. Plants will similarly be transformed with empty versions of the IR462 and 463 vectors to provide controls for the transformations and also to provide suitable equivalents for the subsequent BPH bioassays to reduce any effects that may be a result of unintended effects from the transformation procedure.

Chapter 9. Conclusion

9.1. Identification of brown planthopper-induced genes in the susceptible rice variety TN1 by suppression subtractive hybridisation

Differentially expressed genes in TN1 rice under planthopper infestation were classified into 10 functional categories (Fig 9.1). Percentages represent the percentages of genes belonging to a particular functional group, including those of unknown functions (Fig 9.1). Interestingly, those genes with functions concerned with the wound response accounted for the largest functional category (29%), while those involved in the stress response and oxidative stress accounted for 9% and 6%, respectively. Those involved in electron transport represented 9%, ABA/WDS induced proteins represented 6%, and those involved in signalling pathways accounted for 6%. Those involved in aromatic metabolism, ribonuclease T2 and metabolic processes represented 6%, 6% and 3%, respectively. Those of unknown functions represented 17% (Fig 9.1).

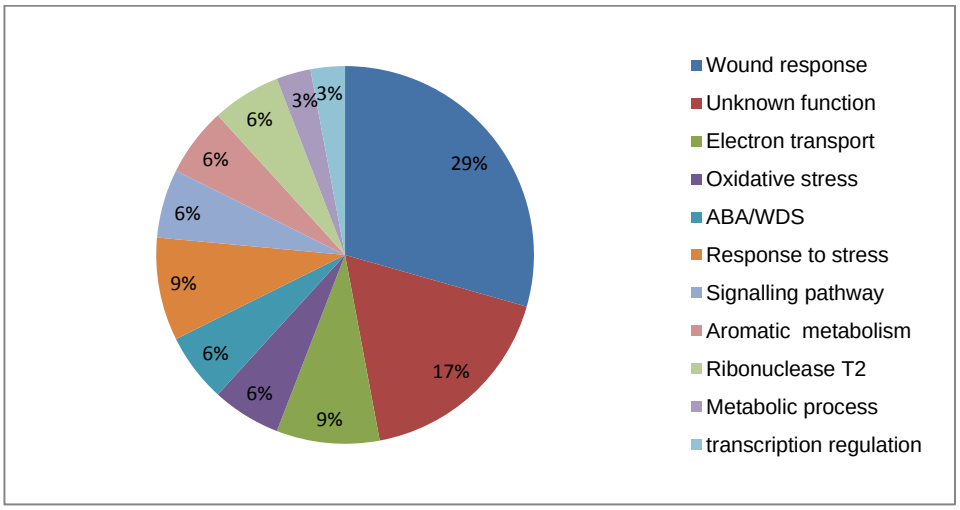

Fig 9.1. Specific expression of rice genes induced by brown planthopper classified by functionality.

Results from the subtractive library demonstrated that a number of wound response genes were differentially expressed under planthopper attack. Interestingly, β-1,3-glucanase 1, 2 and 5 genes were differentially expressed in response to BPH feeding. These are classified as pathogen related genes (PR)

(Frye et al., 2001; Senthilkumar et al., 1999) and play an important role in plant resistance in response to fungal infestation by hydrolysing fungal cell walls (Leubner-Metzger and Meins, 1999).

In addition to expression of the β-1,3-glucanase genes, callose/glucan synthase genes were also expressed in TN1 in response to BPH feeding. Callose is a polysaccharide, β-1, 3-glucan, with some β-1, 6-branches and it occurs in the cell walls of a wide range of higher plants and as stated above plays an important role not only in plant defence in response to biotic and abiotic stresses, but also in a wide variety of processes during plant development. Callose synthase genes GSL1, 3 and 5 were differentially expressed in response to BPH feeding.
GTP binding protein is a small protein, which regulates callose synthase (Qadota et al., 1996) and in the present study was expressed in response to BPH attack in the susceptible cultivar TN1.

9.2. Rice –Brown planthopper interaction

9.2.1. Resistance mechanism in rice plants in response to BPH feeding

First, the BPH acts on the plant by penetrating its tissues, injecting saliva into its cells, and sucking up phloem sap. In response to BPH feeding, the plant up-regulates expression of its callose synthase and β-1,3-glucanase genes. Consequently, callose deposition occludes the sieve tubes and prevents the BPH from ingesting the phloem sap. However, β-1,3-glucanases that decompose the deposited callose and thereby facilitate the BPH's continued feeding from the phloem are strongly induced in susceptible plants, but much more weakly induced in resistant plants. Thus, differential expression of β-1,3-glucanases can account for between-plant differences in resistance levels.

This study has shown that feeding by the BPH can induce callose synthesis and deposition on the sieve plates of rice plants. Callose deposition affects phloem transportation and plays an important role in preventing the BPH from ingesting the phloem sap. The presented results show that not only callose deposition is sufficient for resistant plants to defend themselves against the BPH, but also that β-1,3-glucanases genes Gns2 and Gns5 which are active callose-

decomposing enzymes, are induced by BPH activity and might play a role in susceptibility of TN1 plants (Fig 9.2). The differential expression of these enzymes may result in different resistance levels in rice plants.

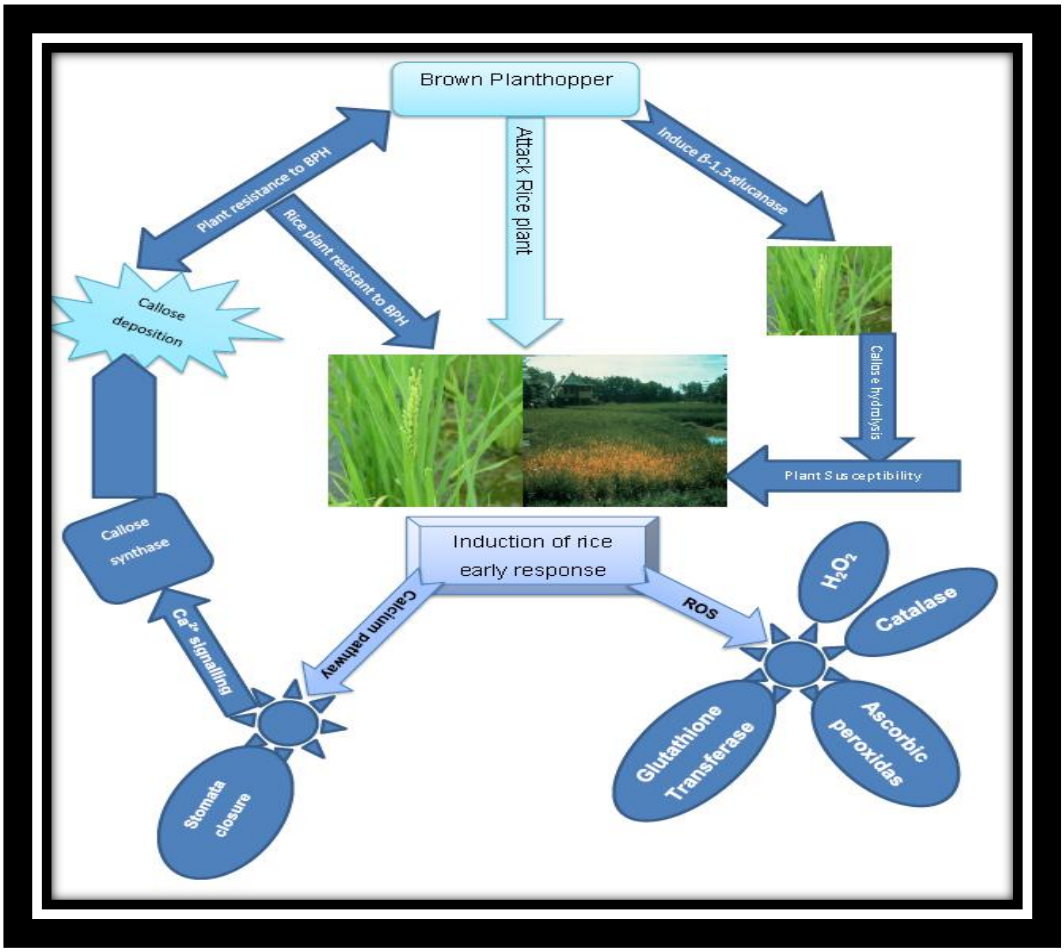

Fig 9.2. Schematic diagram showing predicted BPH-rice interaction. First, the BPH acts on the plant by penetrating its tissues, injecting saliva into its cells, and sucking up phloem sap. In response to BPH feeding, the plant up-regulates expression of its callose synthase and β-1,3-glucanase genes. Consequently, callose deposition occludes the sieve tubes and prevents the BPH from ingesting the phloem sap. However, β-1,3-glucanases that decompose the deposited callose and thereby facilitate the BPH's continued feeding from the phloem are strongly induced in susceptible plants, but much more weakly induced in resistant plants. Thus, differential expression of β-1,3-glucanases can account for between-plant differences in resistance levels. ROS and Ca^{2+} as second messenger signalling play important role in plant defence in response to insects feeding. ROS function as a secondary messenger in response to insect (BPH) feeding. Signal propagation is complemented by the accumulation of reactive oxygen species (ROS) in the extracellular spaces between cells and by rapid expression of ROS-responsive transcripts. Different ROS antioxidant enzymes glutathione, catalase, hydrogen peroxide and Ascorbic peroxidase play important role in clearing excess free radicals. Reactive Oxygen

Species especially SOD is the important free radical scavenger in the plants. SOD can clear the excessive free oxygen radicals such as O_2, H_2O_2 and OH^- which are dangerous to plant cells. Calcium signalling plays an important role in plant defence. Ca^{2+} signalling can be stimulated by ROS (H_2O_2). Thereafter, calcium triggers callose synthase followed by callose deposition in response to insects wound.

9.3. Oxidative signal-inducible (Oxi1) protein kinase regulates important developmental processes and defence responses in plants

The *Arabidopsis* mutant Oxi1 protein kinase has shown resistance to aphid feeding. The resistance was a result of regulation of important developmental and defence responses in plants (Fig 9.3). Oxi1 mutants have shown resistance to aphid feeding and shift in the growth rate in both adults and nymphs compared to Col-0 wild type. β-1,3-glucanase genes Gns1, Gns2, Gns3 and Gns5 were not expressed in Oxi1 mutant. However, Gns2 was expressed to high level in Col-0 in response to aphid feeding. In addition, the expression level of Callose synthase GSL5 in Oxi1 mutant was significantly higher than the wild type Col-0. GSL5 is normally induced in response to insect wound (Jacobs et al., 2003). The Oxi1 null mutant showed an increase in plant susceptibility compared to wild-type *Arabidopsis* following aphid infestation.

In *Arabidopsis*, Oxi1 plays a major role in plant "immunity" against insect attack. NADPH-produced ROS is revealed to initiate expression of Oxi1 protein kinase during plant–insect interaction. Interestingly, expression levels of Oxi1 appear important in mediating suitable defence response, down-regulation and overexpression of Oxi1 result in enhanced susceptibility to biotrophic pathogens (Fig 9.3). Oxi1 MAPK has emerged as a powerful key player linking Reactive Oxygen Species (ROS) accumulation to disease resistance in response to virulent *Hyaloperonospora parasitica* attack (Anthony et al., 2006; Rentel et al., 2004).

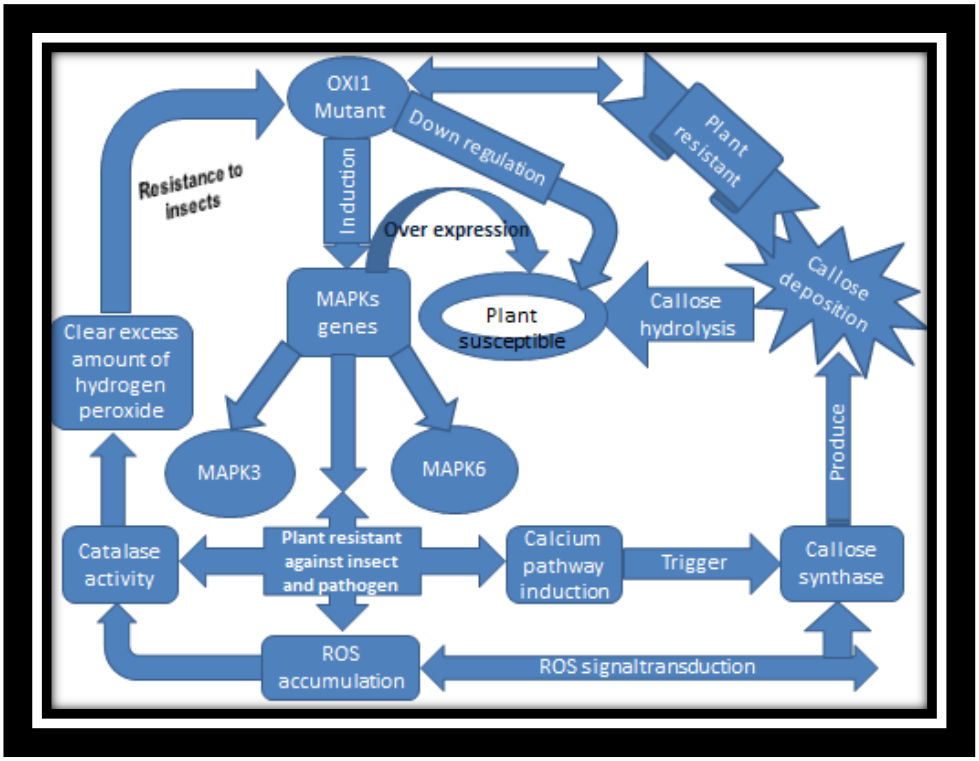

Fig 9.3. Schematic diagram showing predicted signalling pathway in Oxi1 protein kinase mutant. Oxi1 required for full activation of MAPK3 and MAPK6 (Rentel et al., 2004). Activation of MAPK cascades trigger Ca^{2+} signalling pathway which in turn stimulate callose synthase followed by callose deposition in response to insect (Aphid/BPH) feeding. Over expression or down regulation of Oxi1 MAP kinases resulted in plant susceptibility enhancement in response to biotic and abiotic stresses (Rentel et al., 2004).

9.4. Hydrogen peroxide plays a key role in plant defence mechanism

Hydrogen peroxide (H_2O_2) is a key signalling molecule involved in numerous processes including cell wall rigidification, transcription of defence-related genes and hypersensitive (programmed) cell death (Levine et al., 1994; Neill et al., 2002). Not only does hydrogen peroxide play an important regulatory role, but it has also been reported to be a toxic cellular metabolite (Gadjev et al., 2008). In addition to the above, hydrogen peroxide functions as a signalling molecule in plants to different stimuli (Gadjev et al., 2008).

Hydrogen peroxide plays a dual role in plants: at low levels it acts as a messenger molecule involved in mediating signalling pathways which triggers tolerance against various biotic and abiotic stresses (Dat et al., 2003). However, at high concentrations, it coordinates programmed cell death (Dat et al., 2003).

Application of 10mM hydrogen peroxide induced Oxi1 MAPKs especially MAPK3 and MAPK6. Oxi1 required for full activation of MAPK3 and MAPK6 (Rentel et al., 2004). Activation of MAPK cascades triggers Ca^{2+} signalling pathway which in turn stimulate callose synthase followed by callose deposition in response to insect (Aphid/BPH) feeding (Figs 8.4, 8.5). However, application of 10mM hydrogen peroxide in Oxi1 mutant resulted in over expression of Oxi1 MAPK kinase protein. Over expression or down regulation of Oxi1 MAPKs kinase protein resulted in plant susceptibility enhancement in response to biotic and abiotic stresses (Rentel et al., 2004). Also over expression of Oxi1 MAPK protein kinases caused down regulation for ROS signal transduction which led to ROS accumulation followed by cell death (Peterson et al., 2009).

Despite the strong correlation between ROS accumulation and insect or disease resistance, current understanding of the discriminators of ROS signalling is sorely limited. The oxidative inducible signalling (Oxi1) protein kinase has emerged as a potential player linking ROS accumulation to disease resistance in response to virulent *H. parasitica* attack (Rentel et al., 2004).

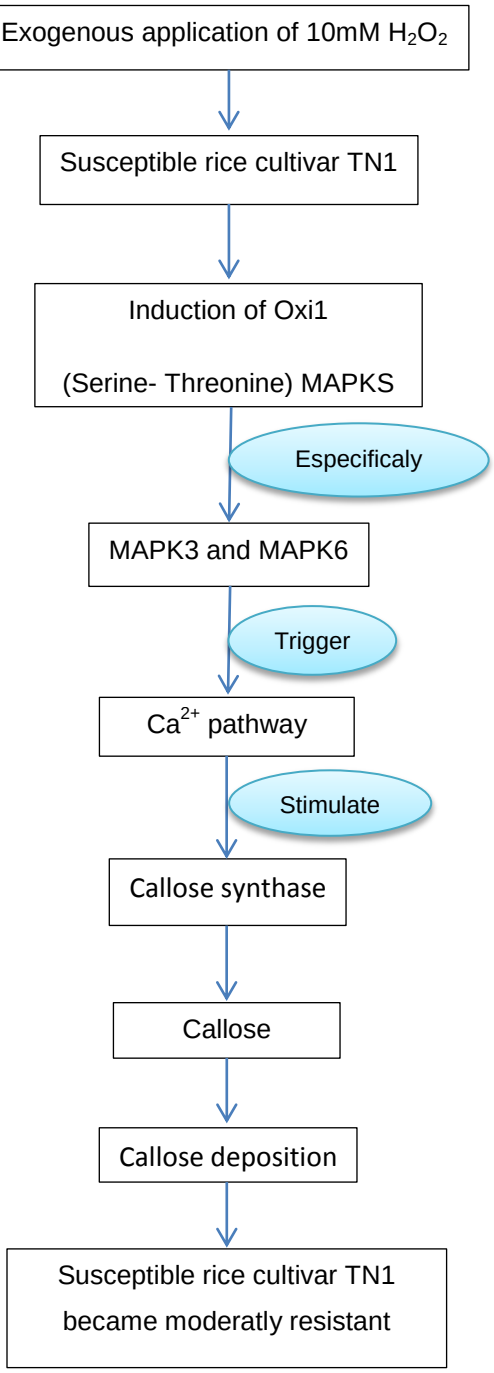

Fig 9.4. Schematic diagram showing predicted signalling pathway in susceptible rice cultivar TN1 in response to hydrogen peroxide treatment. Application of 10mM hydrogen peroxide, in rice cultivar TN1 followed by BPH infestation, induced Oxi1 protein kinase. Induction of Oxi1 protein kinase is required for full activation of MAPK3 and MAPK6. Thereafter, MAPKs trigger Ca^{2+} signalling pathway which in turn stimulate callose synthase followed by callose deposition in response to BPH feeding. As a result of hydrogen peroxide application, susceptible rice cultivar TN1 to BPH insects became moderate resistant.

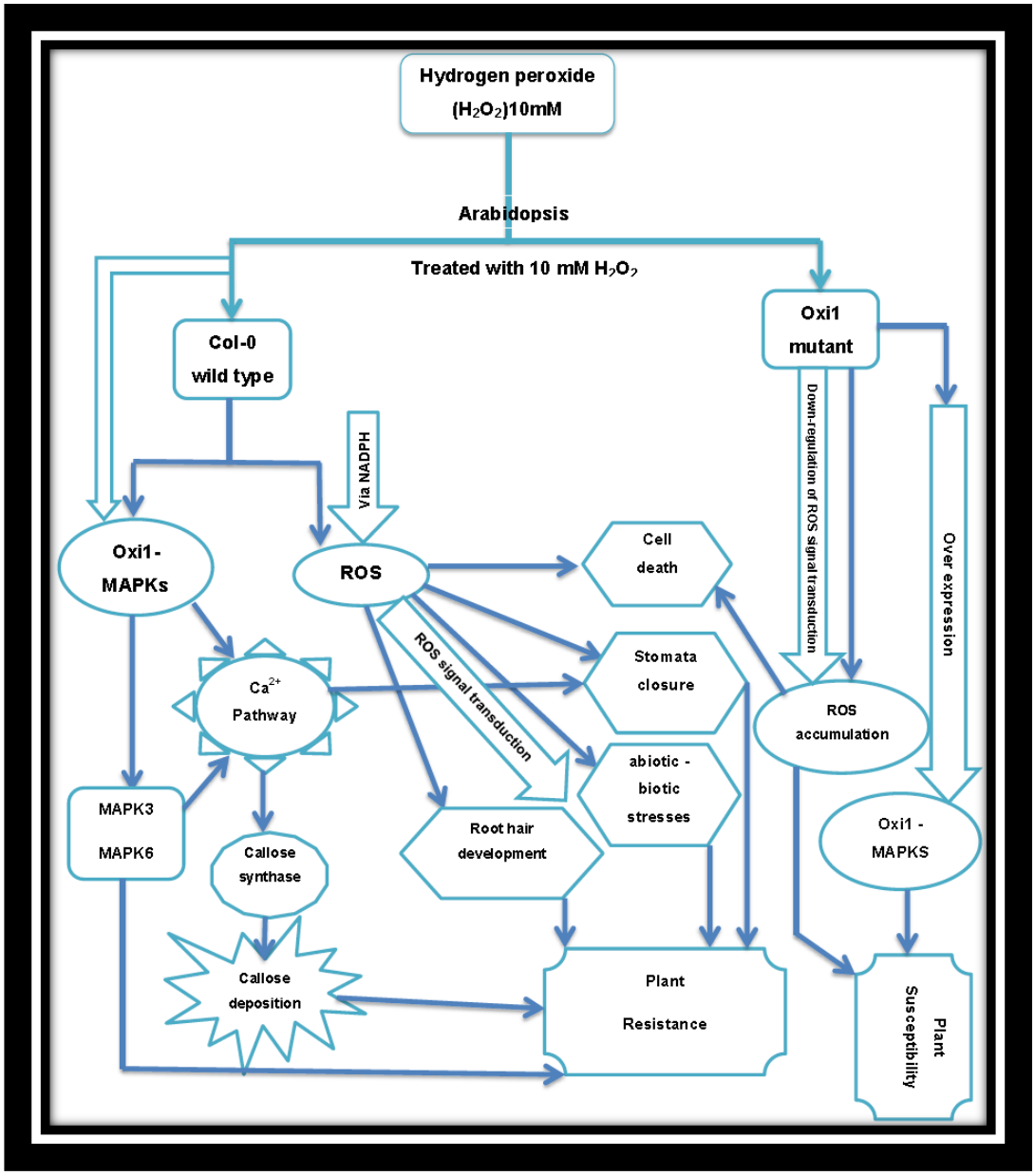

Fig 9.5. Schematic diagram showing predicted signalling pathway in the *Arabidopsis* wild type (Col-0) and Oxi1 mutant in response to hydrogen peroxide treatment. In the Arabidopsis wild type Col-0, application of 10mM hydrogen peroxide followed by aphid infestation, induced Oxi1 MAPKs especially MAPK3 and MAPK6. Oxi1 protein kinase is required for full activation of MAPKs 3 and 6 that triggers calcium signalling pathway, which in turn stimulate callose synthase leading to callose deposition in the sieve elements in response to aphid feeding. On the other hand, exogenous application of hydrogen peroxide followed by aphid infestation in the *Arabidopsis* Oxi1 mutant may affect different signalling pathways which results in plant susceptibility.

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