# Molecular biology studies on resistance and susceptibility of rice to sheath blight disease caused by *Rhizoctonia solani* Kuhn

(イネにおける紋枯病抵抗性および感受性に関する分子生物学的研究)

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**2012**

**Acknowledgements**

The work described here and the dissertation itself would not have been possible without the valuable contributions of Professor Akihiro Nose, Tropical Crops Science Laboratory, Faculty of Agriculture, Saga University. I express my sincere appreciation for his painstaking guidance and patience during the study and preparation of manuscripts and thesis. I am also deeply indebted to him for allowing me to carry out the study here.

Secondly, I appreciate the invaluable contributions of Mr. Kenzo Oshima for his support and trouble-shooting during the study and in the course of my stay in Japan. I also deeply appreciate the contributions of Prof. Jun Takeda for his friendship and advice during my stay, as well as Dr. Yosuke Okimoto for his support. I also wish to thank the Doctorate, Graduate and Undergraduate students of Tropical Crop Science Laboratory for their support and encouragement.

I am also sincerely grateful for the support and encouragements I got from my wife and son, my wife's family members, and my family in Kenya together with my friends both in Japan and Kenya. Finally, I express my indebtedness to the Ministry of Education, Science and Culture (MONBUSHO) Japan for providing me with the scholarship to carry out this study.

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**Abbreviations**

| | |
|---|---|
| 1,3BPG, | 1,3-bisphosphoglycerate |
| 2-PG, | 2- Phosphoglycerate |
| 3-PG, | 3-Phosphoglycerate |
| ADP, | Adenosine diphosphate |
| ATP, | Adenosine triphosphate |
| DHAP, | Dihydroxyacetone phosphate |
| E-4-P, | Erythrose-4-phosphate |
| F-1,6-$P_2$, | Fructose-1,6-bisphosphate |
| F-2,6-$P_2$ | Fructose-2,6-bisphosphate |
| F-6-P, | Fructose-6-phosphate |
| FW, | Fresh weight |
| G-1-P, | Glucose-1-phosphate |
| G-6-P, | Glucose-6-phosphate |
| GAP, | Glyceraldehyde-3-phosphate |
| MARs, | Mass-action ratios |
| NAD, | Nicotinamide adenine dinucleotide |
| NADH, | Nicotinamide adenine dinucleotide (reduced form) |
| OPPP, | Oxidative pentose phosphate pathway |
| PAL, | Phenylalanine ammonialyase |
| PEP, | Phosphoenolpyruvate |
| PFK, | ATP-dependent phosphofructokinase |
| PFP, | Pyrophosphate-dependent phosphofructokinase |
| PPi, | Pyrophosphate |
| *R. solani,* | *Rhizoctonia solani* Kuhn |
| RPPC, | Reductive pentose phosphate cycle |
| TCA, | Tricarboxylic acid cycle |

# CHAPTER 1

## General Introduction

There is no problem confronting humans more fundamental than feeding its expanding populations. The greater proportion of our food is derived from relatively few plant species among which, cereal crops are main. Rice (*Oryza sativa* L.) one of the 'big three' cereals, is the principal food for 60 % of the worlds' people. It is a staple in parts of Africa, Asia, South America and to some extent the United States (Janick *et al.,* 1981). To increase production, high-yielding cultivars have been introduced in many countries. However, rice is subject to diseases that often place major biological constraints on its production. Of these, rice sheath blight is one of the most prevalent causing great damage to rice yield and quality worldwide (Lee and Rush, 1983).

Rice sheath blight was first reported in Japan in 1910 and is reported to occur through out the temperate and tropical production areas and is most prominent where rice is grown under intense production systems (Lee and Rush, 1983). The causal agent of rice sheath blight is *Rhizoctonia solani* Kuhn a fungus that survives either as sclerotia or mycelia in plant debris, floats to the surface of floodwater, germinates and infects the rice plants (Marchetti, 1983). Yield losses as large as 50 % occur in susceptible cultivars when all the leaf sheaths and leaf blades are affected (Lee and Rush, 1983). The disease causes lesions on sheaths of lower leaves. After initial infection, mycelia move up the plant by surface hyphae and develop new infection structures (infection cushions) and bigger lesions over the entire plant (Groth and Nowick, 1992). The use of high yielding rice varieties, which are susceptible to the disease, has contributed to

increased disease incidence (Bonman, 1992). Most long-grain cultivars of rice are susceptible or very susceptible to sheath blight whereas medium- and short-grain cultivars tend to be moderately susceptible to moderately resistant (Lee and Rush, 1983). However, there are problems associated with screening for *R. solani* resistance especially in field conditions. These include its polyxenous character with hosts that includes rice, tobacco, potato and tomato, poor and erratic sheath blight development caused by less relative humidity in widely spaced fields, and low heritability on an individual plant basis (Groth and Nowick, 1992). Another factor that influence the development of *R. solani* is high temperature (Lee and Rush, 1983).

Because of the economic importance of rice sheath blight disease, research has been focused on development of resistant cultivars (Wasano and Hirota, 1986). Resistance has been investigated by researchers leading to reports evaluating disease pressures obtained by various inoculation techniques in the greenhouse or field tests (Lee and Rush, 1983; Wasano *et al.,* 1983). Cultivar performance was reported to be greatly influenced by physiological, morphological and ecological factors with young plants being more resistant than old plants and greatest susceptibility being at panicle emergence (Lee and Rush, 1983). In the interaction between plants and most pathogens, two types of plant resistance have been reported i.e., major gene and polygene resistance (Wasano and Dhanapala, 1982).

Major gene resistance is based on the model of gene-for-gene theory in which single corresponding genes in the host and pathogen condition race-cultivar specificity (Ellingboe, 1976). Classical genetics has shown that the resistant trait in a cultivar showing race-specific resistance is often inherited in a simple Mendelian fashion as

though it were conditioned by single dominant genes (Collinge and Slusarenko, 1987). Typically, the dominant allele confers resistance in the host and a recessive allele confers virulence in the pathogen. The host is resistant only if it carries at least one copy of the resistance allele and the pathogen carries one copy of the avirulence allele (Kniskern and Rausher, 2006). Over past decades, molecular dissection of plant–pathogen interactions exhibiting this type of resistance has revealed that, in most instances, the host-resistance locus (R-gene) codes for a protein that recognizes the product of the pathogen avirulence gene directly, leading to initiation of a cascade of physiological changes in the plant that ultimately results in the hypersensitive response and systemic acquired resistance (Dangl and Jones, 2001). Major gene resistance has been adopted as a model in agricultural systems because the genetics of gene-for-gene interactions are simple. This type of resistance has been reported in interactions between potato and *Phytophthora infestans* (Malcomson and Black, 1966), oats and crown rust disease (Dinoor, 1977), tall morning-glory (*Ipomoea purpurea*) and *Coleosporium ipomoeae* a common fungal rust pathogen (Kniskern and Rausher, 2006) among others.

However, the history of crop improvement shows that a breakdown of host resistance occurs after several years owing to appearance of new virulent strains of the pathogens. In deed, such a breakdown was reported in rice improved to resist bacterial leaf blight disease where rice varieties with major gene resistance to the disease lost host resistance when the pathogen developed adaptive capacity to overcome it (see Wasano and Dhanapala, 1982). Similarly, rapeseed (*Brassica napus*) with major gene resistance to blackleg caused by *Leptosphaeria maculans* lost their resistance in Australia in 2002 only three years after their commercial production started (Sprague

*et al*., 2006). Nevertheless, crops with major gene resistance against pathogens have had significant positive impacts on agriculture. However, even with these reported positive impacts, and the known yield losses caused by sheath blight disease, no major gene resistance has been reported against the *R. solani* pathogen (Che *et al*., 2003).

Polygenic or quantitative resistance involves resistance that acts as a quantitative trait (Wasano *et al*., 1986). In this model, level of resistance is governed by allelic composition at several to many loci and may or may not be independent of the genotype of the pathogen (Geiger and Heun, 1989). Although most studies reported that the source of immunity or of high level resistance to sheath blight disease was lacking, Wasano *et al.* (1985) identified materials that showed field resistance through classical crossbreeding. The lines identified by Wasano *et al.* (1985) were hybrids obtained by crossing Tetep and $CN_4$-4-2. Tetep is a primitive cultivar from Vietnam, which was reported as having some resistance to sheath blight disease (Marchetti, 1983). $CN_4$-4-2 was a hybrid of Chugoku 45 and Nipponbare, a susceptible japonica variety. Wasano and Hirota (1986) reported that the resistant rice line showed more resistance than the parent, Tetep. Resistance based on polygenic characters is desirable because it involves a self-defence mechanism and it is stable over a long time (Wasano and Dhanapala, 1982). Sheath blight disease resistance is inherited as a dominant character (Wasano and Hirota, 1986).

Interactions between a plant and a pathogen cause substantial diversion of carbon resources in the whole plant. These interactions result in activation of metabolic pathways (Jwa *et al.,* 2006) leading to generation of products that enhance adaptation

mechanisms. In bean (*Phasoelus vulgaris* L.) plants infected by *R. solani*, induction of defence-related genes phenylalanine ammonia lyase (PAL), chalcone synthase, chalcone isomerase, and hydroxyproline-rich glycoprotein suggested activation of the phenylpropanoid pathway (Guillon et al., 2002). Indeed when bean hypocotyls infected by *R. solani* were examined, induction of peroxidase and polyphenoloxidase led to lignification around lesion areas and limited their expansion (Stockwell and Hanchey, 1987). More aggressive tactics have been reported to include synthesis of toxic antimicrobial compounds (phytoalexins) in tobacco (Broglie *et al*., 1991), and activation of chitin-hydrolyzing enzymes belonging to the PR protein groups 3, 4, 8, and 11 in potato (Lehtonen *et al.*, 2008). Although very little is known about host–fungus interaction of rice and *R. solani* at the molecular level, studies that have investigated this interaction have shown that rice plants mobilize a complex network of active defence mechanisms, which include induction of pathogenesis-related genes, *PR1b* and *PBZ1* (Zhao *et al*., 2008), and lipoxygenase (*LOX*) gene (Taheri and Tarighi, 2010). Moreover, it has been shown that glycolysis is at the core of carbon allocation to various biosynthetic pathways in *R. solani*-infected rice plants (Danson *et al*., 2000; Mutuku and Nose, 2010, 2011, 2012). However, regulation of this carbon allocation was not investigated. As crop production shifts from the tedious classical crossbreeding to a type of breeding that involves biotechnology, important questions about the possibility of using methods like metabolic engineering to enhance resistance response mechanisms in rice and other crops infected by *R. solani* have to be examined.

Metabolic engineering involves the targeted and purposeful alteration of a specific metabolic pathway (Plaxton, 2004). Since it is now possible to manipulate nucleic

acids and gene expression, an important goal of metabolic engineering is to enhance the output of specific biosynthetic pathways (Plaxton, 2004). However, for metabolic engineering to be established, identification of the target genes, their expression, enzyme protein amount and activity, and the metabolite content have to be analysed (Birkemeyer *et al*., 2005). In the course of this analysis, mechanisms of metabolic control, i.e., coarse and fine controls have to be considered (Plaxton, 2004). Coarse control is a long-term energetically expensive response that is achieved through changes in the total cellular population of enzyme molecules. Thus, any alteration in the rates of gene expression i.e., transcription, translation, mRNA processing or degradation, or proteolysis can be considered as coarse metabolic control (Plaxton, 2004). On the other hand, fine metabolic controls are generally fast and modulate the activity of a regulatory enzyme. Mechanisms of fine metabolic control include variation in substrate concentration and pH, allosteric effectors and covalent modification operating primarily on regulatory enzymes (ap Rees *et al*., 1977; Plaxton and Podesta, 2006). Therefore, during manipulation, different approaches are required if either coarse or fine metabolic control is to be achieved. For example, during response to disease infection, coarse control might be achieved by allowing interaction between transcription factors, RNA polymerase and promoters therefore increasing transcription of a desirable mRNA, whereas fine control might be achieved by modulating the activity of a regulatory enzyme in the pathway by genetic manipulation (ap Rees and Hill, 1994). Moreover it is necessary to establish whether metabolic engineering should target one particular step in a pathway or a series of steps. These issues indicate there are many challenges that need to be addressed before a successful strategy to improve rice and indeed other plants infected by *R. solani* is achieved. From studies of biochemical mechanisms of sheath blight disease

resistance (Groth and Nowick, 1992; Danson *et al*., 2000*a*; Nose *et al*., 2002*a*,*b*; Zhao *et al*., 2008; Mutuku and Nose, 2010, Taheri and Tarighi, 2010; Mutuku and Nose, 2011, 2012), it is clear that the important chemical interactions involve (a) substances present prior to contact in both host and pathogen (b) substances released after contact, and (c) substances synthesized after contact.

The object of this study was to investigate regulation of cellular carbon allocation that result in activation of resistance response mechanisms against sheath blight disease caused by *R. solani* in rice. At first we investigated the changes in carbon distribution leading to activation of glycolysis, oxidative pentose phosphate (OPP), shikimate, TCA and phenylpropanoid pathways. By studying these changes, we were able to find out how carbon resource distribution occurred after *R. solani* infection in rice as shown in Chapter two. The findings showed that glycolytic pathway was activated and that it was linked to activation of OPPP, TCA, shikimate and phenylpropanoid pathway. These studies suggested that regulation of carbon allocation in the glycolytic pathway was an important resistance response mechanism that was linked to activation of phenylpropanoid pathway. This as shown in Chapter three was important because we were able to find out four reactions whose enzymes could be modulated to enhance glycolysis and consequently enhance generation of the products of phenylpropanoid pathway in *R. solani*-infected rice plants. One of the goals of our future studies is to completely elucidate the regulation glycolysis. However, we acknowledge the complications arising from compartmentation of glycolysis and as a result we designed studies to examine regulation and how it is affected by compartmentation. As an example, Chapter four discusses the investigation we did on the reaction catalyzed by PFP/PFK, one of the regulatory reactions of glycolysis in

leaf sheaths of *R. solani*-infected rice plants. Finally as shown in Chapter five, we investigated the link between regulation of glycolysis at four reactions and lignin deposition because lignification was shown to be linked to resistance and susceptibility to sheath blight disease. In the General Discussion, we discussed and summarized these studies on regulation of carbon allocation in leaf sheath of *R. solani*-infected rice plants. In addition, we presented a proposal for using metabolic engineering as a strategy to modulate the glycolysis of infected plants to enhance their resistance response. We also explored the possibility that this could be re-produced in other plants that are infected by *R. solani*.

# CHAPTER 2

## *Rhizoctonia solani* infection in two rice lines increases expressions of metabolic enzyme genes in glycolytic, oxidative pentose phosphate pathways and secondary metabolism.

### 1. Introduction

*Rhizoctonia solani* infection has been reported to increase glucose and fructose concentrations in the leaf blades of inoculated plants (Danson *et al.,* 1999*a*), activation of glucose-6-phosphate dehydrogenase (G6PDH) (Danson *et al.,* 2000*a*; Nemoto and Sasakuma, 2000; Dong, 2004) and production of oxidised phenolics (Groth and Nowick, 1992). In previous studies, it was reported that the increase in activity of G6PDH, 6-phosphogluconate dehydrogenase, phosphoenolpyruvate phosphatase, phosphoenolpyruvate carboxylase led to the activation of the pentose phosphate and glycolytic pathways in leaf sheaths of *R. solani*-infected rice plants (Danson *et al.,* 2000*b*). Further studies also showed that the activity of rice leaf sheath pyrophosphate-dependent phosphofructokinase (PFP) (Mutuku and Nose, 2012) and contents of glyceraldehyde-3-phosphate and phosphoenolpyruvate were high in leaf sheaths of *R. solani*-infected plants (M. Mutuku and A. Nose, unpublished). However the effects of *R. solani* infection on expressions of metabolic enzyme genes in these metabolic pathways had not been examined. The current study examined the time course of the changes in gene expression of the glycolytic pathway, OPPP, RPPC, secondary metabolism and TCA cycle using primers that were complementary to common regions of enzyme genes in these pathways. Although there are post-translational modifications and problems associated with isozymes, mRNA profiling

provides a wealth of information for genetic studies of disease resistance (Wang *et al.,* 1989; Pfaffl, 2001; Tian *et al.,* 2004). Furthermore, analysis of the changes in gene expression in plants responding to stress conditions is necessary to identify common promoter regions of genes that can be used in other methods of genetic improvement like QTL analysis.

To avoid problems associated with isozymes, in the present study, primers were designed so as to include common regions among isozymes. To deal with the problems of subcellular localization, primers were selected from either cytosolic or plastidial forms. Although in the present study, the problems arising from post-transcriptional modifications had not been considered, we recognized that addressing discrepancies associated with post-transcriptional, translational, or post-translational regulation is necessary.

Against this background, a study to investigate the changes in the mRNA expression in *R. solani*-resistant and susceptible rice lines after *R. solani* infection was carried out. In the present study examined gene expression was examined for 30 metabolic enzyme consisting of eight metabolic enzyme genes that participate in the glycolytic pathway; three metabolic enzyme genes of the oxidative pentose phosphate pathway (OPPP) and; six metabolic enzyme genes participating in the reductive pentose phosphate cycle (RPPC); sucrose phosphatase (SPase, EC 3.1.3.24) participating in sucrose synthesis; ADP-glucose pyrophosphorylase (ADPase, EC 2.7.7.9) and starch synthase (SS, EC 2.4.1.21) which participate in starch synthesis; six metabolic enzyme genes of the tricarboxylic acid (TCA) cycle and four metabolic enzyme genes of the secondary metabolism. Figure 2.1 shows the location of these metabolic

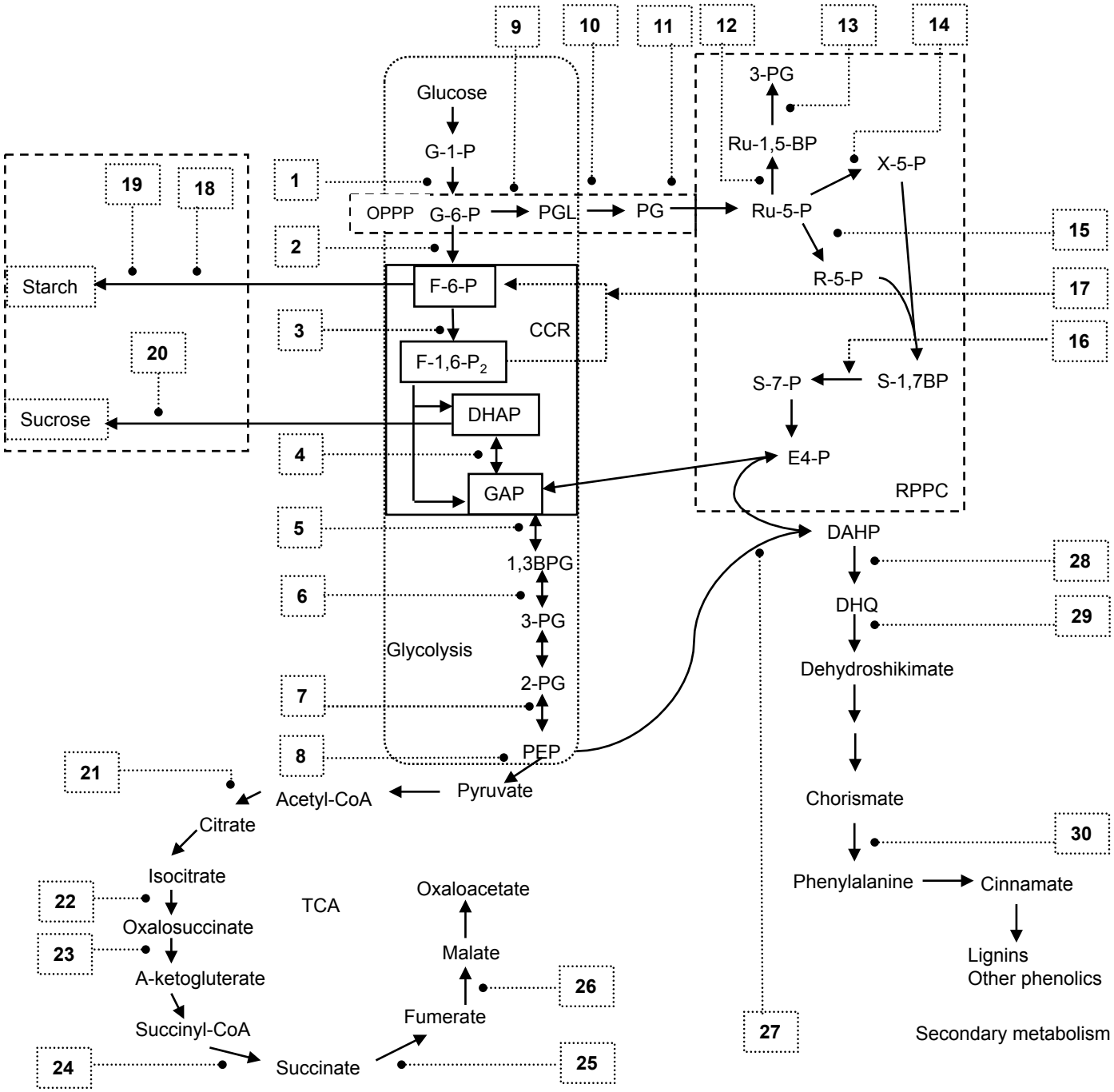


Figure 2.1. Glycolytic, oxidative and reductive pentose phosphate, shikimate, phenylpropanoid and TCA metabolic pathways showing the position of the metabolic enzyme genes examined in the present study. **Glycolytic pathway: 1:** phosphoglucomutase (PGM, EC 2.7.5.1), **2**: phosphoglucose isomerase (PGI, EC 5.3.1.9), **3**: 6-phosphofructokinase (PFK, EC 2.7.1.11), **4**: triosephosphate isomerase (TPI, EC 5.3.1.1), **5**: glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), **6**: phosphoglycerate kinase (PGK, EC 2.7.2.3), **7**: enolase (EC 4.2.1.11), **8**: pyruvate kinase (PK, EC 2.7.1.40); **Oxidative pentose phosphate pathway (OPPP): 9:** glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), **10:** 6-phosphogluconolactonase (PGLase, EC 3.1.1.31), **11:** 6-phosphogluconate dehydrogenase (PGDH, EC 1.1.1.43); **Reductive pentose phosphate cycle (RPPC)**: **12:** phosphoribulokinase (PRK, EC 2.7.1.19), **13:** ribulose-bisphosphate carboxylase (Rubisco, EC 4.1.1.39), **14:** ribulose-phosphate-3-epimerase (RPE, EC 5.1.3.1), **15:** ribose-5-phosphate isomerase (RPI, EC 5.3.1.6), **16:** sedoheptulose-1,7-bisphosphatase (SBPase, EC 3.1.3.37), **17:** fructose-1,6 -bisphosphatase (FBPase, EC 3.1.3.11), **Starch synthesis: 18:** starch synthase (SS, EC 2.4.1.21), **19:** ADP-glucose pyrophosphorylase (ADPase, EC 2.7.7.9), **Sucrose synthesis: 20:** sucrose phosphatase (SPase, EC 3.1.3.24); **TCA: 21:** citrate synthase (CS, EC 2.3.3.1), **22:** isocitrate dehydrogenase (IDH, EC 1.1.1.41), **23:** oxoglutamate dehydrogenase (ODH, EC 1.2.42), **24:** succinyl-CoA synthetase (SCS, EC 6.2.1.4), **25:** succinate dehydrogenase (SDH, EC 1.3.5.1), **26:** fumarate hydratase (FH, EC 4.2.1.2); **Secondary metabolism: 27:** 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHP synthase, EC 2.5.1.54), **28:** 3-Dehydroquinate synthase (DHQ synthase, EC 4.2.3.4), **29:** 3-dehydroquinate hydratase (DHQase, EC 4.2.1.10), **30:** Phenylalanine ammonialyase (PAL, EC 4.3.1.24). Regarding the subcellular localization, glycolysis and sucrose synthesis take place in the cytosol, starch synthesis and secondary metabolism take place in the plastids while TCA cycle occurs in the mitochondria. PPP, pentose phosphate pathway, comprises oxidative and reductive pentose phosphate pathways. Adapted from Nose *et al.* (2002*a*).

enzymes genes in the metabolic pathways.

Gene expressions in leaf sheaths of *R. solani*-infected plants of *R. solani*-resistant and susceptible rice lines was compared to that of their respective control plants and the changes in the time course of expressions were recorded at 1 day post-inoculation (dpi), 2 dpi and 4 dpi.

## 2. Materials and Methods

### 2-1. Sample preparation

Samples used for this experiment consisted of the $2F_{18}$-7-32 (32) and $2F_{21}$-21-29 (29) rice lines, which were selected for their resistance and susceptibility to sheath blight disease, respectively (Wasano *et al.*, 1985). The two lines were grown in pots under 16 h of light and 8 h of darkness at 25 $^{o}$C. The pots contained 1:1 ratio of peat moss to vermiculite. The plants were transferred to an incubator at 28 $^{o}$C when they reached the 7-leaf growth stage.

The use of potato-sucrose-agar medium (PSA), inoculum preparation and inoculation followed the description of Wasano *et al.* (1983). Briefly, sheath blight fungus isolate Rck-1-2, a member of anastomosis group AG-1 from Kyushu Agricultural Experiment Station, Chikugo, Japan, was grown on PSA at 28 °C for about 4 days. It was then chopped into small pieces, introduced into a syringe and 0.25 mL of the fungal suspension was injected into the interstices between the second and the third leaf sheaths from the flag leaf. Control plants were treated under the same conditions except that the pathogen preparation was replaced with distilled water. It was then

chopped into small pieces, introduced into a syringe and 0.25 mL of the fungal suspension was injected into the interstices between the second and the third leaf sheaths from the flag leaf and control plants, and immediately plunged into liquid nitrogen after one day, two days and four days post- inoculation (dpi). Time points were selected based on the findings reported by Danson (2000*c*) who noted that in a sheath blight resistant rice line, at 1 dpi, the activity of the enzymes responsible for resistance to *R. solani* infection increased and that the activity was maximum at 4 dpi. The samples were then ground into a fine powder using a pestle and mortar pre-cooled in liquid nitrogen and stored at -80 $^{o}$C until use.

**2-2. Total RNA isolation**

Total RNA was isolated from the leaf sheaths using an extraction buffer consisting of 6 M guanidine isothiocynate, 0.75 M sodium citrate pH 7, 10 % sarcosyl and diethylpyrocarbonate (DEPC) water, phenol and *ß*-mecarptoethanol. Total RNA extraction was performed as follows; 1.2 mL of extraction buffer was pipetted into 2 mL Eppendorf tubes, after which about 0.2 g of ground sample was added. These were vortexed and incubated for 10 min at room temperature (RT). To the mixture, 300 µL of a chloroform - isoamyl alcohol solution was added; the mixture was vortexed and incubated for 15 min at RT. This mixture was then centrifuged at 28, 000 X *g* for 20 min, using a refrigerated centrifuge (TOMY High Speed Refrigerated Micro Centrifuge, MX-150, TOMY Tech., Japan) at 4 $^{o}$C. About 0.4-0.5 mL of the supernatant was transferred into new 1.5 mL Eppendorf tubes. RNA precipitation was achieved by the addition of 0.4 mL of isopropanol and 40 µL of 2 M sodium acetate. The mixture was inverted slowly several times and incubated for 10 min at RT and

then centrifuged at 28, 000 X *g* for 20 min at 4 $^{o}$C. The liquid phase was decanted and the pellet was rinsed by the addition of 1.0 mL of 75 % ethanol and centrifuged at 28, 000 X *g* for 5 min at 4 $^{o}$C. Ethanol was completely removed by centrifuging the samples at RT for 3 min, using a centrifugal evaporator (RD 400, Yamato, Japan) and about 30 µL of distilled water was added to dissolve the pellet. The resultant RNA was quantified using GeneQuant$^{TM}$ RNA/DNA calculator (Amersham Pharmacia Biotech), the tubes were labelled and stored at -80 $^{o}$C until use.

**2-3. Optimisation of RT-PCR**

The RNA was treated with deoxyribonuclease (DNase I) (Stratagene) to remove contaminating DNA and used for RT-PCR in a two-step reaction with a kit from Toyobo (Toyobo, Japan), according to the manufacturer's instructions. The reverse transcription step was performed in a 20 µL volume containing 3 µg of DNase I-treated RNA, 0.5 µg of oligo $(dT)_{20}$ primer, 50 U of reverse transcriptase, first strand buffer, 0.5 mM dNTP mix and 40 U of ribonuclease inhibitor. This mixture was incubated at 42 $^{o}$C for 90 min and at 94 $^{o}$C for 5 min. Synthesized cDNA was then used in PCR where fragments corresponding to the genes of interest were amplified using a Bio-rad iCycler Thermal cycler (Bio-rad, USA). Each RNA sample was tested for DNA contamination by replacing the reverse transcriptase in the RT-PCR with distilled de-ionised water.

**2-4. Optimisation of PCR**

Optimisation of PCR was carried out as described by Pfaffl (2001) with some modifications. The primer sequences of the reference gene, Actin, were 5′-TCGTCGTACTCAGCCTTGGCAAT-3′ for the reverse primer and 5′-CTCTCTGTATGCCAGTGGTCGTA-3′ for the forward primer. Actin PCR cycle was optimised to 95 $^{o}$C as initial denaturation for 2 min, denaturation at 95 $^{o}$C for 30 sec, annealing at 58 $^{o}$C for 30 sec, extension at 72 $^{o}$C for 1 min, repeated for 27 cycles. The final extension was performed at 72 $^{o}$C for 5 min.

Conditions for all the other PCRs were optimised in a Bio-rad iCycler Thermal cycler (Bio-rad, USA) with regard to *Taq* DNA Polymerase (Promega, USA), forward and reverse primers, $MgCl_2$ concentrations (Toyobo, Japan), dNTP concentration (Takara, Japan) and various annealing temperatures (55-65 $^{o}$C). The reaction products were electrophoresed in 1.2 % (w/v) agarose/ TAE gels and visualized by ethidium-bromide staining. Levels of mRNA expression were determined by normalization to Actin, as described by Hirose et al. (2006). The expression values obtained in different cDNAs were standardized so that the sum of the values in the expression level of Actin in these cDNAs was equal to one. This set of cDNAs was used to obtain the primer-specific expression patterns of each metabolic enzyme gene. Levels of mRNA expression of the metabolic enzyme genes were given as fold changes relative to the levels of expression of Actin gene. Metabolic enzymes genes were selected from the Kyoto Encyclopaedia of Genes and Genomes (http://www.genome.jp/kegg/) and Cellular Function and Metabolic Map (Japan Biochemistry Society, 1997). Using NCBI Blast (http://blast.ncbi.nlm.nih.gov/) and

GENETEX-WIN genetic information processing software (Software Development, Tokyo, Japan) metabolic enzymes with *Oryza sativa* genetic information were analyzed to identify regions of highest homology to other plant species. This information was then used in Primer-3 (http://frodo.wi.mit.edu/primer3/) to determine base pair sequences of primer pairs.

Data did not deal with the changes in the levels of mRNA expression due to changes such as post-translational modifications e.g. phosphorylation and feedback regulation, etc. As described by Wang *et al.* (1989), Pfaffl (2001), Tian *et al.* (2004), mRNA profiling alone provides a wealth of information for gene expression studies, although addressing discrepancies associated with posttranscriptional, translational, or posttranslational regulation is sometimes necessary. Metabolic enzymes sequences were selected as common sequences among the various isozymes in subcellular compartments in order to reduce the problems associated with compartmentation.

**2-5. Data analysis**

Gels were analyzed using Kodak 1D Image Analysis Software version 3.5 (Scientific Imaging Systems Eastman Kodak Co. NY), according to the manufacturer's manual. Statistical analyses were performed using Excel (Microsoft, USA) and significant levels were tested at $P < 0.05$ using the Student's *t*-test.

**2-6. Direct sequencing of PCR products**

DNA used for the reactions described above was stored and used for direct sequencing. The following method was adopted: for a 20 μL reaction, 15-150 ng of PCR product was mixed with 1.5 pmol of primer, 2 μL of BigDye® sequencing buffer (Applied Biosystems, USA), 4 μL of terminator reaction mix (Applied Biosystems, USA) and water. These were incubated in a PCR reaction consisting of denaturation at 96 $^{o}$C for 1 min, annealing at 58 $^{o}$C for 30 sec and extension at 60 $^{o}$C for 4 min for a total of 30 cycles. Ethanol precipitation was achieved by transferring the products obtained into new 1.5 mL Eppendorf tubes and mixing with 0.25 mM EDTA, 100 mM ammonium acetate and 50 μL of 100 % ethanol. The mixture was then vortexed and incubated at RT for 15 min. After incubation, the mixture was centrifuged at 28, 000 *g* for 20 min at RT to form a pellet. Ethanol was decanted and 200 μL of 75 % ethanol was added to the pellet. This mixture was centrifuged at 28, 000 *g* for 5 min at RT to wash the pellet. Ethanol was decanted and the pellet was dried using a centrifugal evaporator (RD 400, Yamato, Japan), dissolved in 25 μL of Hi-Di ™ formamide (Applied Biosystems, UK), and vortexed. The samples were heat-shocked for 5 min in a heating block (Dry ThermoUnit DTU-1B, Taitec Corp., Japan) set at 98 $^{o}$C and kept on ice until they were transferred to the Applied Biosystems ™ 310 sequencing machine. Sequencing data were copied to Rice Annotation Project Database (RAP-DB) (http://rapdb.dna.affrc.go.jp) to confirm gene names, description and RAP-DB numbers, as listed in Tables 2.1, 2.2 and 2.3.

Table 2.1. Metabolic enzyme genes of glycolytic pathway and oxidative pentose phosphate pathway (OPPP) examined in the study. The sequences were used to obtain gene names and description from the Rice Annotation Project Database (RAP-DB) (http://rapdb.dna.affrc.go.jp)

| Description | Sequences | RAP-DB Number |
|---|---|---|
| **Glycolysis** | | |
| 1: Phosphoglucomutase (PGM, EC 2.7.5.1) | Forward TCGCAACATGATTCTGGGTA<br>Reverse TTATCGCCTCCAAGGTTGTC | Os03g0712700 |
| 2: Phosphoglucose isomerase (PGI, EC 5.3.1.9) | Forward AGTGGAGGCACACCTGAAAC<br>Reverse AGCATAAATCCCCACAGCAC | Os09g0465600 |
| 3: 6-Phosphofructokinase (PFK, EC 2.7.1.11) | Forward CTGGGTACATGGCAACTGT<br>Reverse CCAGCAACGAACAGAAATGA | Os08g0345700 |
| 4: Triosephosphate isomerase (TPI, EC 5.3.1.1) | Forward ATGGGCCGCAAGTTCT<br>Reverse TGATTCAGCAACCTCTGCAC | Os01g0147900 |
| 5: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) | Forward TCAAATGCTAGCTGCACCAC<br>Reverse GCTGTAACCCCACTCGTTGT | Os08g0440800 |
| 6: Phosphoglycerate kinase (PGK, EC 2.7.2.3) | Forward TCTTGTTCCACGCTTGTCTG<br>Reverse GCATATTCCCTGGGCTTTGTA | Os02g0169300 |
| 7: Enolase (EC 4.2.1.11) | Forward TTGCTGGAAACAAGCAACTG<br>Reverse CCAATTTCAGCGGTCATCTT | Os10g0167300 |
| 8: Pyruvate kinase (PK, EC 2.7.1.40) | Forward TGGAGGTAGATGCCGTTTTC<br>Reverse AGCGACAAAACAGTTCATGG | Os07g018100 |
| **Oxidative Pentose Phosphate Pathway (OPPP)** | | |
| 9: Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) | Forward ACGCAGCAAGATGACAGATG<br>Reverse TGAGAAGCGAAGGACAGACA | Os07g0406300 |
| 10: 6-Phosphogluconolactonase (PGLS, EC3.1.1.31) | Forward TCTTATGCTTCTGGGGATGG<br>Reverse TCCAAGCGCATGACAAATAC | Os08g0547100 |
| 11: 6-Phosphogluconate dehydrogenase (PGDH, EC 1.1.1.43) | Forward ATCTTGGGATGGGTGTTTCC<br>Reverse GGCAAGTTGCTGGAGAAGTC | Os06g0111500 |

Table 2.2. Metabolic enzyme genes of reductive pentose phosphate cycle (RPPC), starch and sucrose synthesis, and TCA cycle examined in the study. The sequences were used to obtain gene names and description from the Rice Annotation Project Database (RAP-DB) (http://rapdb.dna.affrc.go.jp)

| Description | Sequences | RAP-DP Number |
|---|---|---|
| **Reductive Pentose Phosphate Cycle (RPPC)** | | |
| 12: Phosphoribulokinase (PRK, EC 2.7.1.19) | Forward CAGCAGGTAGAAAGGCCAGA<br>Reverse TTGATGCTTTCAAGGCTGTG | Os02g0698000 |
| 13: Ribulose-bisphosphate carboxylase (Rubisco, EC 4.1.1.39) | Forward TTCATGGACAAGCTCGTC<br>Reverse TTGACGTTCTCCTGCTCCTT | Os11g0707000 |
| 14: Ribulose-phosphate-3-epimerase (RPE, EC 5.1.3.1) | Forward TTTGGTTGTTGATGCTCTGC<br>Reverse CCCAGCCTTCAATGACCTTGT | Os03g0169100 |
| 15: Ribose-5-phosphate isomerase (RPI, EC 5.3.1.6) | Forward GCTCAGGGAGAAGATGATC<br>Reverse GTCGCTGATCTCGTTGAGGT | Os04g0306400 |
| 16: Sedoheptulose-1,7-bisphosphatase (SBPase, EC 3.1.3.37) | Forward CTCTTGATGGGTCCAGCATT<br>Reverse ACCGGATGATCTCGTTCTTG | Os04g0234600 |
| 17: Fructose-1,6-bisphosphatase (F-1,6BPase, EC 3.1.3.11) | Forward TCGGAGGAGGAGGACGTG<br>Reverse CCGTTCTTGCTCTTCTGGTC | Os03g0267300 |
| **Starch and Sucrose Synthesis** | | |
| 18: ADP-glucosepyrophosphorylase (ADPase, EC 2.7.7.9) | Forward AGATCACCTTTACCGCATGG<br>Reverse CTTCGAAGGAGGCAAGTGTC | Os08g0345800 |
| 19: Starch synthase (SS, EC 2.4.1.21) | Forward TGATGGTTGTAATGCCGAGA<br>Reverse AATTGACTGCCTCACCCTTG | Os06g0160700 |
| 20: Sucrose phosphatase (SPase, EC 3.1.3.24) | Forward CTTAGGTTCCAGGCGCTATG<br>Reverse TCTGCATCATTGCCAGAGTC | Os05g0144900 |
| **TCA** | | |
| 21: Citrate synthase (CS, EC 2.3.3.1) | Forward ATGGTTTAGCTGGACCGTT<br>Reverse TCCCAAATGAGCTGAGATC | Os02g0194100 |
| 22: Isocitrate dehydrogenase (IDH, EC 1.1.1.41) | Forward GCTGGAGGAGTTGCTTTGTC<br>Reverse TGTGTGCAAGTCCTCTGGTC | Os01g0654500 |
| 23: Oxoglutamate dehydrogenase (ODH, EC 1.2.42) | Forward TTGGCTGAGGGATAGGATT<br>Reverse CCATCACCATGCAACAAGA | Os04g0390000 |
| 24: Succinyl-CoA synthetase (SCS, EC 6.2.1.4) | Forward CCATCACCCTTGACAGGAAT<br>Reverse AATGCTTCCACAACCTGTC | Os02g0621700 |
| 25: Succinate dehydrogenase (SDH, EC 1.3.5.1) | Forward CCACATGTTCGTCATCAAGG<br>Reverse AATTATTCGTCAGGCGATGG | Os08g0120000 |
| 26: Fumarate hydratase (FH, EC 4.2.1.2) | Forward AATTGGGCGTACACATACC<br>Reverse CCCAGGCTCATTTTCAGGTA | Os03t03379001 |

Table 2.3. Metabolic enzyme genes of secondary metabolism examined in the study. The sequences were used to obtain gene names and description from the Rice Annotation Project Database (RAP-DB) (http://rapdb.dna.affrc.go.jp)

| Description | Sequences | RAP-DB Number |
|---|---|---|
| **Secondary metabolism** | | |
| 27: 3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHP synthase, EC 2.5.1.54) | Forward TACACGTGAGGATTCCACCA<br>Reverse GTTCAGCCTTGGGTCACAGT | Os07t0622200 |
| 28: 3-Dehydroquinate synthase (DHQ synthase, EC 4.2.3.4) | Forward AGGCATGTTCATGGGAAGAG<br>Reverse ACACTGTGGCTGGTAGAACG | Os09g0539100 |
| 29: 3-Dehydroquinate hydratase (DHQase, EC 4.2.1.10) | Forward GAGGCCAGTACGATGGTGAT<br>Reverse TTGGGCTCTTGCTCTGTTTT | Os01g0375200 |
| 30: Phenylalanine ammonialyase (PAL, EC 4.3.1.24) | Forward GTGTTCTGCGAGGTGATGAA<br>Reverse CATGAGCTTGAGGATGTCCA | Os05g0427400 |

## 3. Results

Figure 2.2 shows the changes in the time course of expression of glycolytic metabolic enzyme genes: phosphoglucomutase (PGM, EC 2.7.5.1), phosphoglucose isomerase (PGI, EC 5.3.1.9), 6-phosphofructokinase (PFK, EC 2.7.1.11), triosephosphate isomerase (TPI, EC 5.3.1.1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), phosphoglycerate kinase (PGK, EC 2.7.2.3), enolase (EC 4.2.1.11), pyruvate kinase (PK, EC 2.7.1.40) in leaf sheaths of *R. solani*-infected and control plants at 1, 2 and 4 dpi. The results showed that there were significant differences ($P < 0.05$) in the levels of expression of PFK, PGK, enolase and PK between the *R. solani*-infected plants of the resistant rice line and those of the susceptible rice line. In the all cases, higher gene expressions were detected in the *R. solani*-infected plants of the resistant rice line compared to those of the susceptible rice line. In addition, expressions of PFK, PGK, enolase and PK was significantly higher ($P < 0.05$) in the *R. solani*-infected plants of both resistant and susceptible rice lines at 1 to 4 dpi, compared to those of the control plants. On the other hand, the time course of expressions of PGM and GAPDH revealed that the expression were lower in the *R. solani*- infected plants of both resistant and susceptible rice lines, compared to those of the control plants. Time course of expression of PGI revealed that the expression was similar between the *R. solani*-infected plants and that of the control plants of both resistant and susceptible rice lines. Time course of expression of TPI showed that the expression increased at 4 dpi in leaf sheaths of *R. solani*-infected plants of both resistant and susceptible rice lines, compared to 1 dpi levels.

Figure 2.3 shows the changes in the time course of expressions of OPPP metabolic enzyme genes: glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49),

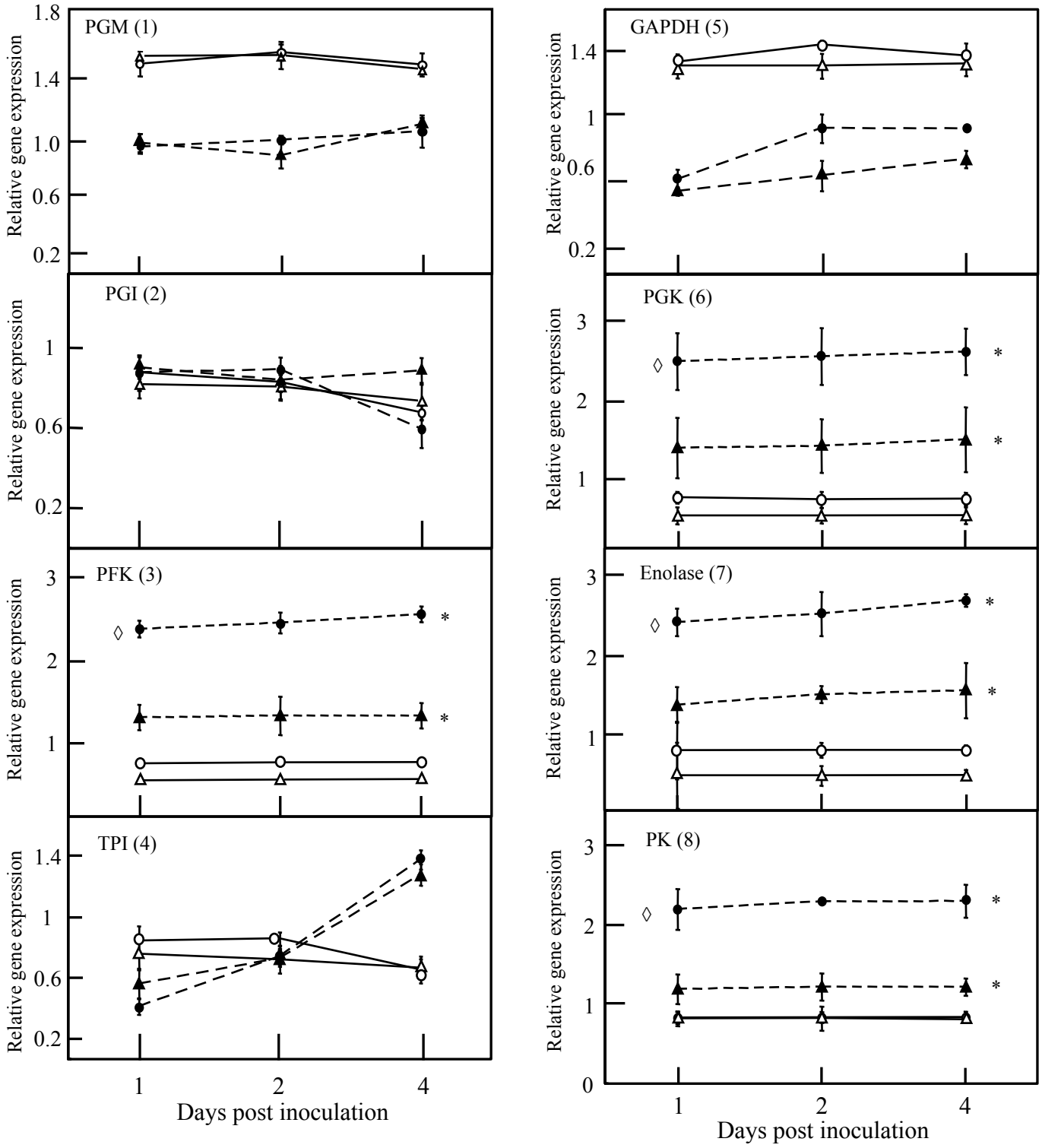


Figure 2.2. Time course changes in relative mRNA expression of glycolytic metabolic enzyme genes at 1 dpi, 2 dpi and 4 dpi in *R. solani*-infected samples of the *R. solani*-resistant and susceptible rice lines (closed circles, closed triangles), respectively, and those of control samples (open circles, open triangles), respectively. Relative mRNA expression was measured by normalization to Actin gene, as described in Materials and Methods. Values were means ± SD of three separate determinations including 50 samples. ***** Relative mRNA expression was significantly different ($P < 0.05$) between the *R. solani*-infected samples of both rice lines compared to that of the control samples at 1 dpi, 2 dpi and 4 dpi. ◊ Relative mRNA expression was significantly different ($P < 0.05$) between the *R. solani*-infected samples of the resistant rice line and that of the susceptible rice line. **PGM**: phosphoglucomutase (PGM, EC 2.7.5.1); **PGI**: phosphoglucose isomerase (PGI, EC 5.3.1.9); **PFK**: 6 -phosphofructokinase (PFK, EC 2.7.1.11); **TPI**: triosephosphate isomerase (TPI, EC 5.3.1.1); **GAPDH**: glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12); **PGK**: phosphoglycerate kinase (PGK, EC 2.7.2.3); enolase (EC 4.2.1.11); **PK**: pyruvate kinase (PK, EC 2.7.1.40). The numbers following the enzyme name abbreviations in the figures are the same as those showing the enzyme location in Figure 2.1.

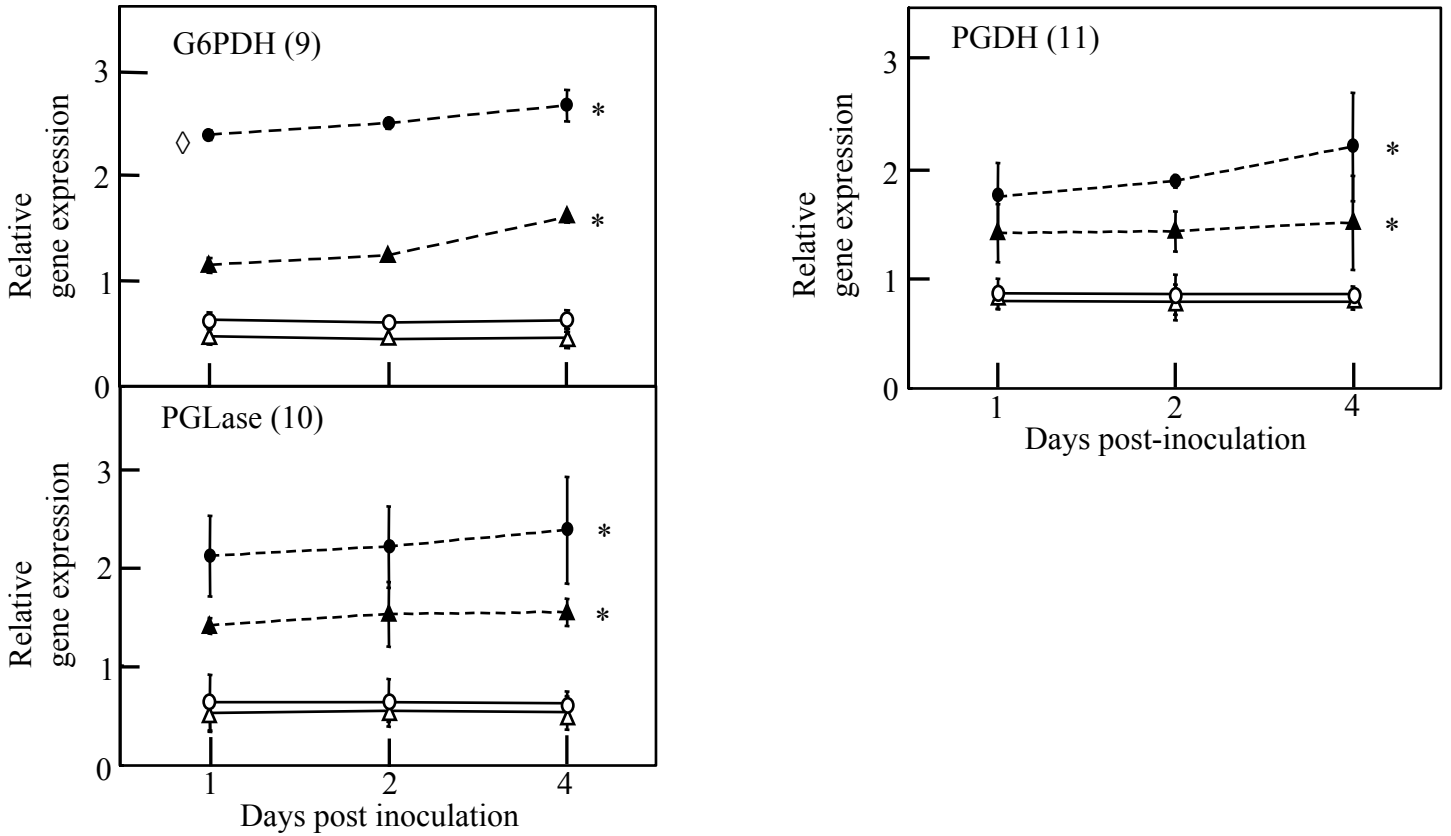


Figure 2.3. Time course changes in relative expression of oxidative pentose phosphate (OPPP) metabolic enzyme genes at 1 dpi, 2 dpi and 4 dpi in leaf sheaths of *R. solani*-infected plants of the *R. solani*-resistant and susceptible rice lines (closed circles, closed triangles), respectively, and those of control plants (open circles, open triangles), respectively. Relative expression was measured by normalization to Actin gene, as described in Materials and Methods. Values were means ± SD of three separate determinations including 50 plants. ***** Relative expression was significantly different ($P < 0.05$) between the *R. solani*-infected plants of both rice lines, compared to that of the control plants at 1 dpi, 2 dpi and 4 dpi. $^{\lozenge}$ Relative expression was significantly different ($P < 0.05$) between the *R. solani*-infected plants of the resistant rice line and that of the susceptible rice line. **G6PDH**: glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49); **PGLase**: 6-phosphogluconolactonase (PGLase, EC 3.1.1.31), **PGDH**: 6-phosphogluconate dehydrogenase (PGDH, EC1.1.1.44). The numbers following the enzyme name abbreviations in the figures are the same as those showing the enzyme location in Figure 2.1.

6-phosphogluconolactonase (PGLase, EC 3.1.1.31) and 6-phosphogluconate dehydrogenase (PGDH, EC 1.1.1.44) after *R. solani* infection in both resistant and susceptible rice lines. Expression of G6PDH was significantly higher ($P < 0.05$) in leaf sheaths of *R. solani*-infected plants of the resistant rice line, compared to that of the susceptible rice line. Also, expressions of G6PDH, PGLase and PGDH were significantly higher ($P < 0.05$) in leaf sheaths of *R. solani*-infected plants of both resistant and susceptible rice lines, compared to those of the respective control plants.

Figure 2.4 shows the changes in time the course of expressions of RPPC metabolic enzyme genes: phosphoribulokinase (PRK, EC 2.7.1.19), ribulose-bisphosphate carboxylase (Rubisco, EC 6.3.4.), ribulose-phosphate-3-epimerase (RPE, EC 5.1.3.1), ribose-5-phosphate isomerase (RPI, EC 5.3.1.6), sedoheptulose-1,7-bisphosphatase (SBPase, EC 3.1.3.37) and fructose-1,6-bisphosphatase (F-1,6BPase, EC 3.1.3.11) in leaf sheaths of *R. solani*-infected and control plants at 1 to 4 dpi. Changes in the time course of expressions showed that expressions of these reductive pentose phosphate cycle metabolic enzyme genes were lower in leaf sheaths of *R. solani*-infected plants of both resistant and susceptible rice lines, compared to those of the control plants at 1 to 4 dpi. Expressions of PRK, Rubisco and RPI were significantly lower ($P < 0.05$) in the *R. solani*-infected plants, compared to those of the control plants at 1 to 4 dpi.

Figure 2.5 shows the changes in the time course of expressions of metabolic enzyme genes, ADP-glucose pyrophosphorylase (AGPase, EC 2.7.7.27), starch synthase (SS, EC 2.4.1.21) and sucrose phosphatase (SPase, EC 3.1.3.24). Changes in expressions after infection showed that SPase was significantly higher ($P < 0.05$) in leaf sheaths of *R. solani*-infected plants of the resistant rice line, compared the susceptible rice line.

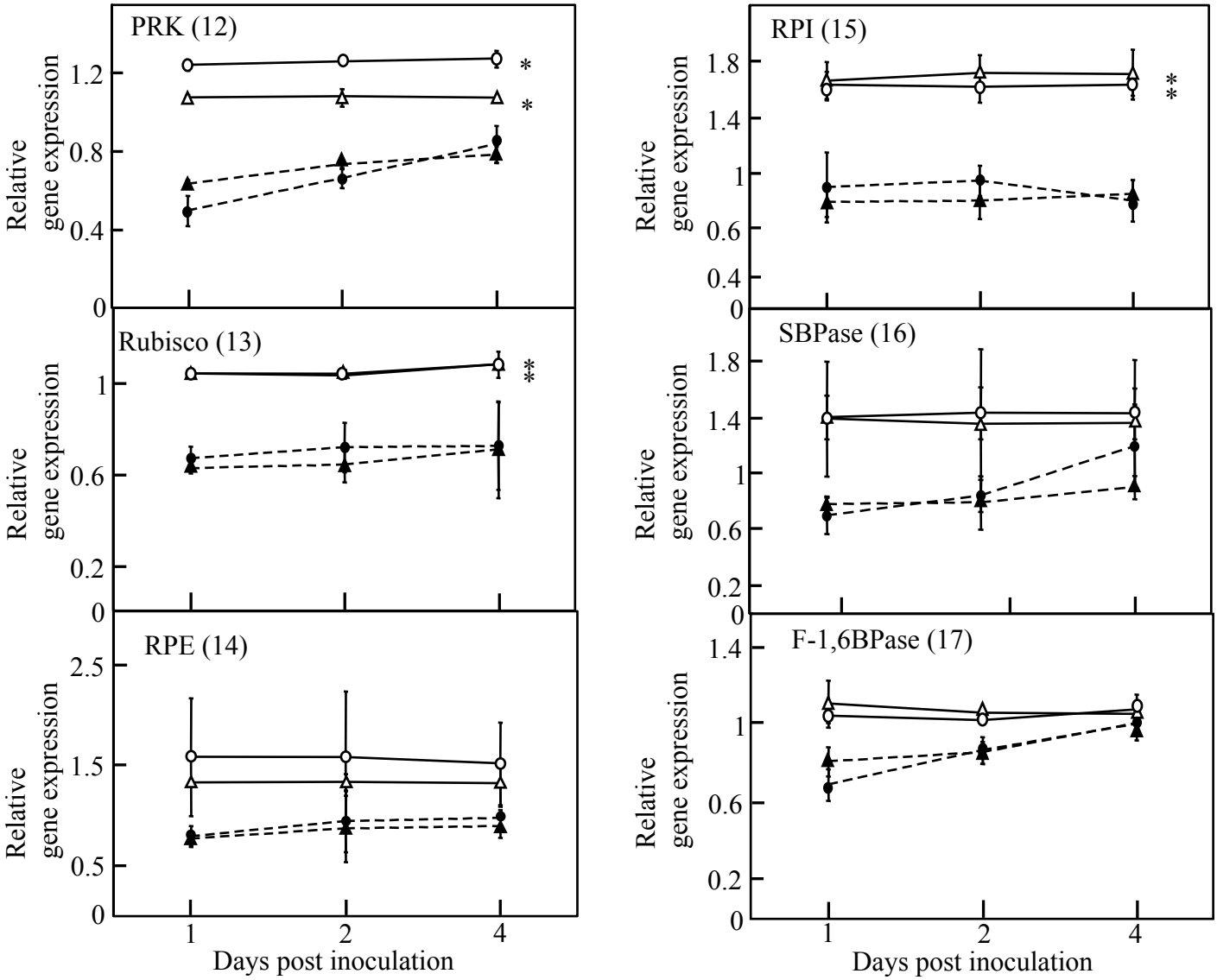


Figure 2.4. Time course changes in relative expression of reductive pentose phosphate cycle (RPPC) and non-oxidative PPP metabolic enzyme genes at 1 dpi, 2 dpi and 4 dpi in leaf sheaths of *R. solani*-infected plants of the *R. solani*-resistant and susceptible rice lines (closed circles, closed triangles), respectively, and those of control plants (open circles, open triangles), respectively. Relative expression was measured by normalization to Actin gene, as described in Materials and Methods. Values were means ± SD of three separate determinations including 50 plants. ***** Relative expression was significantly different ($P < 0.05$) between the *R. solani*-infected plants of both rice lines compared to that of the control plants at 1 dpi, 2 dpi and 4 dpi. **F-1,6BPase**: fructose-1,6 -bisphosphatase (F-1,6BPase, EC 3.1.3.11); **PRK**: phosphoribulokinase (PRK, EC 2.7.1.19); **Rubisco**: ribulose-bisphosphate carboxylase (Rubisco, EC 6.3.4.); **RPE**: ribulose-phosphate-3 -epimerase (RPE, EC 5.1.3.1); **RPI**: ribose-5-phosphate isomerase (RPI, EC 5.3.1.6); **SBPase**: sedoheptulose-1,7-bisphosphatase (SBPase, EC 3.1.3.37). The numbers following the enzyme name abbreviations in the figures are the same as those showing the enzyme location in Figure 2.1.

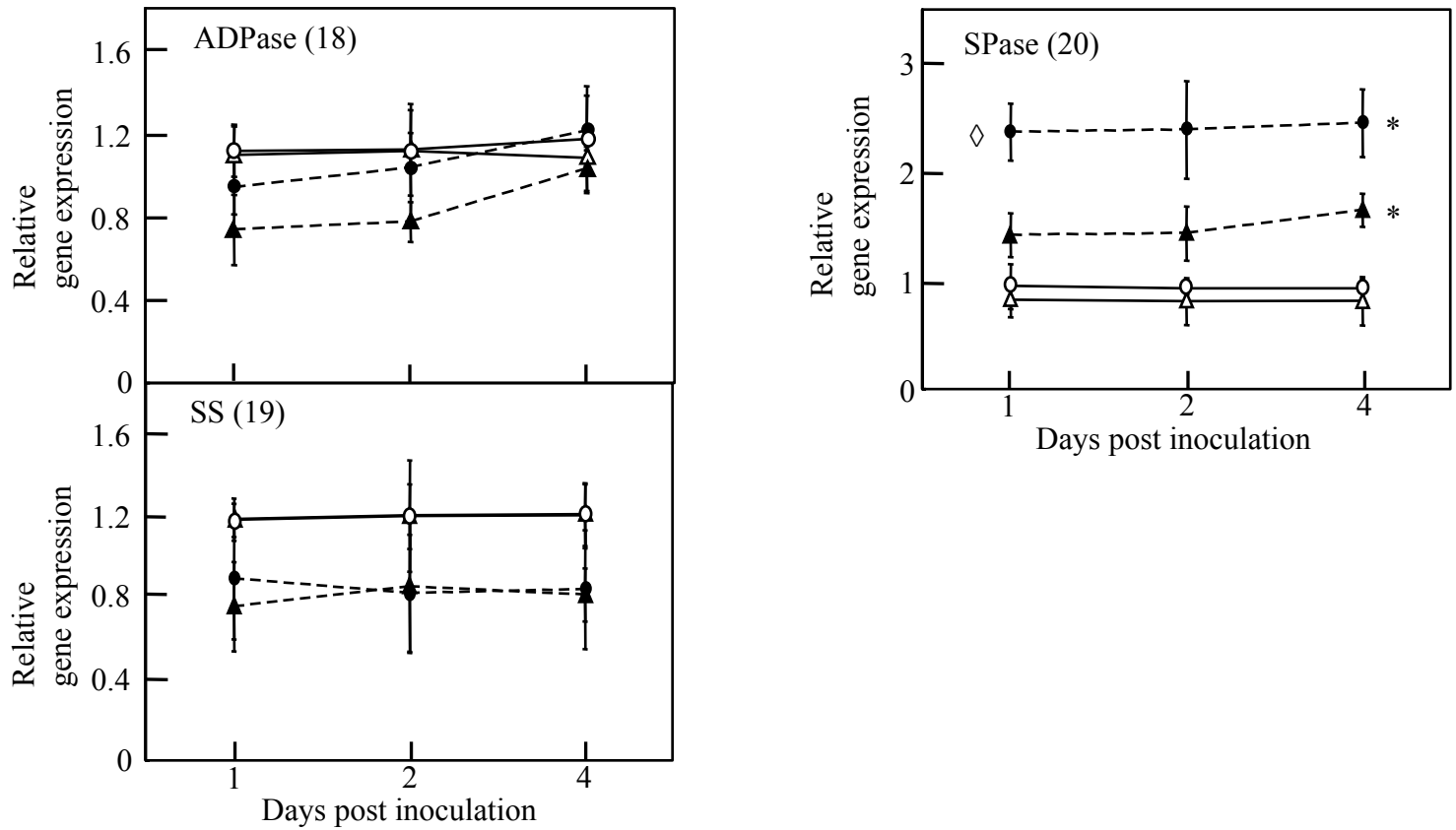


Figure 2.5. Time course changes in relative expression of starch and sucrose synthesis metabolic enzyme genes at 1 dpi, 2 dpi and 4 dpi in leaf sheaths of *R. solani*-infected plants of the *R. solani*-resistant line and susceptible rice lines (closed circles, closed triangles), respectively, and those of control plants (open circles, open triangles), respectively. Relative expression was measured by normalization to Actin gene, as described in Materials and Methods. Values were means ± SD of three separate determinations including 50 plants. ***** Relative expression was significantly different ($P < 0.05$) between the *R. solani*-infected plants of both rice lines compared to that of the control plants at 1 dpi, 2 dpi and 4 dpi. ◊ Relative expression was significantly different ($P < 0.05$) between the *R. solani*-infected samples of the resistant rice line and that of the susceptible rice line; **ADPase**: ADP-glucose pyrophosphorylase (ADPase, EC 2.7.7.27) and **SS**: starch synthase (SS, EC 2.4.1.21); **SPase**: sucrose phosphatase (SPase, EC 3.1.3.24). The numbers following the enzyme name abbreviations in the figures are the same as those showing the enzyme location in Figure 2.1.

Furthermore, the expression of SPase was higher in leaf sheaths of *R. solani*-infected plants of both resistant and susceptible rice lines, compared to the respective control plants. The expressions of AGPase and SS were lower in leaf sheaths of *R. solani*-infected plants, compared to the control samples at 1 to 4 dpi.

Figure 2.6 shows the changes in the time course of expression of the metabolic enzyme genes of the TCA cycle: citrate synthase (CS, EC 4.1.3.7), isocitrate dehydrogenase (IDH, EC 1.1.1.41), oxoglutamate dehydrogenase (ODH, EC 1.1.1.42), succinyl-CoA synthetase (SCS, EC 6.2.1.4), succinate dehydrogenase (SDH, EC 1.2.4.2) and fumarate hydratase (FH, EC 4.2.1.2) in leaf sheaths of *R. solani*-infected plants of the resistant and susceptible rice lines and the respective control plants at 1, 2 and 4 dpi.

The changes in the time course showed that expression of CS was higher in leaf sheaths of *R. solani*-infected plants of the resistant and susceptible rice lines, compared to that of the control plants, with the resistant rice line showing a significantly higher ($P < 0.05$) expression than the susceptible rice line at 1 to 4 dpi. The expressions of IDH, ODH, SCS, SDH and FH were significantly higher ($P < 0.05$) in leaf sheaths of *R. solani*-infected plants of both rice lines, compared to control plants.

Figure 2.7 shows the changes in the time course of expression of the metabolic enzyme genes of secondary metabolism: 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHP synthase, EC 2.5.1.54), 3-dehydroquinate synthase (DHQ synthase, EC 4.2.3.4), 3-dehydroquinate dehydratase (DHQase, EC 1.1.1.25) and

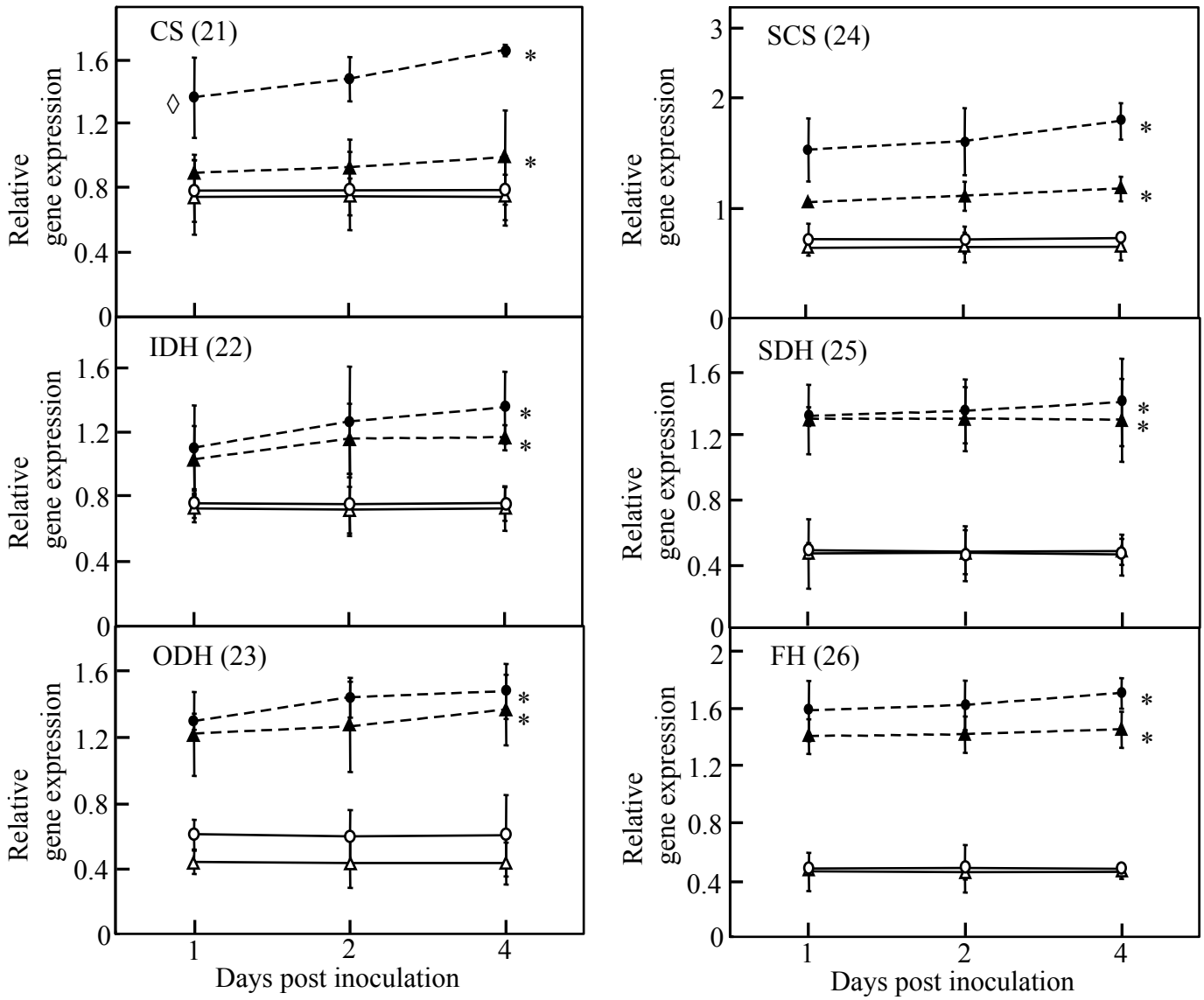


Figure 2.6. Time course changes in relative expression of TCA metabolic enzyme genes at 1 dpi, 2 dpi and 4 dpi in leaf sheaths of *R. solani*-infected plants of the *R. solani*-resistant line and susceptible rice lines (closed circles, closed triangles), respectively, and those of control plants (open circles, open triangles), respectively. Relative expression was measured by normalization to Actin gene, as described in Materials and Methods. Values were means ± SD of three separate determinations including 50 plants. ***** Relative expression was significantly different ($P < 0.05$) between the *R. solani*-infected plants of both rice lines compared to that of the control plants at 1 dpi, 2 dpi and 4 dpi. ◊ Relative expression was significantly different ($P < 0.05$) between the *R. solani*-infected plants of the resistant rice line and that of the susceptible rice line. **CS**: citrate synthase (CS, EC 4.1.3.7), **IDH**: isocitrate dehydrogenase (IDH, EC 2.3.3.8), **ODH**: oxoglutamate dehydrogenase (ODH, EC 1.1.1.42), **SCS**: succinyl-CoA synthetase (SCS, EC 6.2.1.4), **SDH**: succinate dehydrogenase (SDH, EC 1.2.4.2), and **FH**: fumarate hydratase (FH, EC 4.2.1.2). The numbers following the enzyme name abbreviations in the figures are the same as those showing the enzyme location in Figure 2.1.

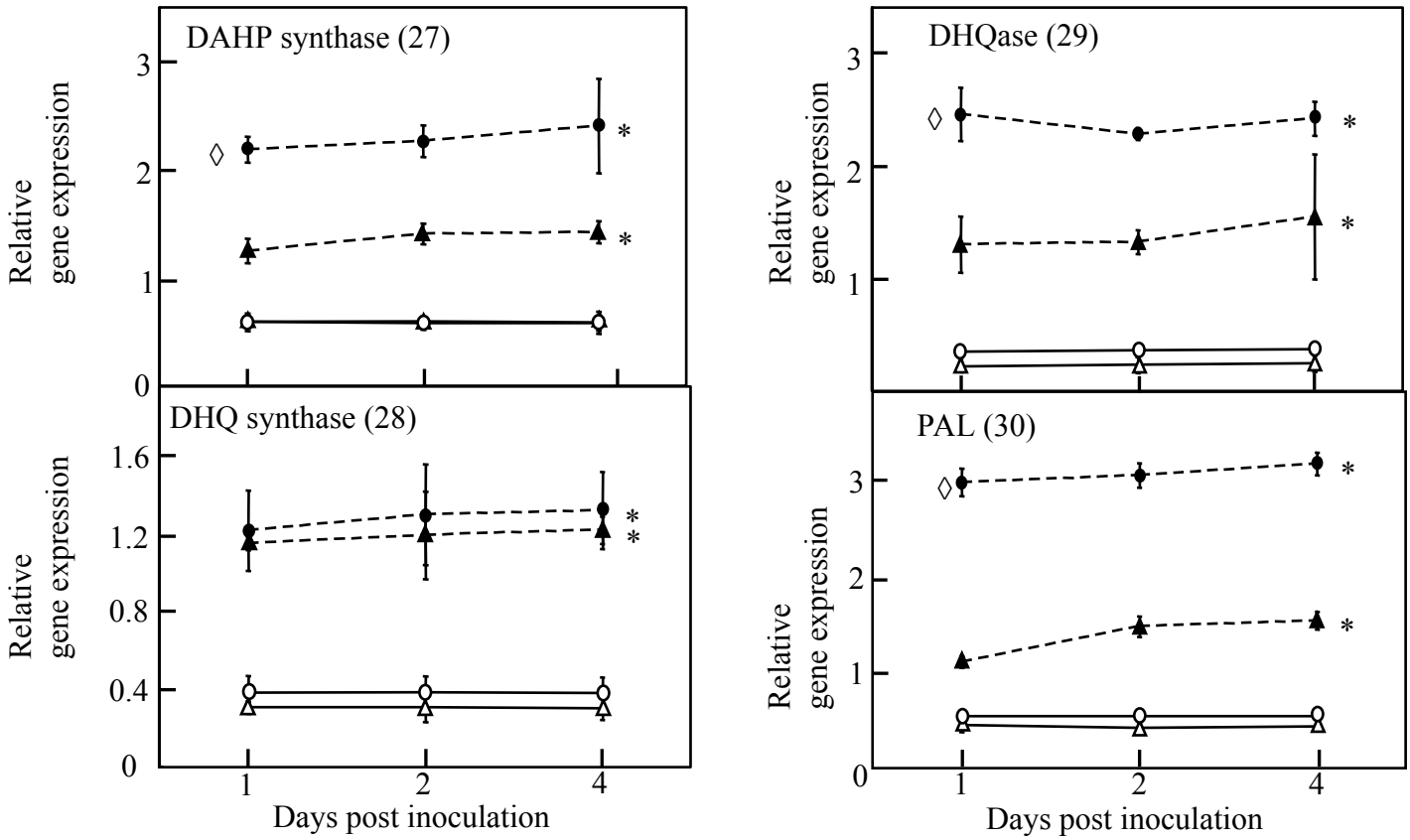


Figure 2.7. Time course changes in relative expression of metabolic enzyme genes of secondary metabolism at 1 dpi, 2 dpi and 4 dpi in leaf sheaths of *R. solani*-infected plants of the *R. solani*- resistant and susceptible rice lines (closed circles, closed triangles), respectively, and those of control plants (open circles, open triangles), respectively. Relative expression was measured by normalization to Actin gene, as described in Materials and Methods. Values were means ± SD of three separate determinations including 50 plants. * Relative expression was significantly different ($P < 0.05$) between the *R. solani*-infected plants of both rice lines compared to that of the control plants at 1 dpi, 2 dpi and 4 dpi. ◊ Relative expression was significantly different ($P < 0.05$) between the *R. solani*-infected plants of the resistant rice line and that of the susceptible rice line. mRNA expression of **DAHP synthase**: 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHP synthase, EC 2.5.1.54), **DHQ synthase**: 3-dehydroquinate synthase (DHQ synthase, EC 4.2.3.4), **DHQase:** 3-dehydroquinate hydratase (DHQase, EC 4.2.1.10) and **PAL**: phenylalanine ammonialyase (EC 4.3.1.5). The numbers following the enzyme name abbreviations in the figures are the same as those showing the enzyme location in Figure 2.1.

phenylalanine ammonialyase (PAL, EC 4.3.1.24) in leaf sheaths of *R. solani*-infected plants of the resistant and susceptible rice lines and those of the respective control plants at 1, 2 and 4 dpi.

Changes after infection showed that the expressions of DAHP synthase, DHQ synthase and PAL were significantly higher ($P < 0.05$) in leaf sheaths of *R. solani*-infected plants of the resistant rice line, compared to the susceptible rice line at 1 to 4 dpi. The expressions of DAHP synthase, DHQ synthase, DHQase and PAL were significantly higher ($P < 0.05$) in leaf sheaths of *R. solani*-infected plants of both resistant and susceptible rice lines, compared to the control plants at 1, 2 and 4 dpi. Changes in expression of DHQ synthase after infection were similar in the infected samples of both resistant and susceptible rice lines.

**4. Discussion**

Time course of changes in expressions showed that PFK, PGK, enolase, PK, G6PDH, SPase, CS, DAHP synthase, DHQase and PAL in leaf sheaths of *R. solani*-infected rice plants of the resistant rice line were significantly higher ($P < 0.05$) at 1 dpi, compared to those of the susceptible rice line. Comparison between the *R. solani*-infected plants and the control plants, showed that expressions of all the enzyme genes studied were significantly higher ($P < 0.05$) in leaf sheaths of *R. solani*-infected plants, compared to those of the control plants, except for PGM, PGI, TPI, GAPDH, enzyme genes of RPPC, ADPase and SS. The expressions of TPI in leaf sheaths of *R. solani*-infected plants of both rice lines increased significantly at 4 dpi, compared to those of the control plants. These results were in agreement with the findings that the

enzymes responsible for the *R. solani* resistance mechanisms showed increased activity by 1 dpi, with maximum activity at 4 dpi (Danson, 2000*c*). Higher expressions of PFK, PGK, enolase and PK in leaf sheaths of *R. solani*-infected plants suggested increased glycolytic activity, which in higher plants, begins with sucrose (Kubota and Ashihara, 1990; Nose *et al.,* 2002*a*) and generates PEP and pyruvate.

The expression of SPase was higher in the resistant rice line, compared to that of the susceptible rice line. These results and the findings by Danson *et al.* (1999*a*), which reported that sucrose concentration increased after *R. solani* infection suggested that sucrose synthesis was activated after infection. In contrast, lower expression of ADP-glucose pyrophosphorylase (ADPase) and starch synthase (SS) in leaf sheaths of *R. solani*-infected plants of both rice lines, compared to those of the control plants, suggested that starch synthesis was low. Starch concentration was also lower in the *R. solani*-infected plants of both rice lines, compared to that of control plants (data not shown). These results agree with the findings that in cvs. Hinohikari and Sasanishiki, *R. solani*-resistant and susceptible rice cultivars, respectively, starch concentration decreased to a third of that of the control plants after *R. solani* infection (Danson *et al.,* 1999*a*).

The results also showed that the expressions of cytosolic G6PDH, PGLase and PGDH, which participate in the oxidative pentose phosphate pathway (OPPP) were significantly higher ($P < 0.05$) in leaf sheaths of *R. solani*-infected plants of both rice lines (Fig. 2.3). These results were in agreement with the findings of Danson *et al.* (2000*b*), which showed that the enzymatic activity of G6PDH and PGDH increased in the rice plants infected with *R. solani,* suggesting activation of OPPP after infection

and possibly increased production of erythrose-4-phosphate (E-4-P), a substrate of the secondary metabolism. E-4-P is used together with PEP, a product of the glycolytic pathway, as substrates in the shikimate pathway.

The expressions of the metabolic enzyme genes of the shikimate pathway in leaf sheaths of *R. solani*-infected rice plants of both rice lines were significantly high ($P < 0.05$) (Fig. 2.7). These high expressions of DAHP synthase, DHQ synthase and DHQase in leaf sheaths of *R. solani*-infected plants of both rice lines indicated the activation of the shikimate pathway, especially in the resistant rice line. The expressions of DAHP synthase and DHQase in leaf sheaths of *R. solani*-infected plants of the resistant line were more than 1.6-fold higher than those of the susceptible rice line. In other reports, it was observed that DHQase-specific transcripts accumulated in tomato cells after exposure to a fungal elicitor (Bischoff *et al.* 2001) for the synthesis of proteins. The shikimate pathway may also regulate the phenylpropanoid pathway by controlling the supply of phenylalanine (Fig. 1) (Yao *et al.,* 1995).

The expression of PAL in leaf sheaths of *R. solani*-infected plants of both rice lines was significantly higher ($P < 0.05$), compared to that of the control samples (Fig 2.7). Because the activity of PAL determines the extent to which phenylalanine enters the phenylpropanoid pathway (Lavola *et al.,* 2000), high PAL expression after *R. solani* infection suggested the activation of the phenylpropanoid pathway, which was in agreement with the findings that PAL was stimulated by pathogenic attack (Dixon and Paiva, 1995; Ritter and Schulz, 2004). Furthermore, PAL expression was more than 2.1-fold higher in leaf sheaths of *R. solani*-infected plants of the resistant rice line,

compared to that of the susceptible rice line. In addition, PAL enzyme activity was more than 5-fold and 3-fold higher in leaf sheaths of *R. solani*-infected plants of the resistant and susceptible rice lines, respectively, compared to that of the respective control plants (data not shown). This suggested that high PAL expression was necessary for resistance to infection, because, as reported elsewhere, suppression of PAL gene expression increased disease susceptibility of transgenic tobacco plants to *Cercospora nicotianae* while over-expression resulted in the slow development of disease lesions (Maher *et al.,* 1994).

Expressions of TCA enzymes genes suggested the existence of a high TCA cycle activity. The TCA cycle is an essential metabolic network that provides precursors for anabolic processes and reducing factors (NADH) from acetyl CoA, a product obtained by the decarboxylation of pyruvate (Mailloux *et al.,* 2007). Although there are few reports on the TCA cycle in disease-infected plants, we can speculate that since this cycle links the carbon and nitrogen metabolism, disruption of this cycle may adversely affect the plant carbon and nitrogen metabolism and cellular biosynthetic processes. On the other hand, the results showed that the expressions of the RPPC enzyme genes decreased after *R. solani* infection. This may be linked to the low carbon fixation in rice leaf sheaths, which are mostly etioplasts or to the low starch synthesis mentioned earlier, since starch synthesis in chloroplasts proceeds via RPPC.

Based on the sub-cellular localization in glycolysis, OPPP, RPPC and secondary metabolism, fructose-6-phosphate (F-6-P) in a series of steps was converted to starch in plastids and to sucrose in the cytosol, starting with dihydroxyacetone phosphate (DHAP). On the other hand, glyceraldehyde-3-phosphate (GAP) obtained from

fructose-1,6-bisphosphate (F-1,6-$P_2$) was converted to phosphoenolpyruvate (PEP) and E-4-P, which are used as substrates in the secondary metabolism, starting with the shikimate pathway. That is, F-6-P, F-1,6-$P_2$, DHAP and GAP and the respective enzymes appear to be involved in the regulation of carbon distribution between the primary and secondary metabolism. Nose *et al.* (2002*a*, *b*) postulated that these carbon branches in the plant cell act as a carbon crossroad.

Based on the results, the high expressions of the enzymes of the glycolytic pathway; PFK, TPI, PGK, and enolase after *R. solani* infection suggested the activation of the glycolytic pathway and possibly higher generation of PEP. The high expression of the enzymes of OPPP; G6PDH, PGLase and PGDH suggested the activation of OPPP while the lower expressions of the reductive phase of the pentose phosphate cycle suggested that E-4-P might have been derived from GAP. The results showed that sucrose synthesis was activated unlike starch synthesis. Higher expressions of the genes of the secondary metabolism suggested that the production of the substrates, PEP and E-4-P increased in the primary metabolism. These findings indicated that carbon distribution between the primary and secondary metabolism relating to resistance and susceptibility to sheath blight disease in rice, was regulated in the carbon crossroad postulated by Nose *et al.* (2002*a*, *b*).

Expression of all the enzyme genes except PGI, TPI, and ADPase changed at 1 dpi. The expression of PFK, PGK, enolase, PK, enzymes of the OPPP, SPase, enzymes of the TCA cycle and enzymes of the secondary metabolism was significantly higher ($P < 0.05$) in leaf sheaths of *R. solani*-infected plants at 1 dpi, while that of PGM, GAPDH, genes of RPPC, ADPase and SS was lower. These results showed that

significant changes in the mRNA expression of enzyme genes occurred at 1 dpi and indicated that studies to elucidate mRNA transcription within hours after infection should be carried out.

Taken together, these findings showed that the resistance to sheath blight disease in the rice line $2F_{18}$-7-32 (32) was associated with a high activation of the glycolytic pathway, OPPP and secondary metabolism and also with a low starch synthesis, and suggested that most of the genetic responses to *R. solani* infection occurred within at least one day

## 5. Summary

Using two rice lines, namely $2F_{18}$-7-32 (32) a *Rhizoctonia solani* - resistant rice line and one, $2F_{21}$-21-29 (29) a susceptible rice line, changes in the time course of mRNA expression of 30 metabolic enzyme genes in *R. solani*-infected samples were examined. These consisted of 8 metabolic enzyme genes of the glycolytic pathway; 3 metabolic enzyme genes of the oxidative pentose phosphate pathway (OPPP), and 6 metabolic enzyme genes of the reductive pentose phosphate cycle (RPPC); sucrose phosphatase (SPase, EC 3.1.3.24), participating in sucrose synthesis; ADP-glucose pyrophosphorylase (ADPase, EC 2.7.7.9) and starch synthase (SS, EC 2.4.1.21) which are involved in starch synthesis; 6 metabolic enzyme genes of the tricarboxylic acid (TCA) cycle and 4 metabolic enzyme genes of the secondary metabolism. The results showed that significant changes in the time course of mRNA expression occurred at 1 dpi and that mRNA expression of glycolytic enzymes; 6-phosphofructokinase, phosphoglycerate kinase, enolase and pyruvate kinase and secondary metabolism

enzymes; 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase and phenylalanine ammonialyase was significantly higher ($P < 0.05$) in the *R. solani*-infected samples of the resistant rice line, compared to that of the susceptible rice line. The results suggested that *R. solani* infection led to activation of the glycolytic pathway, OPPP, TCA cycle and secondary metabolism. Time course of mRNA expression of RPPC genes, ADPase and SS suggested that starch synthesis was low in *R. solani*-infected samples of both *R. solani*-resistant and susceptible rice lines. It appeared that *R. solani* infection was associated with the activation of the glycolytic pathway, OPPP, secondary metabolism and TCA cycle and low starch synthesis.

# CHAPTER 3

## Changes in metabolites and enzyme activities in two rice lines infected with *Rhizoctonia solani* (Kuhn): activation of glycolysis and connection to secondary metabolism.

### 1. Introduction

To prevent loss due to rice sheath blight disease, research is focused on development of resistant cultivars, which also enable the possibility of producing crops without using agricultural chemicals (Wasano and Hirota, 1986). Two types of resistance have been reported, polygene and major gene resistance. The history of crop improvement by major gene resistance based on Mendelian genetics shows that a breakdown of host resistance could occur after several years owing to appearance of new virulent strains of the pathogens. For example, rice varieties with major gene resistance to leaf blight disease lost host resistance when the pathogen developed adaptive capacity to overcome it (Wasano and Dhanapala, 1982).

Although most studies reported that the source of immunity or of high level resistance to sheath blight disease was lacking, Wasano *et al.* (1985) identified materials that showed field resistance through classical crossbreeding, which is difficult and only possible over many years (Groth and Nowick, 1992). The rice lines were hybrids of Tetep and $CN_4$-4-2. Tetep is a primitive indica cultivar that was reported to have some resistance to sheath blight disease (Marchetti, 1983). $CN_4$-4-2 was a hybrid of Chugoku 45 and Nipponbare, a susceptible japonica variety. When the resistant rice

line was exposed to sheath blight disease, it showed more resistance than Tetep (Wasano and Hirota, 1986). Currently, improvement for sheath blight disease resistance is based on the analysis of quantitative trait loci (QTL) (Gaire *et al.,* 2011). Resistance based on polygenic characters is desirable because it involves a self-defence mechanism and it is stable over a long time (Wasano and Dhanapala, 1982). However, interpretation of polygenic quantitative trait loci studies is limited by the lack of information on metabolic pathways (McMullen *et al.,* 1998). Also, in order to utilise QTL analysis in clarifying the role of specific genes in a biochemical pathway, it is necessary to identify the enzyme genes to be targeted (Kliebestein, 2009).

During stress response plant defence mechanisms display coordinated and integrated set of metabolic alterations in an attempt to adapt to stress (Broglie *et al.,* 1991). For example, studies of the interaction between rice and *R. solani* at the molecular level have shown that glycolytic pathway is activated accompanied by the activation of phenylpropanoid pathway (Danson *et al.,* 2000*a*; Nose *et al.,* 2002*a,b*). Specific glycolytic enzymes that were highly expressed in leaf sheaths of *R. solani*-infected rice plants were found to include phosphofructokinase (PFK), triosephosphate isomerase (TPI), phosphoglycerate kinase (PGK), enolase, and pyruvate kinase (PK). We have previously shown that the increased expression of these glycolytic enzymes was accompanied by activation of oxidative pentose phosphate pathway (OPPP), TCA, shikimate and phenylpropanoid pathway (Mutuku and Nose, 2010). However, the low expressions of genes of the non-oxidative PPP raised questions about the source of E-4-P utilised in the shikimate pathway. Taken together these studies suggested that the glycolytic pathway was at the core of carbon allocation for pathways such as OPPP, TCA, shikimate and phenylpropanoid pathway in leaf

sheaths of *R. solani*-infected rice plants. This led to the proposal that regulation of glycolysis in leaf sheaths of *R. solani*-infected rice plants was directly involved in regulation of carbon allocation for the other pathways and it was an important resistance response mechanism. However, glycolytic regulation was not investigated.

To study regulation, gene expression, enzyme activity, and metabolite contents have to be analysed. In the course of this analysis, mechanisms of metabolic control affecting the pathway, i.e., coarse and fine controls have to be considered (Plaxton, 2004). Any alteration of gene expression i.e., transcription, translation, mRNA processing or degradation can be considered as coarse metabolic control (Plaxton, 2004). On the other hand, fine metabolic controls are generally fast and modulate the activity of a regulatory enzyme (Plaxton and Podesta, 2006). Mechanisms of fine metabolic control include variation in substrate concentration and pH, allosteric effectors and covalent modification operating primarily on regulatory enzymes (ap Rees *et al.,* 1977).

Gene expression and *in vitro* enzyme activity can be determined and the results used to show reactions where coarse control is exerted. However, they cannot be used to determine *in vivo* regulation of the metabolic pathway. The availability of metabolite data is quite relevant to determining how and where flux control of a specific metabolic pathway is exerted i.e., probable regulatory enzymes (Plaxton, 2004). This can be done by calculating mass-action ratios and comparing them with apparent equilibrium constants (*K´*) (Connett, 1985). If the mass-action ratio of a reaction is markedly smaller than *K*', it can be concluded that this reaction is a non-equilibrium reaction *in vivo* (Kubota and Ashihara, 1990; Morandini, 2009) suggesting that there

are other factors for example, fine control affecting regulation of the reaction. However, elucidation of fine control of plant glycolysis is complicated by the alternative glycolytic reactions in the plant cytosol (Plaxton, 1996). Cytosolic glycolysis is an adaptive pathway whose maximum enzymatic activities vary with a variety of phenomena such as changing biological and physical environments (Black *et al.,* 1987). This implies that its regulation varies according to the specific tissue and external environment. Nevertheless, fine control of glycolysis is reported to be exerted by hexokinase, PFK and PK (Faiz-ur-Rahman *et al.,* 1974; Kubota and Ashihara, 1990).

Taken together, our previous studies (Danson *et al.,* 2000*a*; Nose *et al.,* 2002*a*; Mutuku and Nose, 2010) have shown that glycolytic pathway might be a target to identify specific defence-related enzymes for enhancement using methods such as metabolic engineering. However, since its regulation has not been investigated, it is not clear, (a) which steps might be targeted to enhance this pathway, (b) whether regulation of this pathway maybe done through coarse or fine control, (c) the implications of regulation of glycolysis to other pathways leading to generation of desirable products associated with resistance response. One of the basic goals of metabolic engineering in plants is to enhance the production of a desired compound during defence response (Bolton, 2009). Strategies for achieving this goal include upregulating several consecutive enzymes in a primary metabolic pathway (Capell and Christou, 2004; Katagiri, 2004). To address these questions, we determined reactions where coarse control was exerted by examining enzymatic activities and comparing them with expression data obtained from our previous studies (Mutuku and Nose, 2010). In the interpretation of the metabolite measurements made in this

study, the reactions of glycolysis were classified according to their displacement from equilibrium, on the premise that only those enzymes that catalyse reactions far from equilibrium can control metabolic pathways (ap Rees *et al.,* 1977; Kubota and Ashihara, 1990; Plaxton, 1996). Then we examined activation of phenylpropanoid pathway by investigating PAL and peroxidase activity together with $H_2O_2$ generation as part of the resistance responses that relied on the regulation of glycolysis after *R. solani* infection in rice. For better understanding, the biochemical reactions involved in glycolysis, pentose phosphate pathway (PPP), TCA, shikimate and phenylpropanoid pathway are as shown in Fig.ure 3.1. This study used two near-isogenic rice lines i.e., *R. solani*-resistant ($2F_{18}$-7-32) and *R. solani*-susceptible ($2F_{21}$-21-29) developed by Wasano *et al*. (1985).

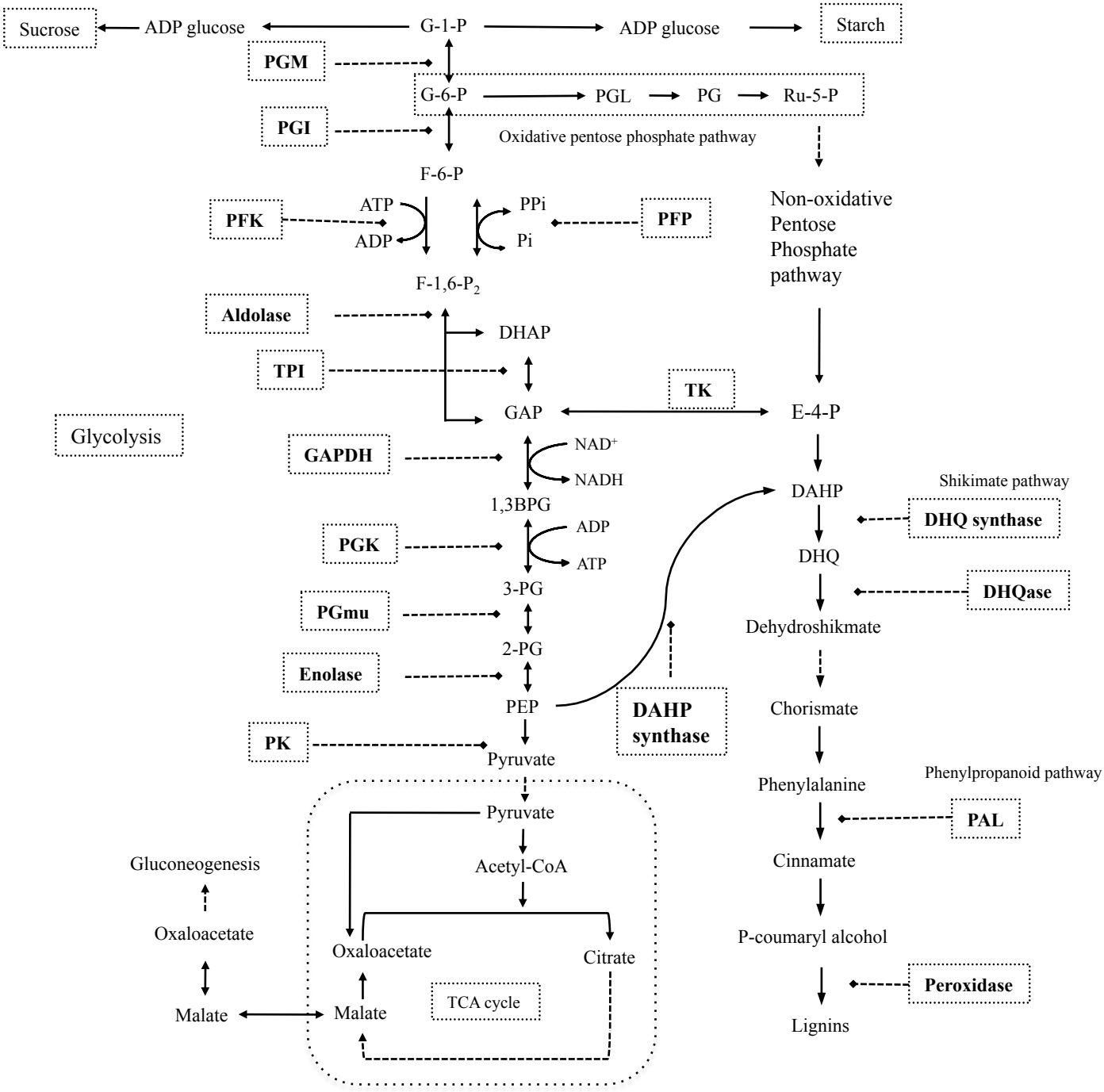


Figure 3.1. Simplified diagram showing the positions of metabolites and enzymes examined in the glycolytic pathway and secondary metabolism. In rice leaf sheaths, glycolysis occurs in the plastid and it is central in the allocation of carbon resources for sucrose and starch synthesis, pentose phosphate, TCA, shikimate and phenylpropanoid pathways. In non-stress conditions, F-6-P is phosphorylated to F-1,6-$P_2$ by 6-phosphofructokinase (**PFK**, EC 2.7.1.11). However, we have recently shown that the additional establishment of glycolysis in the cytosol after *R. solani* infection in rice leaf sheaths was accompanied by the activation pyrophosphate-dependent PFK (**PFP**, EC 2.7.1.90) (Mutuku and Nose, 2012). **PGM**, phosphoglucomutase (EC 2.7.5.1); **PGI**, phosphoglucose isomerase (EC 5.3.1.9); aldolase (EC 4.1.2.13); **TPI**, triosephosphate isomerase (EC 5.3.1.1); **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); **PGK,** phosphoglycerate kinase (EC 2.7.2.3); **PGmu**, phosphoglycerate mutase (EC 2.7.5.3); enolase (EC 4.2.1.11); **PK**, pyruvate kinase (EC 2.7.1.40); **TK**, transketolase (EC 2.2.1.1); **PAL**, phenylalanine ammonialyase (EC 4.3.1.24); peroxidase (EC 1.11.1.14). G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; F-1,6-$P_2$, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceradehyde-3-phosphate; 1,3BPG,1,3-Bisphosphoglycerate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phoshoglycerate; PEP, phosphoenolpyruvate. Adapted from Nose et al. (2002*a*) and Mutuku and Nose (2010).

## 2. Materials and Methods

Samples and fungus preparations were done as previously described (Mutuku and Nose, 2010). Briefly, plants were grown under greenhouse conditions until they attained the seven-leaf growth stage. After inoculation, rice leaf sheaths (at least 5 cm long to include the infection site) were obtained at 10 am, one day, two days and four days post inoculation, ground in liquid nitrogen and stored at -80 $^{o}$C until use.

### 2-1. Extraction and determination of metabolites

Metabolites were extracted according to Lowry and Passonneau (1972) and Du *et al*. (1998). About 0.5 g of ground rice leaf sheaths and 1.0 mL of ice-cold 5 % $HClO_4$ were pulverized together in a liquid nitrogen-cooled mortar and pestle. The mixture was transferred to 2.0 mL eppendorf tube kept in ice and was allowed to thaw slowly. The mortar and pestle were rinsed twice with 0.5 mL of 5 % $HClO_4$. The combined extract was kept on ice for 30 min and centrifuged at 3, 000 *g* for 10 min using a refrigerated centrifuge (TOMY High Speed Refrigerated Micro Centrifuge, MX-150, TOMY Tech., Japan) at 4 $^{o}$C. The supernatant was retained and the pellet was washed with 1.0 mL of 5 % $HClO_4$ and centrifuged as above. The supernatants from the two centrifugations were combined and 14 mg $mL^{-1}$ charcoal was added to the combined solution. The tubes were kept on ice for 5 min and centrifuged at 10, 000 *g* for 5 min at 4 $^{o}$C. The supernatant was neutralized with 2.5 M $K_2CO_3$ to about pH 6.0 and centrifuged at 10, 000 *g* for 5 min at 4 $^{o}$C after which it was used immediately for determinations of metabolites using a Jasco V-550 UV/VIS spectrophotometer (Jasco Corporation, Japan).

### 2-1-2. Measurements of Glucose-6-phosphate (G-6-P), glucose-1-phosphate (G-1-P) and fructose-6-phosphate (F-6-P)

G-6-P, G-1-P and F-6-P were measured as described by Du *et al*. (1998) with some modifications by following changes in absorbance at 340 nm. The assay mixture (1.0 mL) contained 100 mM Tris-HCl pH 8.5, 10 mM $MgCl_2$, 0.25 mM NADP and an aliquot of extract. To the mixture, 0.5 units $mL^{-1}$ (U $mL^{-1}$) of glucose-6-phosphate dehydrogenase, 1.0 U $mL^{-1}$ phosphoglucomutase and 1.0 U $mL^{-1}$ phosphoglucose isomerase, were added sequentially. The contents in nmol $g^{-1}$ FW of G-6-P, G-1-P and F-6-P were estimated at 25 $^{o}$C.

### 2-1-3. Measurements of Fructose-1,6-bisphosphate (F-1,6-$P_2$), dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP)

F-1,6-$P_2$, DHAP and GAP were measured by a method modified from Wirtz *et al*. (1980) in an assay mixture of 1.0 mL containing 100 mM Tris-HCl pH 8.1, 5 mM $MgCl_2$, 0.2 mM NADH and an aliquot of extract. To the mixture, 0.5 U $mL^{-1}$ of glyceraldehyde-3-phosphate dehydrogenase, 5.0 U $mL^{-1}$ triosephosphate isomerase and 0.2 U $mL^{-1}$ of aldolase were added sequentially. F-1,6-$P_2$, DHAP and GAP were measured at 340 nm. Measurements were done at 25 $^{o}$C.

### 2-1-4. Measurement of 3-phosphoglycerate (3-PG)

3-PG was measured in two-step method described by Usuda (1985) with some modifications. The assay mixture (1.0 mL) contained 50 mM Tris-HCl pH 8.0, 10

mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM ATP, 0.2 mM NADH and extract. The extract was incubated in the assay mixture for 20 min at room temperature before addition of NADH. Thereafter, NADH was added and measured at 340 nm. Then, 1.0 U $mL^{-1}$ each of phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase was added. 3-PG (nmol $g^{-1}$ FW) was determined as the difference between the values before and after addition of the enzymes.

### 2-1-5. Measurements of 2-phosphoglycerate (2-PG), phosphoenolpyruvate (PEP) and pyruvate

2-PG, PEP and pyruvate were assayed as described by Chen *et al*. (2002) with some modifications. To the reaction mixture, containing 200 mM Tris-HCl pH 7.6, 3.0 mM EDTA-NaOH pH 7.0, 0.15 mM NADH and an aliquot of extract, 2.0 U $mL^{-1}$ lactate dehydrogenase (LDH) were added; pyruvate content (nmol $g^{-1}$ FW) was determined as the difference in absorbance at 340 nm between before and after addition of LDH. After this, 1.25 mM ADP, 10 mM $MgSO_4$, 37 mM KCl, 18 U $mL^{-1}$ LDH and 10 U $mL^{-1}$ pyruvate kinase (PK) were added to the reaction mixture whose final volume was 1.0 mL. PEP content (nmol $g^{-1}$ FW) was determined as the difference in absorbance at 340 nm between before and after addition of both LDH and PK. The 2-PG was determined as the difference in absorbance at 340 nm between before and after addition of both 20 U $mL^{-1}$ enolase in the PEP reaction above (Lamprecht and Heinz, 1984).

### 2-1-6. Measurement of Erythrose-4-phosphate (E-4-P)

Measurement of E-4-P was done as described by Racker (1965) with modifications. E-4-P was measured in a volume of 1 mL containing 100 $\mu$L extract, 25 mM glycylglycine pH 7.4, 0.12 mM NADH, 0.3 mM F-6-P. After optical density was measured at 340 nm (A1), 23 U mL $^{-1}$ TPI and 2 U mL $^{-1}$ of $\alpha$-glycerol-3-phosphate dehydrogenase were added and the reaction was let to run for 8 min to obtain A2. After 1.0 unit mL $^{-1}$ transaldolase was added, the reaction was let to run again for 8 min and A3 was recorded. The difference between A1 and A2 corresponds to the triose phosphate content of the samples (Racker, 1965). E-4-P was measured as the difference between A2 and A3.

### 2-1-7. Measurements of NAD and NADH

Measurement of NAD and NADH was performed as described by Tezuka *et al.* (1994). Plant samples homogenized with 0.1 M HCl (for NAD) or 0.1 M NaOH (for NADH) at 95 °C were cooled in an ice bath, and then the pH was adjusted to 6.5 with NaOH (for NAD) or 7.5 with HCl (for NADH). After the addition (0.5 mL) of 0.2 M glycylglycine (pH 6.5 or 7.5) to the oxidized or reduced coenzyme fractions, respectively, the volume of each fraction was measured. Each fraction was centrifuged (10,000 *g* for 20 min at 4 °C), and the resulting supernatants were immediately used for the measurement of NAD and NADH. For measurements, samples were added to the reaction mixture containing 50 mM glycylglycine (pH 7.4), 20 mM nicotinamide, 1 mM phenazine methosulfate (PMS), 1 mM thiazolyl blue (MTT), and alcohol dehydrogenase (final concentration, 40 $\mu$g mL $^{-1}$). After placing

the cuvette containing the reaction mixture in a UV-visible spectrophotometer for measurement at 570 nm, 40 $\mu$L of 80 % ethanol was added to start the reaction.

### 2-1-8. Measurements of ADP and ATP

Measurement of ATP and ADP was done as described by Stitt *et al*. (1989) with some modifications. ATP was measured in a volume of 1 mL containing 100 $\mu$L extract plus 100 mM Tris-HCl (pH 8.1), 5 mM $MgCl_2$, 0.25 mM NADP, 1 mM glucose, 0.7 U $mL^{-1}$ G6PDH, 0.7 U $mL^{-1}$ PGI. After 2 min incubation, 0.6 U $mL^{-1}$ hexokinase was added. ADP was measured in a volume of 1 mL containing 100 $\mu$L extract plus 100 mM Tris-HCl (pH 8.1), 2 mM $MgCl_2$ 10 mM KCl, 60 $\mu$M NADH, 1.5 mM PEP, 2.5 U $mL^{-1}$ LDH. After 2 min incubation, 4 U $mL^{-1}$ PK and 15 U $mL^{-1}$ myokinase were added.

### 2-1-9. The recovery of metabolites

The recoveries of metabolites were carried out as described by Leegood and Furbank (1984) with modifications. Frozen rice leaf sheath tissues were weighed and measured amounts of intermediate (1,000 nmol) was added to the pestle and mortar and pulverised with the leaf sheath tissue. Table 3.1 shows the recoveries of metabolites concur with percentages shown by Du *et al*. (1998) and demonstrates that there were no serious losses during extraction and analysis.

Table 3.1. Recoveries of metabolites from rice leaf sheaths of *R. solani*-infected plants. The recoveries are expressed as a percentage of the amount added (1,000 nmol) and are the averages of three separate determinations. Recoveries were estimated as described in Material and methods.

| Metabolites | Recoveries (% ± SD) |
|---|---|
| Glucose-1-phosphate (G-1-P) | 104 ± 5 |
| Glucose-6-phosphate (G-6-P) | 84 ± 2 |
| Fructose-6-phosphate (F-6-P) | 72 ± 3 |
| Fructose-1,6-bisphosphate (F-1,6-$P_2$) | 88 ± 6 |
| Dihydroxyacetone phosphate (DHAP) | 89 ± 4 |
| Glyceraldehyde-3-phosphate (GAP) | 83 ± 6 |
| 3-phosphoglycerate (3-PG) | 87 ± 7 |
| 2-phosphoglycerate (2-PG) | 103 ± 7 |
| Phosphoenolpyruvate (PEP) | 83 ± 6 |
| Pyruvate | 79 ± 4 |
| Erythrose-4-phosphate (E-4-P) | 90 ± 8 |
| ADP | 99 ± 10 |
| ATP | 99 ± 3 |
| NAD | 97 ± 8 |
| NADH | 98 ± 7 |

**2-2. Enzyme assays**

Rice leaf sheath tissues (0.5 g) was prepared as above and pulverised in 5 volumes of extraction buffer (100 mM Tris-HC1, pH 8.0; 2 mM $MgCI_2$; 1 mM EDTA; 28 mM $\beta$-mercaptoethanol; 1 mM phenylmethylsulphonyl fluoride) together with 250 mg polyvinyl polypyrollidone using a pestle and mortar chilled in liquid nitrogen. The mixture was then centrifuged for 10 min at 10,000 *g* at 4 $^{o}$C and the supernatant was assayed at once at 25 $^{o}$C for 20 min in the following 1.0 mL, reaction mixtures according to the accompanying references: **phosphoglucomutase** (EC 5.4.2.2), 50 mM Hepes (pH 7.6), 1 mM $MgCI_2$, 0.25 mM G-1-P, 0.024 mM glucose 1,6-bisphosphate, 0.4 mM NAD and 1.5 U mL $^{-1}$ G6PDH (Sweetlove *et al*., 1996); **glucose-6-phosphate isomerase** (EC 5.3.1.9), 75 mM glycylglycine (pH 8.5), 10 mM $MgCI_2$, 0.5 mM NAD, 1 mM F-6-P, 0.5 U mL $^{-1}$ G6PDH (Simcox *et al*., 1977); **aldolase** (EC 4.1.2.13), 50 mM Hepes (pH 7.0), 2 mM $MgCl_2$, 0.5 mM NADP, 0.1 mM F-1,6-$P_2$, 10 U mL $^{-1}$ each of PGI and G6PDH (Holaday, *et al*., 1992); **triosephosphate isomerase** (EC 5.3.1.1), 100 mM Hepes (pH 8.0), 5 mM EDTA, 0.2 mM NADH, 1.5 mM GAP, 1 U mL $^{-1}$ GDH (Burrell *et al*., 1994); **glyceraldehyde-3-phosphate dehydrogenase** (EC 1.2.1.12) 100 mM N-tris (hydroxymethyl)methyl-3-aminopropane-sulfonic acid (Taps; pH 8.6), 20 mM $NaH_2PO_4$, 1 mM NAD, 6 mM cysteine, 1.5 mM GAP **(**Burrell *et al*., 1994); **phosphoglycerate kinase** (EC 2.7.2.3), 100 mM Hepes (pH 7.6), 1 mM EDTA, 2 mM $MgSO_4$, 0.2 mM NADH, 6.5 mM 3-PG, 1 mM ATP, 3.3 U mL $^{-1}$ GAPDH (Burrell *et al*., 1994); **phosphoglycerate mutase** (EC 2.7.5.3), 60 mM Tris (pH 7.6), 4 mM $MgSO_4$, 0.23 mM ADP, 0.075 mM NADH, 3 mM 3-PG, 1 U mL $^{-1}$ enolase, 3 U mL $^{-1}$ PK, 0.15 U mL $^{-1}$ LDH (ap Rees *et al*., 1975); **enolase** (EC 4.2.1.11), 100 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 0.1 mM

NADH, 2.7 mM ADP, 0.5 mM 2-PG, 5 U mL $^{-1}$ PK, 6 U mL $^{-1}$ LDH (Miernyk and Dennis, 1984); **pyruvate kinase** (EC 2.7.1.40); 50 mM 3-(Nmorpholino) propanesulfonic acid (pH 7.0), 100 mM KCl, 15 mM $MgCl_2$, 0.15 mM NADH, 5.0 mM PEP, 1.0 mM ADP, 0.15 U mL $^{-1}$ LDH (Burrell *et al*., 1994). **Transaldolase** (EC 2.2.1.2), 10 mM $MgCl_2$, 0.1 mM NADH, 0.66 mM F-6-P, 0.02 mM E-4-P, 13 U mL$^{-1}$ TPI, 3.5 U mL $^{-1}$ GDH, and TES-NaOH pH 7.5 in a total volume of 1 mL. **Transketolase** (EC 2.2.1.1) was measured in a manner similar to transaldolase except 20 $\mu$M thiamine pyrophosphate was added and the sugar phosphates were 0.13 mM xylulose-5-phosphate and 0.33 mM ribose-5-phosphate (Simcox *et al*., 1977).

### 2-2-1. Phenylalanine ammonialyase (PAL) activity

The ground tissue (0.5 g) was placed into 1 mL of 10 mM sodium phosphate buffer pH 6.0. After the mixture was centrifuged at 10,000 *g* for 5 min at 4 °C, 200 $\mu$L of the supernatant was used to measure PAL activity using the method of Redman *et al*. (1999). The reaction mixture, in a final volume of 1 mL, consisted of 250 mM of Tris-HCl buffer pH 8; 200 $\mu$L of enzyme preparation; 6 $\mu$M of L-phenylalanine. The enzyme reaction was started by the addition of enzyme extract and after incubation for 60 min at 37 ˚C, the reaction was stopped by the addition of 50 $\mu$L of 5N HCl. The amount of trans-cinnamic acid formed was determined by measuring absorbance at 290 nm.

### 2-2-2. Peroxidase activity

Peroxidase activity was determined as described by Redman *et al*. (1999) with some modifications. The ground tissue (0.1 g) was placed into 1 mL of 0.01 M sodium phosphate buffer (pH 6.0). After the sample was centrifuged at 10,000 *g* for 5 min at 4 °C, peroxidase activity was determined with 0.25 % (v/v) guaiacol and 0.3 % (v/v) $H_2O_2$ in 1 mL of 0.01 M sodium phosphate buffer (pH 6.0). The reaction was initiated by the addition of 0.5 $\mu$L of the supernatant extract to 999.5 $\mu$L of the reaction mixture. Activity was measured as a change in the absorbance at 470 nm.

### 2-3. $H_2O_2$ detection by the 'DAB-uptake method'

*R. solani*-infected rice leaf sheaths from both resistant and susceptible lines were cut and placed in 1 mg mL $^{-1}$ 3,3'-diaminobenzidine (DAB)-HCl pH 3.8 (Sigma, Japan) following a modification of the 'DAB-uptake method' described by Thordal-Christensen *et al*. (1997). Rice leaf sheath of about 5 cm in length (including the infection site) were washed with distilled water and incubated in 1 mg mL $^{-1}$ DAB-HCl pH 3.8 (a low pH is necessary in order to solubilize DAB) for 8 h after which, photographs were taken using an Olympus BH2 microscope equipped with a camera (Olympus, Japan).

### 2-4. Peroxidase detection

*In-situ* peroxidase activity was detected in rice leaf sheaths following pretreatment in 1 mg mL$^{-1}$ DAB-HCl (as above, but re-buffered to pH 5.8 using NaOH immediately

before use), 0.1 % Triton-X 100 for 5 min following a method described by Thordal-Christensen *et al*. (1997). After incubation, $H_2O_2$ was added to the final concentrations of 10, 1.0, 0.1, 0.01 mM and incubation was performed by mild agitation at RT for 10 min.

### 2-5. Data analysis

Statistical analysis was done using Excel (Microsoft, USA) and significant levels were tested at $P < 0.05$ using the Student's *t*-test.

## 3. Results

### 3-1. Metabolites contents in leaf sheaths of *R. solani*-infected rice plants

Figure 3.2, 3.3, 3.4 and 3.5 show the time course of changes in G-1-P, G-6-P, F-6-P, F-1,6-$P_2$, DHAP, GAP, 3-PG, 2-PG, PEP and pyruvate contents in leaf sheaths of *R. solani*-infected and control plants of the resistant and susceptible rice lines at 1, 2 and 4 dpi. This study showed that there were significant changes in metabolites contents in leaf sheaths of *R. solani*-infected rice plants. These changes revealed that there was uneven distribution of carbon between the upper and the lower part of the glycolytic pathway (Fig. 2). G-1-P and G-6-P contents in leaf sheaths of *R. solani*-infected rice plants of both lines appeared to be similar, whereas F-6-P content in the resistant line was significantly higher at 1 to 4 dpi. When the lower part of glycolytic pathway was examined, F-1,6-$P_2$, GAP, DHAP, 3-PG and PEP contents in the resistant line were found to be high.

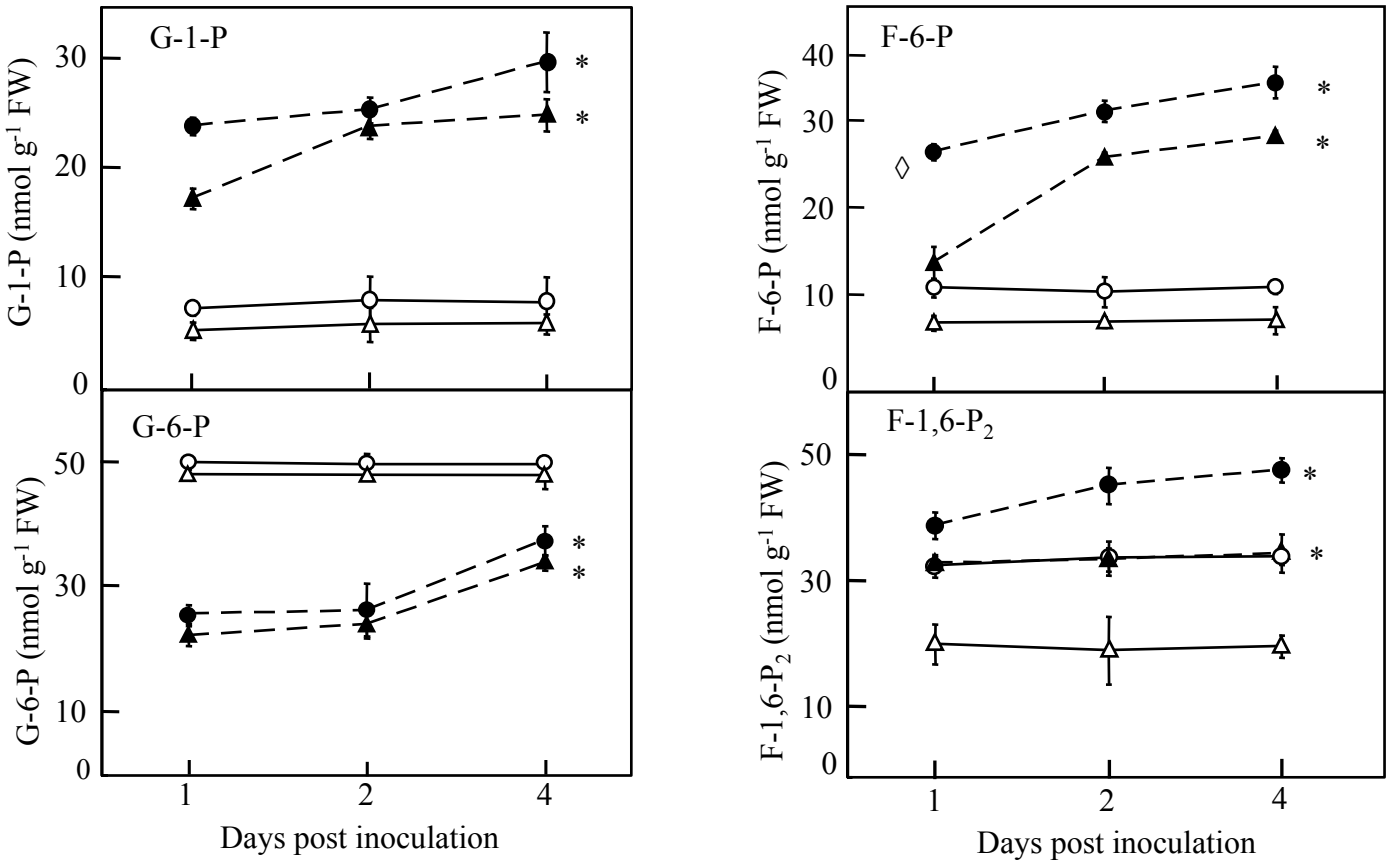


Figure 3.2. The content of glucose-1-phosphate (G-1-P), glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P), fructose-1,6-bisphosphate (F-1,6-$P_2$), in leaf sheaths of *R. solani*-infected plants of the resistant and the susceptible rice lines (closed circles, closed triangles), respectively, and control plants (closed circles, closed triangles), respectively. Values were means ± SD of four separate experiments * Metabolite contents were significantly different ($P < 0.05$) between the *R. solani*-infected plants and those of the control plants. ◊ Metabolite contents were significantly ($P < 0.05$) different between the *R. solani*-infected plants of the resistant rice line and those of the susceptible rice line at 1, 2 and 4 dpi.

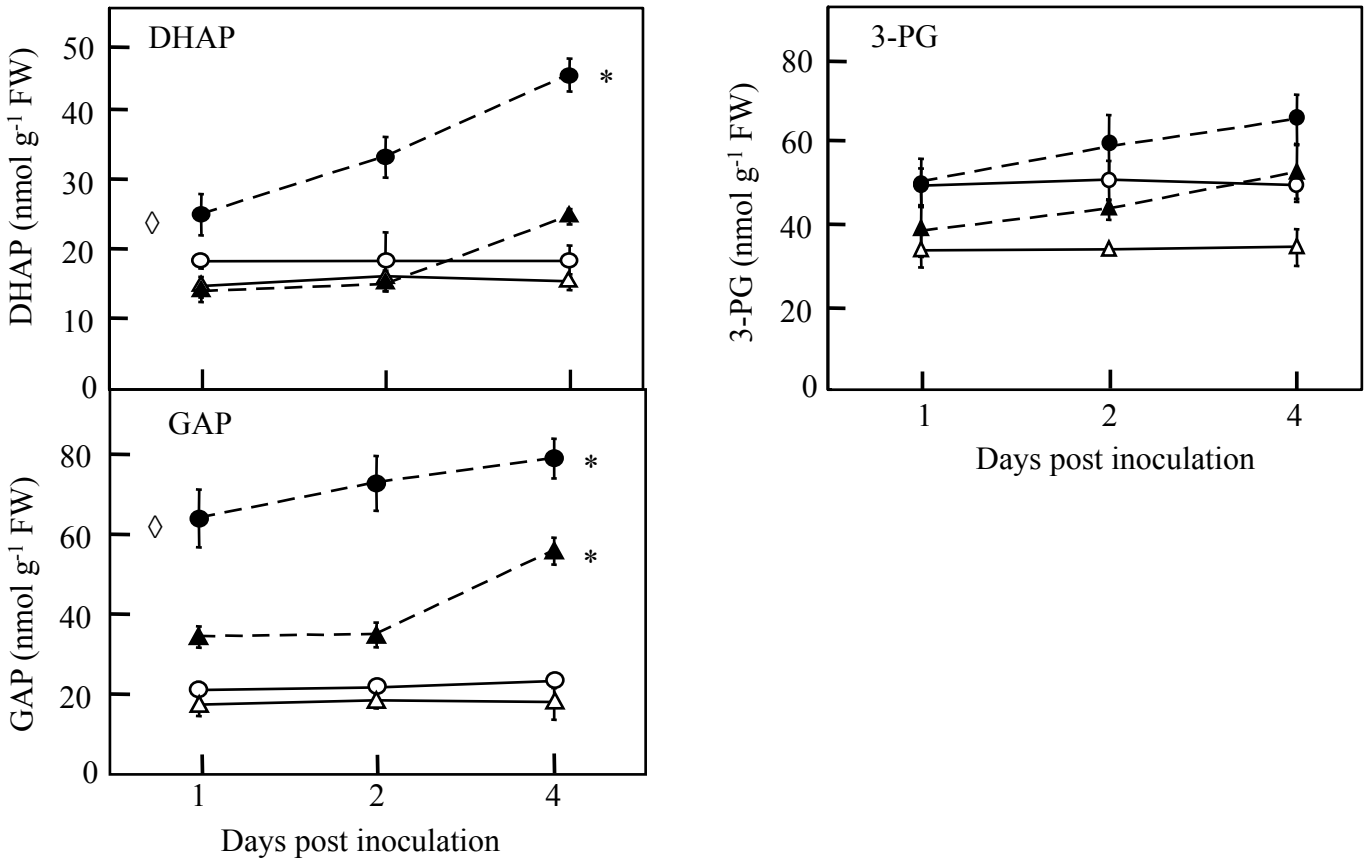


Figure 3.3. Dihydroxyacetone phosphate (DHAP), glyceraldehyde-3-phosphate (GAP), 3-phosphoglycerate (3-PG) in leaf sheaths of *R. solani*-infected plants of the resistant and the susceptible rice lines (closed circles, closed triangles), respectively, and control plants (open circles, open triangles), respectively. Values were means ± SD of four separate experiments * Metabolite contents were significantly different ($P < 0.05$) between the *R. solani*-infected plants and those of the control plants. ◊ Metabolite contents were significantly ($P < 0.05$) different between the *R. solani*-infected plants of the resistant rice line and those of the susceptible rice line at 1, 2 and 4 dpi.

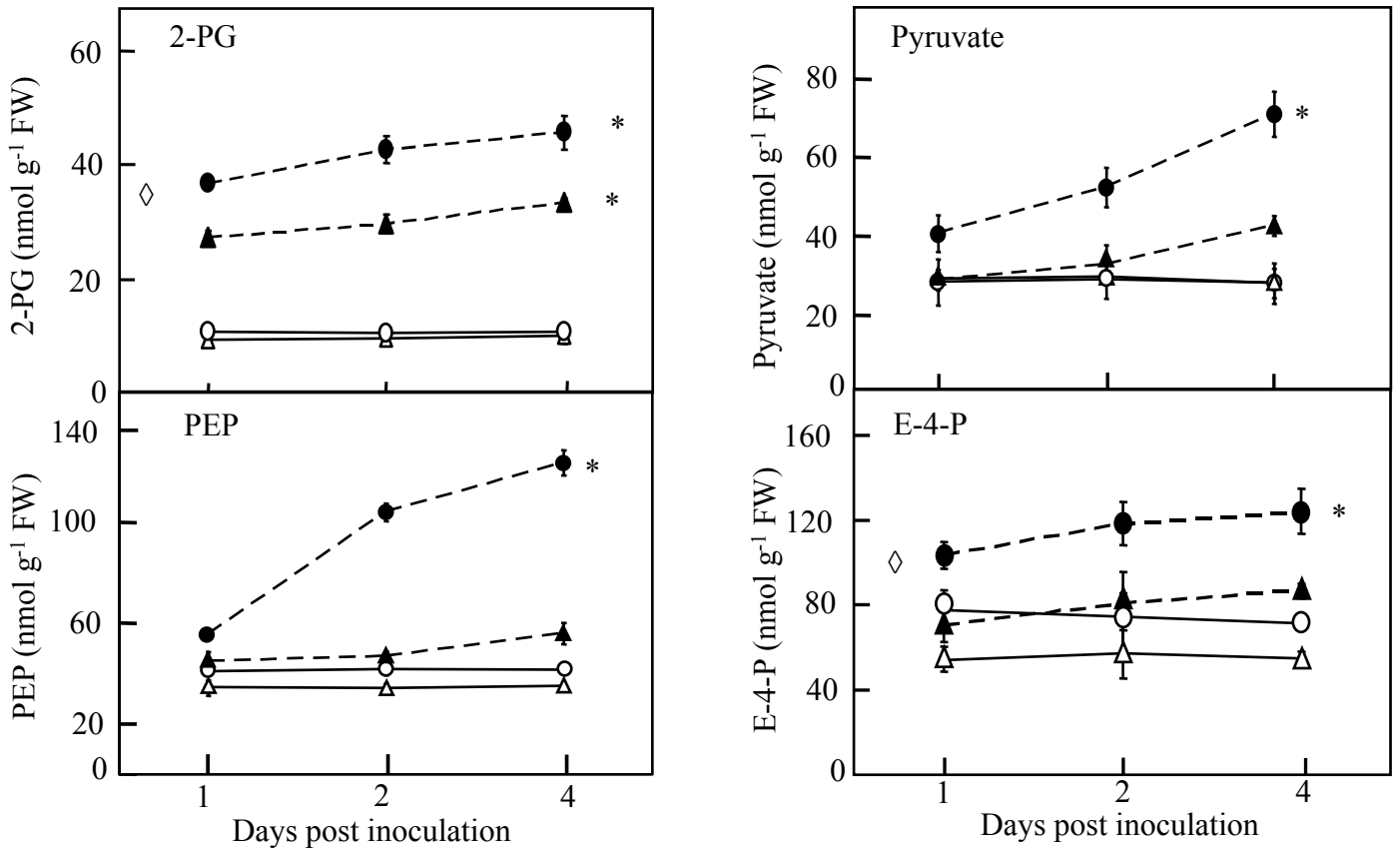


Figure 3.4. 2-phosphoglycerate (2-PG), phosphoenolpyruvate (PEP), pyruvate, erythrose-4-phosphate (E-4-P) in leaf sheaths of *R. solani*-infected plants of the resistant and the susceptible rice lines (closed circles, closed triangles), respectively, and control plants (open circles, open triangles), respectively. Values were means ± SD of four separate experiments * Metabolite contents were significantly different ($P < 0.05$) between the *R. solani*-infected plants and those of the control plants. ◊ Metabolite contents were significantly ($P < 0.05$) different between the *R. solani*-infected plants of the resistant rice line and those of the susceptible rice line at 1, 2 and 4 dpi.

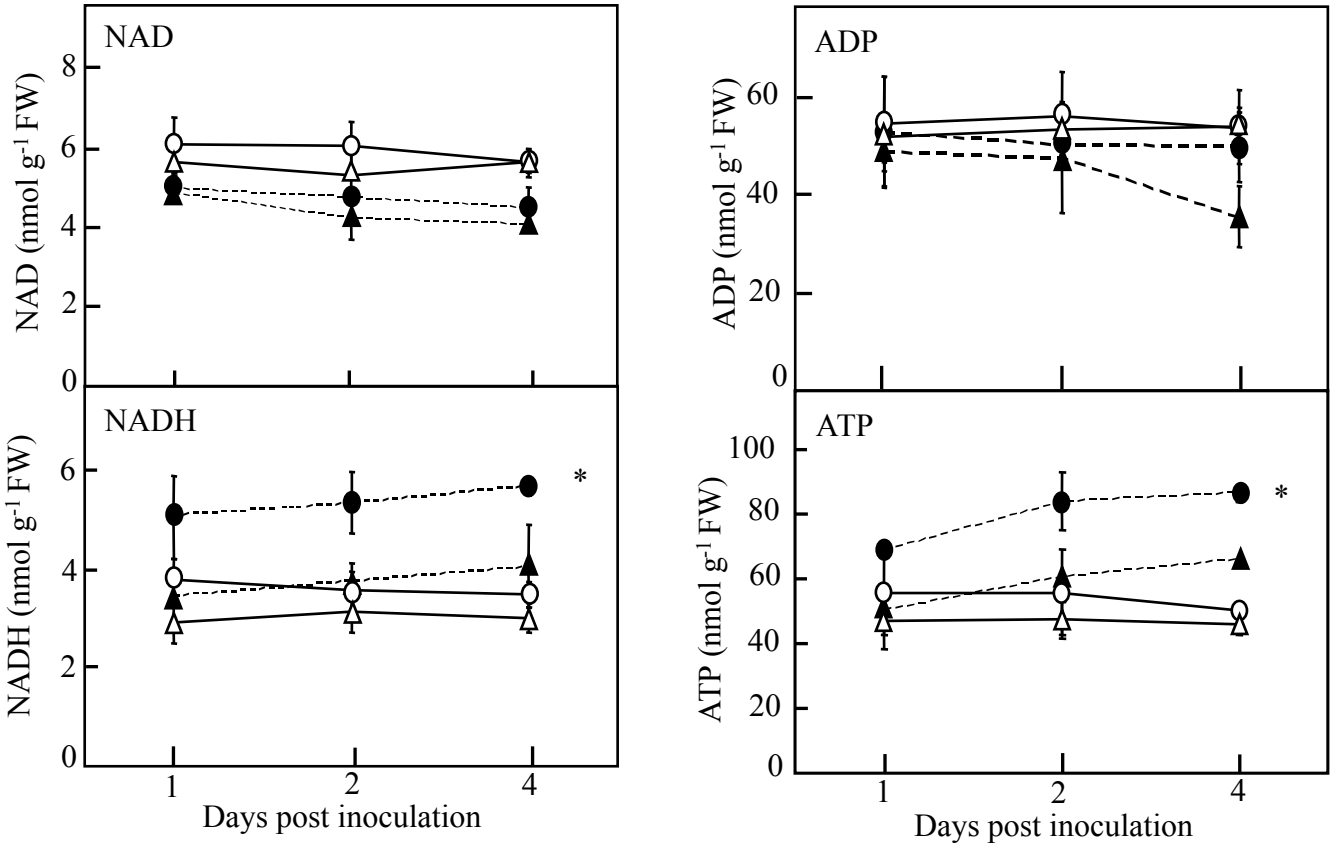


Figure 3.5. Nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide (reduced form) (NADH), adenosine diphosphate (ADP), adenosine triphosphate (ATP) in the *R. solani*-infected plants of the resistant and the susceptible rice lines (closed circles, closed triangles), respectively, and control plants (open circles, open triangles), respectively. Values were means ± SD of four separate experiments.
*The metabolite contents were significantly different ($P < 0.05$) between the *R. solani*-infected plants and those of the control plants.
◊ Metabolite contents were significantly ($P < 0.05$) different between the *R. solani*-infected plants of the resistant rice line and those of the susceptible rice line at 1, 2 and 4 dpi.

The content of G-1-P in infected rice plants of the resistant line increased at 4 dpi and was significantly higher ($P < 0.05$) in infected plants of both rice lines, compared to control plants. Although, G-6-P contents in infected plants of both rice lines increased at 4 dpi, they were significantly lower than those of the control plants. Furthermore, the time course of changes in the contents of G-6-P in the infected plants of both rice lines was similar. F-6-P, F-1,6-$P_2$, DHAP, GAP, 3-PG, PEP and pyruvate content in infected rice plants of the resistant line gradually increased at 1 to 4 dpi. F-6-P, F-1,6-$P_2$, DHAP, GAP, PEP and pyruvate contents were significantly higher ($P < 0.05$) in the resistant line, compared to those of the susceptible line. E-4-P and ATP contents in the infected rice plants of both lines increased at 1 to 2 dpi but did not change at 4 dpi and the time course of changes was similar. The time course of changes in the content of NADH in the infected plants of both rice lines were similar but the content in the resistant line was significantly higher ($P < 0.05$) than that of the susceptible line. ADP and NAD contents in the infected rice plants were slightly lower than those of the control plants. NAD content decreased at 1 to 4 dpi while the NADH: NAD ratio and the ATP: ADP ratio increased at 1 to 4 dpi.

### 3-2. Enzymatic activities in leaf sheaths of *R. solani*-infected rice plants

As shown in Figure 3.6, 3.7 and 3.8 the time course of changes in enzymatic activities showed that PGM in the infected rice plants of the resistant line increased more than 1.4-fold between 1 and 4 dpi. The time course of changes in PGI, TPI and PK activities in infected rice plants of both lines appeared similar. Aldolase, GAPDH, PGmu and enolase activities in the infected rice plants of the resistant line increased at 1 to 4 dpi and were more that 1.2-fold, 1.3-fold, 1.4-fold and 1.1-fold respectively,

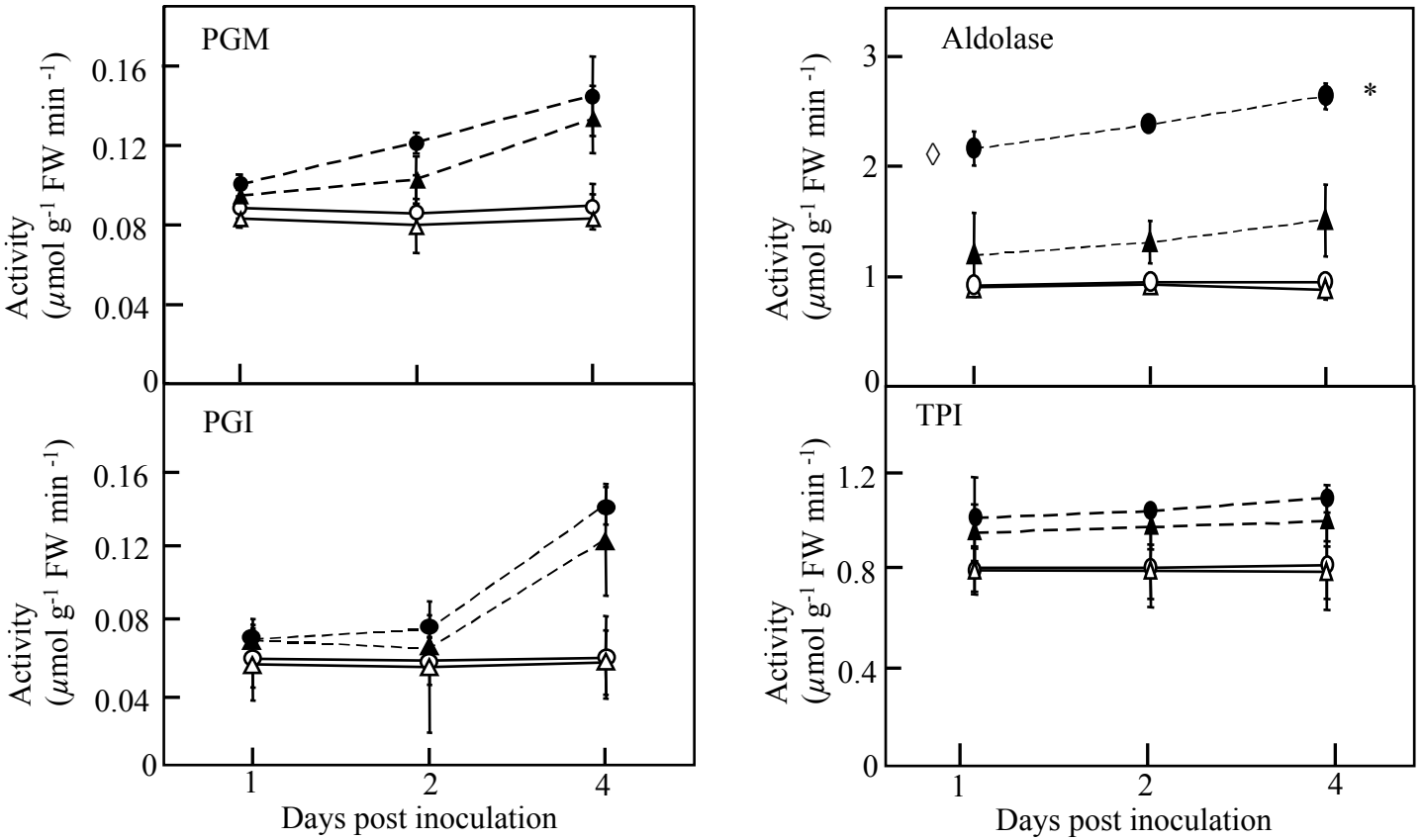


Figure 3.6. Enzyme activity ($\mu$mol g$^{-1}$ FW min $^{-1}$) in the *R. solani*-infected plants of the resistant and the susceptible rice lines (closed circles, closed triangles), respectively, and control plants (open circles, open triangles), respectively, at 1, 2 and 4 dpi. PGM, phosphoglucomutase (EC 5.4.2.2); PGI, phosphoglucose isomerase (EC 5.3.1.9 ); aldolase (EC 4.1.2.13); and TPI, triose phosphate isomerase (EC 5.3.1.1). ***** Activity was significantly different between the *R. solani*-infected plants and that of the control plants. ◊ Activity was significantly different between the *R. solani*-infected plants of the resistant rice line and that of the susceptible line. Values were means ± SD of three separate experiments.

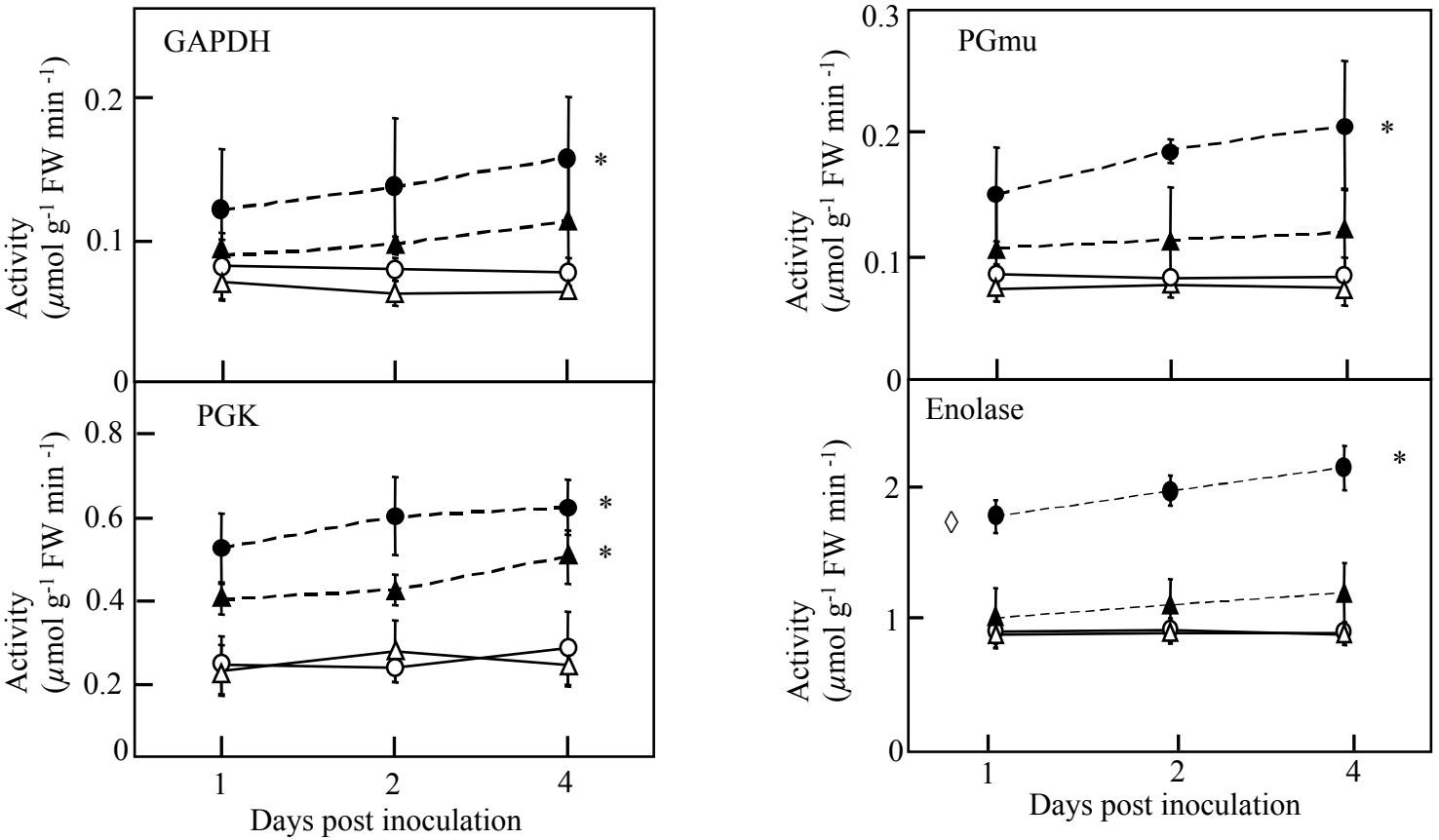


Figure 3.7. Enzyme activity ($\mu$mol g$^{-1}$ FW min $^{-1}$) in the *R. solani*-infected plants of the resistant and the susceptible rice lines (closed circles, closed triangles), respectively, and control plants (open circles, open triangles), respectively, at 1, 2 and 4 dpi. GAPDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12 ); PGK, phosphoglycerate kinase (EC 2.7.2.3); PGmu, phosphoglycerate mutase (EC 2.7.5.3); and enolase (EC 4.2.1.11). ***** Activity was significantly different between the *R. solani*-infected plants and that of the control plants. $^{\lozenge}$ Activity was significantly different between the *R. solani*-infected plants of the resistant rice line and that of the susceptible line. Values were means ± SD of three separate experiments.

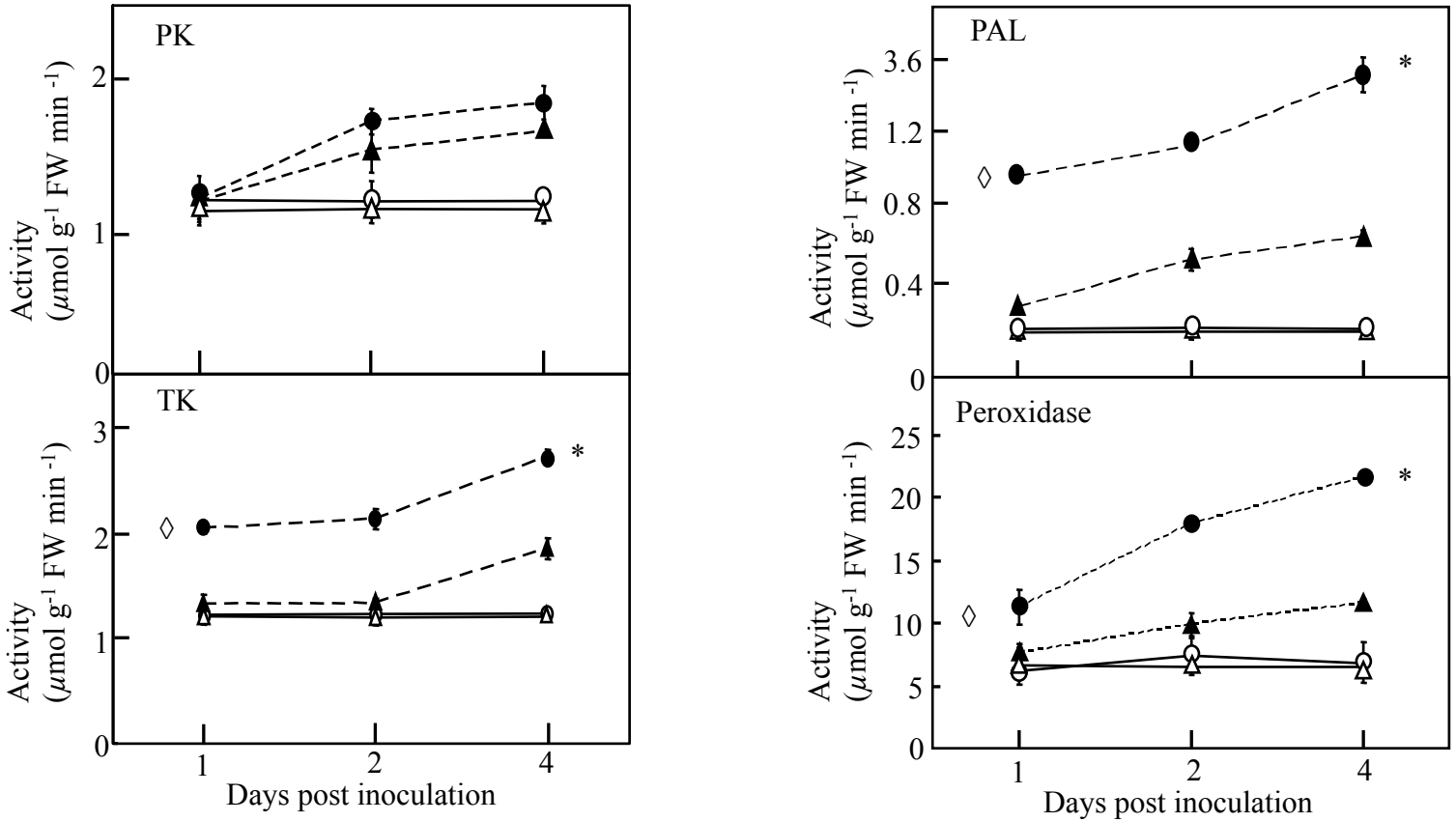


Figure 3.8. Enzyme activity ($\mu$mol g$^{-1}$ FW min $^{-1}$) in the *R. solani*-infected plants of the resistant and the susceptible rice lines (closed circles, closed triangles), respectively, and control plants (open circles, open triangles), respectively, at 1, 2 and 4 dpi. PK, pyruvate kinase (EC 2.7.1.40) TK, transketolase (EC 2.2.1.1), PAL, phenylalanine ammonialyase (EC 4.3.1.24) and peroxidase (EC 1.11.1.14). ***** Activity was significantly different between the *R. solani*-infected plants and that of the control plants. ◊ Activity was significantly different between the *R. solani*-infected plants of the resistant rice line and that of the susceptible line. Values were means ± SD of three separate experiments.

higher at 4 dpi compared to 1 dpi levels. Aldolase, GAPDH, PGK, PGmu and enolase activities were found to be high in the infected rice plants of the resistant line at 1 dpi, compared to control plants. In addition, aldolase and enolase activities in the infected rice plants of the resistant line were significantly higher ($P < 0.05$) than those of the susceptible line. Furthermore, aldolase, GAPDH, PGK, PGmu and enolase activities in the infected rice plants of the resistant line at 1 dpi were more than or similar to those of the susceptible line at 4 dpi.

Transketolase (TK), PAL and peroxidase activities in the infected plants of the resistant line were significantly higher ($P < 0.05$) than those of the susceptible line. The time course of changes in the activities showed that TK and PAL in the infected plants of the resistant line increased at 4 dpi while peroxidase increased gradually at 1 to 4 dpi. We attempted to measure transaldolase activity in the infected rice plants but it was too low to be detected. Although the time course of changes in activities showed that TK, PAL and peroxidase activities in the susceptible line increased, the levels at 4 dpi were significantly lower ($P < 0.05$) than those of the resistant line at 1 dpi.

### 3-3. Mass-action ratios, equilibrium constant (*K´*), standard free energy change ($\Delta G^{0}{}'$) and free-energy change ($\Delta G$) *in vivo*

One of the strategies used to determine *in vivo* control points of metabolism is the comparison of apparent equilibrium constants (*K´*) with mass-action ratios (Kubota and Ashihara, 1990). When metabolites contents were corrected using the recovery proportions (Table 3.1), the corrected values indicated changes of between 10-20 % in

the mass-action ratios. For example, when F-6-P content was corrected using the 72 % recovery percentage obtained, mass-action ratios of PGI reduced by 10 %. Similar percentages reported by Du *et al.* (1998) indicated that these losses were not so serious and therefore the recovery proportions were not used to correct these mass-action ratios data. Metabolites concentrations were obtained by assuming 1 g fresh weight of tissue is equal to 1 mL and used to estimate mass-action ratios, standard free energy change ($\Delta G^{0\prime}$) and free-energy changes ($\Delta G$) *in vivo* of PGM, PGI, PFK, aldolase, TPI, GAPDH+PGK, PGmu+enolase and PK in leaf sheaths of *R. solani*-infected and control plants of the resistant and the susceptible rice lines.

Tables 3.2 shows the mass-action ratios calculated from the estimated metabolite concentrations, standard free energy change ($\Delta G^{0\prime}$), free-energy change in *vivo* ($\Delta G$) together with the apparent equilibrium constants ($K'$) cited from the references (see legend in Table 3.2). The calculated mass-action ratios of PGM, PGI, TPI, PGmu+enolase were not far from the apparent equilibrium constants ($K'$), while those of PFK, GAPDH+PGK and PK in the infected rice plants were more than 53,000-, 1900- and 24,000-fold smaller than their respective $K'$ indicating the reactions were far from equilibrium *in vivo*. In the case of the reaction catalyzed by aldolase, the

Tables 3.1 recoveries

Table 3.2. Mass-action ratios (MARs) calculated from estimated metabolites concentrations based on the assumption that 1 g fresh weight of tissue is equal to 1 mL, were compared with equilibrium constants (*K'*), standard free energy change (Δ*G0'*) and free-energy change *in vivo* (Δ*G*) of reactions of phosphoglucomutase (PGM, EC 2.7.5.1), phosphoglucose isomerase (PGI, EC 5.3.1.9), phosphofructokinase (PFK, EC 2.7.1.11), aldolase (EC 4.1.2.13), triosephosphate isomerase (TPI, EC 5.3.1.1), glyceraldehydes-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), phosphoglycerate kinase (PGK, EC 2.7.2.3), phosphoglycerate mutase (PGmu, EC 2.7.5.1), enolase (EC 4.2.1.11) and pyruvate kinase (PK, EC2.7.1.40) in the leaf sheaths of *R. solani*-infected (T) and the control (C) plants of the resistant (32) and the susceptible (29) rice lines at 1 dpi, 2 dpi and 4 dpi.

| | | | **1 dpi** | | | | **2 dpi** | | | | **4 dpi** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | **32T-1** | **32C-1** | **29T-1** | **29C-1** | **32T-2** | **32C-2** | **29T-2** | **29C-2** | **32T-4** | **32C-4** | **29T-4** | **29C-4** |
| PGM | $K'$ =0.05 | MARs | 0.93 | 0.14 | 0.78 | 0.11 | 0.97 | 0.16 | 1.0 | 0.12 | 0.8 | 0.16 | 0.74 | 0.12 |
| | $\Delta G^{0\prime}$ =7.42 | $\Delta G$ | -0.17 | -4.81 | -0.62 | -5.53 | -0.07 | -4.55 | -0.01 | -5.27 | -0.56 | -4.62 | -0.75 | -5.24 |
| PGI | $K'$ =0.36-0.47 | MARs | 1.03 | 0.22 | 0.62 | 0.14 | 1.19 | 0.21 | 1.08 | 0.14 | 0.93 | 0.22 | 0.84 | 0.15 |
| | $\Delta G^{0\prime}$ =2.53 | $\Delta G$ | 0.08 | -3.78 | -1.19 | -4.85 | 0.44 | -3.88 | 0.19 | -4.81 | -0.19 | -3.75 | -0.44 | -4.74 |
| PFK | $K'$ =910 | MARs | 0.017 | 0.018 | 0.020 | 0.021 | 0.016 | 0.018 | 0.017 | 0.020 | 0.015 | 0.020 | 0.017 | 0.020 |
| | $\Delta G^{0\prime}$ =-16.88 | $\Delta G$ | -10.09 | -9.98 | -9.72 | -9.55 | -10.32 | -9.98 | -10.12 | -9.72 | -10.41 | -9.73 | -10.16 | -9.72 |
| Aldolase | $K'$ =6.7 x $10^{-5}$ | MARs | 0.02 | 0.09 | 0.07 | 0.08 | 0.02 | 0.09 | 0.06 | 0.06 | 0.01 | 0.08 | 0.02 | 0.07 |
| | $\Delta G^{0\prime}$ =23.81 | $\Delta G$ | -9.21 | -6.1 | -6.63 | -6.26 | -9.87 | -6.09 | -6.8 | -6.78 | -10.69 | -6.25 | -9.15 | -6.53 |
| TPI | $K'$ =22 | MARs | 0.39 | 0.87 | 0.4 | 0.84 | 0.46 | 0.84 | 0.43 | 0.87 | 0.57 | 0.78 | 0.44 | 0.85 |
| | $\Delta G^{0\prime}$ =-7.66 | $\Delta G$ | -2.34 | -0.35 | -2.26 | -0.44 | -1.95 | -0.43 | -2.12 | -0.34 | -1.4 | -0.6 | -2.03 | -0.4 |
| GAPDH+PGK | $K'$ =1550 | MARs | 0.78 | 2.30 | 1.11 | 1.90 | 0.80 | 2.29 | 1.24 | 1.81 | 0.83 | 2.09 | 0.94 | 1.89 |
| | $\Delta G^{0\prime}$ =-18.20 | $\Delta G$ | -0.62 | 2.04 | 0.23 | 1.57 | -0.56 | 2.03 | 0.52 | 1.46 | -0.49 | 1.80 | -0.18 | 1.55 |
| PGmu+enolase | $K'$ =2 | MARs | 1.11 | 0.83 | 1.18 | 1.02 | 1.78 | 0.83 | 1.08 | 1.01 | 1.90 | 0.84 | 1.07 | 1.01 |
| | $\Delta G^{0\prime}$ =-1.72 | $\Delta G$ | 0.26 | -0.45 | 0.40 | 0.06 | 1.43 | -0.48 | 0.18 | 0.01 | 1.59 | -0.43 | 0.17 | 0.03 |
| PK | $K'$ =20,000 | MARs | 0.81 | 0.71 | 0.66 | 0.77 | 0.64 | 0.69 | 0.87 | 0.82 | 0.76 | 0.64 | 1.28 | 0.75 |
| | $\Delta G^{0\prime}$ =-18.83 | $\Delta G$ | -0.52 | -0.85 | -1.02 | -0.68 | -1.11 | -0.91 | -0.36 | -0.49 | -0.67 | -1.10 | 0.62 | -0.72 |

Mass-action ratios were calculated from data in Figure 1. Apparent equilibrium ($K'$) values are from the following references: PGM (Lunn and ap Rees, 1990), PGI (Kubota and Ashihara, 1990), PFK (Faiz-ur-Rahman et al. 1974), aldolase (Cornish-Bowden, 1997), TPI (Rozovsky and McDermott, 2007), GAPDH+PGK (Rolleston and Newsholme, 1967), PGmu+enolase (Faiz-ur-Rahman et al. 1974), PK (Faiz-ur-Rahman et al. 1974). Mass-action ratios were calculated as follows: PGM, G-1-P/ G-6-P; PGI, F-6-P/ G-6-P; PFK, [ATP*F-6-P]/ [ADP*F-1,6-$P_2$]; aldolase, F-1,6-$P_2$/ [DHAP*GAP]; TPI, DHAP/ GAP; GAPDH+PGK, [NADH+ATP+3-PG]/ [NAD+ADP+GAP]; PGmu+enolase, PEP/ 3-PG; PK, [pyruvate*ATP]/ [PEP*ADP]. Standard free energy change ($\Delta G0'$) was calculated from $\Delta G^{0\prime} = -\boldsymbol{R}T \bullet \ln K'$. Free energy change *in vivo* was calculated from $\Delta G = \Delta G^{0\prime} + \boldsymbol{R}T \bullet \ln l$, Where $l$ is mass action ratio, $\boldsymbol{R}$ is 8.314 J • $mol^{-1}$ • $K^{-1}$ and $T$ is 298 K.

calculated mass-action ratios were bigger than the apparent equilibrium constant ($K'$) and appeared to decrease towards $K'$ when mass-action ratios of infected plants were compared with those of control plants. The standard free energy change ($\Delta G^{0'}$) values of the reactions catalyzed by PGM, PGI and aldolase were positive while those of PFK, TPI, GAPDH+PGK, PGmu+enolase and PK were negative.

**$H_2O_2$ detection in leaf sheaths of *R. solani*-infected rice plants**

The 3,3'-diaminobenzidine (DAB) was readily taken up by the *R. solani*-infected leaf sheaths of both rice lines. $H_2O_2$ produced in the vascular tissue is related to lignification (Olsen and Varner, 1993) and can be detected as a strong reddish-brown colour in leaf sheaths of *R. solani*-infected rice plants of both lines (Fig. 3.9A). The use of the 'DAB-uptake method' for studying $H_2O_2$ *in-situ* and *in vivo* requires the presence of endogenous peroxidase activity in order to ensure the formation of a visible polymer (Thordal-Christensen et al. 1997). A test for required peroxidase activity involves exposure of the target tissue to DAB and $H_2O_2$. As a positive control for uptake and reactivity of DAB, and the sensitivity of *in-situ* DAB-based $H_2O_2$ detection, DAB-polymerization was examined in the leaf sheaths of the control plants of both the resistant and susceptible rice lines in response to addition of $H_2O_2$ and DAB. Figure 3.9B shows the staining reaction in 1 mg $mL^{-1}$ DAB, 10 min after the addition of $H_2O_2$ to 10, 1.0, 0.1, 0.01 mM and the reaction varies from 'strong' to 'not visible'. Dark staining occurred in the vascular tissue of the leaf sheaths of both rice lines as a response to the addition of 10 mM $H_2O_2$ and the colour faded with reducing concentration of $H_2O_2$.

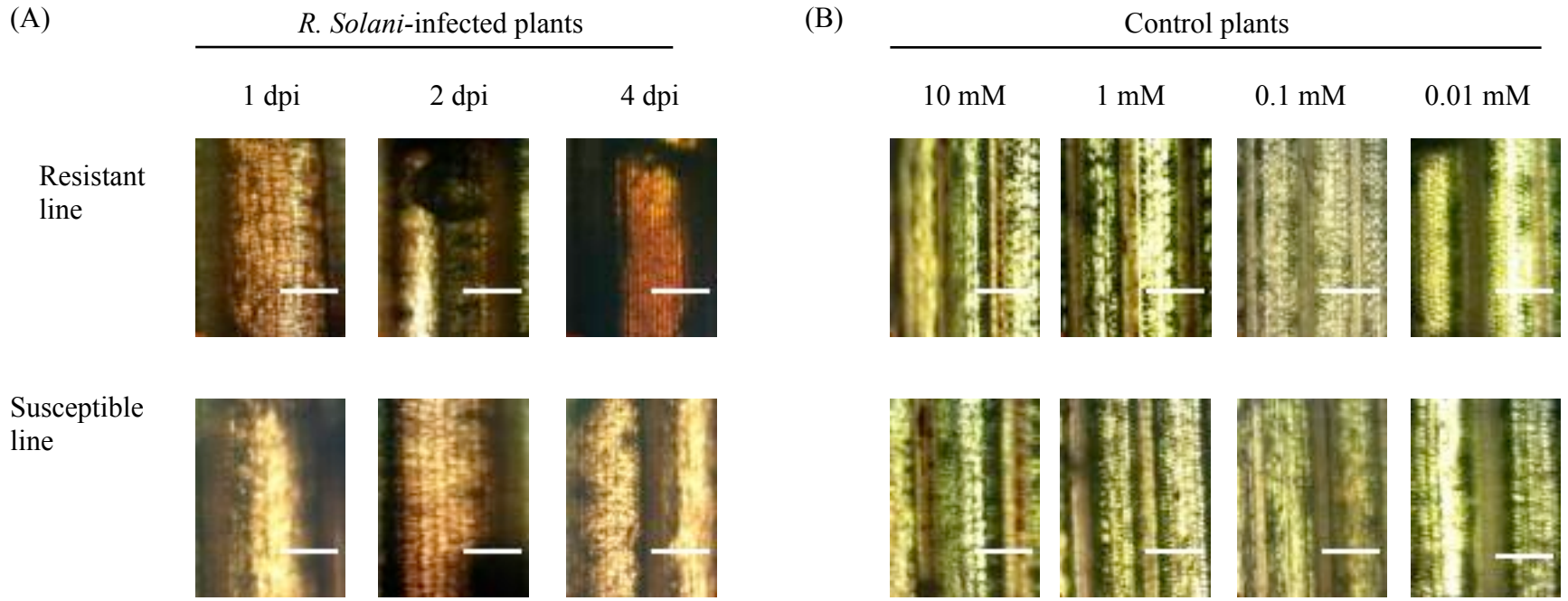


Figure 4. (A) Take up and polymerization of DAB is confirmed by the dark staining (reddish-brown) reaction in the rice leaf sheaths of *R. solani*-infected plants of the resistant and susceptible lines at 1, 2 and 4 dpi. (B) *In vivo* DAB reaction controls. Sensitivity of staining for peroxidase activity in the rice leaf sheaths of control plants of both the resistant and susceptible rice lines. Reactions in rice leaf sheaths after incubation in 1 mg $mL^{-1}$ DAB; 0.1 % Triton-X100 (supplied with $H_2O_2$ to either 10, 1.0, 0.1, 0.01 mM) for 10 min at RT. Notice the dark staining (reddish-brown) became lighter as the concentration of $H_2O_2$ reduced from 10 to 0.01 mM. Magnification X100 and scale bars = 5 mm.

## 4. Discussion

In our previous studies, we found out that in the interaction between rice and *R. solani*, activation of glycolytic pathway was accompanied by the activation of OPPP, TCA, shikimate and phenylpropanoid pathways (Danson *et al*., 2000*a*; Nose *et al*., 2002*a*; Mutuku and Nose, 2010). This suggested that glycolysis in leaf sheaths of *R. solani*-infected rice plants was at the core of carbon allocation for these pathways. The current studies investigated changes in metabolites and enzymatic activities in glycolysis and showed that G-1-P, F-6-P, F-1,6-$P_2$, DHAP, GAP, 3-PG, PEP and pyruvate content as well as activities of all enzymes in infected rice plants of the resistant line increased.

In the current studies, the activities of all enzymes and metabolite contents in leaf sheaths of *R. solani*-infected rice plants of the resistant line increased. Additionally, we previously showed that the expressions of PGM, TPI, GAPDH, enolase and PK in infected plants of the resistant line increased (Mutuku and Nose, 2010). These increases in enzymatic activities, metabolite contents and gene expressions might have occurred because in addition to the maintenance glycolysis in the plastid, establishment of alternative glycolysis in the cytosol compartment occurred as a response to infection. Further evidence for this was recent findings that increase in expression of the PFK plastid isozyme i.e., *Os06g05860* was accompanied by increase in expressions of three cytosolic PFK isozymes i.e., *Os01g09570*, *Os01g53680* and *Os04g39420* in infected rice plants of the resistant line. Furthermore, the increase in expressions of these PFK isozymes localised in plastid and cytosol compartments was strongly correlated with increase in PFK activity in the same plants. Moreover, the

increase in PFP activity in the cytosol was strongly correlated with the increase in expressions of two isozymes i.e., *Os08g25720* ($\alpha$-subunit) and *Os06g13810* ($\beta$-subunit) after infection (Mutuku and Nose, 2012). It has been reported that glycolysis is a network of reactions with possible sites for substrate entry into and out of different compartments (Sung *et al*., 1988; Kammerer *et al*., 1998; Givan, 1999) and that induction of glycolysis in the cytosol compartment occurs to facilitate plant acclimation to environmental stress (Merten, 1991; Plaxton, 1996).

To determine *in vivo* control points of glycolysis in leaf sheaths of *R. solani*-infected rice plants, mass-action ratios were estimated and compared with apparent equilibrium constants ($K'$). The results provided strong evidence that reactions catalysed by PGM, PGI, TPI and PGmu+enolase in infected rice plants were near equilibrium *in vivo* (Table 3.2). Although it is not an invariable rule, enzymes catalysing near-equilibrium reactions are unlikely to produce large effects on metabolites concentration (Fell, 2005). This might explain why for example, contents of G-1-P, G-6-P and F-6-P, which are involved in the reactions catalysed by PGM and PGI appeared similar in infected rice plants of the resistant line. The TPI reaction was different because significant changes in the DHAP/GAP ratios occurred. The equilibrium point of the TPI reaction is at DHAP:GAP= 24:1 (Rozovsky and McDermott, 2007) but in leaf sheaths of *R. solani*-infected rice plants it was DHAP:GAP= 1:1.8 in favour of GAP.

GAP can be utilised in the generation of E-4-P by transketolase. E-4-P can also be generated in the non-oxidative PPP by transaldolase. We could not detect transaldolase activity in leaf sheaths of *R. solani*-infected rice plants. This failure to

detect transaldolase while at the same time detecting transketolase and E-4-P was taken as showing that E-4-P was obtained from GAP by action of transketolase. This helped to clarify the question of the source of E-4-P that we raised previously (Mutuku and Nose, 2010).

In the case of aldolase, the mass-action ratios in infected rice plants of the resistant line were more than *K*'. The aldolase reaction was reported to have a small $\Delta G$ value ($\Delta G$= -1.7) leading to the conclusion that it is at equilibrium *in vivo* (Cornish-Bowden, 1997; Morandini, 2009). The results in Table 3.2 showed that the $\Delta G$ values in infected rice plants of both lines were larger than those reported by Cornish-Bowden (1997) suggesting that this reaction was not at equilibrium *in vivo*. The mass-action ratios of the reactions catalysed by PFK, GAPDH+PGK and PK in infected rice plants of the resistant line were markedly smaller than their respective *K*' indicating the reactions were also far from equilibrium *in vivo*.

It can also be argued that the reactions whose mass-action ratios are smaller than their respective *K*' are either restricted or their reaction products are utilized more than they are being generated. One condition necessary for the latter is that enzymes that utilize the reaction products must be capable of high activity (Cornish-Bowden, 1997). The results showing that the activities of GAPDH, PGK and PK as well as PFK and PFP (Mutuku and Nose, 2012) in infected rice plants of the resistant line were high combined with the results that the negative $\Delta G$ values showed that the forward reactions of PFK, GAPDH, PGK and PK were spontaneous were interpreted as showing that the reactions catalysed by these enzymes were not restricted.

GAPDH exists in plants in three different forms, the $NAD^+$-specific, $NADP^+$-specific, and nonphosphorylating forms (Blakeley and Dennis, 1993). We examined the nonphosphorylating $NAD^+$-dependent GAPDH reaction whose mass-action ratios can be determined as shown in the results. The apparent equilibrium constant of the reaction catalysed by $NADP^+$-GAPDH is unknown, and difficult to determine in non-photosynthetic tissues (Fridlyand et al. 1997). Findings reported by Tsuno *et al*. (1975), Ishimaru *et al*. (2004), Hirose *et al*. (2006) suggest that rice leaf sheaths have no photosynthetic capacity so $NADP^+$-GAPDH was not considered in this study.

PGM, TPI, GAPDH, enolase and PK activities in leaf sheaths of *R. solani*-infected rice plants were compared with the expressions of their genes in the same plants as reported in our previous studies (Mutuku and Nose, 2010) and strong positive correlations (Pearson's correlation coefficient, $r > 0.8$) were observed especially in the resistant line (Fig. 3.10). These increases in enzymatic activities and gene expressions suggested coarse metabolic control was exerted to PGM, TPI, GAPDH, enolase and PK. Perhaps the increase in activities of PGI, aldolase and PGmu as well as PFK and PFP (Mutuku and Nose, 2012) in infected plants especially those of the resistant line might also have been as a result of mechanisms of coarse control. The results showed that the reactions catalysed by PFK, GAPDH+PGK and PK were non-equilibrium *in vivo* suggesting that these reactions exerted fine metabolic control of

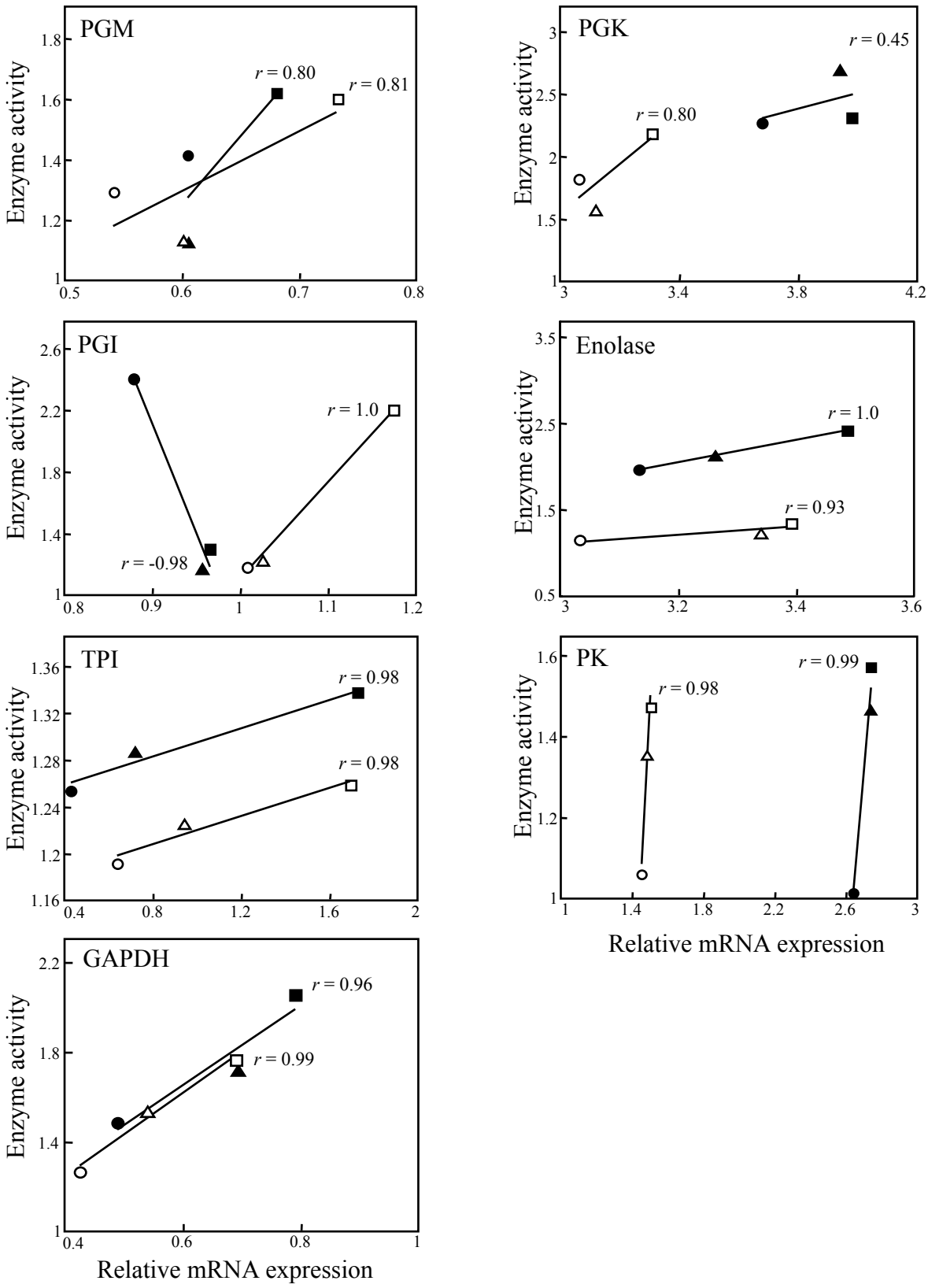


Figure 3.10. The correlation between the mRNA expression and enzyme activity in the *R. solani*-infected rice plants of the resistant line at 1, 2 and 4 dpi (closed circles, closed triangles, closed squares), respectively, and susceptible line at 1, 2 and 4 dpi (open circles, open triangles, open squares), respectively. PGM, phosphoglucomutase (EC 5.4.2.2); PGI, phosphoglucose isomerase (EC 5.3.1.9) ; TPI, triose phosphate isomerase (EC 5.3.1.1); GAPDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12 ); PGK, phosphoglycerate kinase (EC 2.7.2.3); enolase (EC 4.2.1.11); PK, pyruvate kinase (EC 2.7.1.40). The correlation coefficient *r* was calculated using the Pearson's correlation. Relative mRNA expression data was obtained from Mutuku and Nose (2010).

glycolysis. Reactions that exert fine metabolic control can be recognized by the fact that they are greatly displaced from equilibrium *in vivo* (ap Rees and Hill, 1994; Plaxton and Podesta, 2006). These results show that coarse control was exerted to the reactions catalysed by PGM, PGI, TPI and PGmu+enolase whereas, a combination of both coarse and fine controls was exerted to the reactions catalysed by PFK/PFP, aldolase, GAPDH+PGK and PK (Table 3.3).

The results showed that the increase in the levels of enzymatic activities, gene expressions and metabolite contents suggested that additional establishment of glycolysis in the cytosol occurred after infection in the resistant line. Furthermore, coarse control was exerted to the reactions catalysed by PGM, PGI, TPI and PGmu+enolase whereas, a combination of both coarse and fine controls was exerted to the reactions catalysed by PFK/PFP, aldolase, GAPDH+PGK and PK. This suggests that the control of glycolysis in leaf sheaths of *R. solani*-infected rice plants led to increase in the generation of GAP, which was utilized in the generation of E-4-P by transketolase and in down-stream reactions by GAPDH+PGK for generation of PEP.

PEP is reported to be an inhibitor of PFK activity in plants (Plaxton, 1996). PFK has been reported in chloroplast, cytosol (Mustroph et al. 2007) and etioplast (Mutuku and Nose, 2012). PFK located in the chloroplast appears to be very sensitive to PEP because findings reported by Dennis and Greyson (1987) suggest that PEP concentrations greater than 20$\mu$M inhibit its activity. Nevertheless, studies in developing endosperm of rice grain showed that PFK activity was not inhibited by 0.18μmol $g^{-1}$ FW PEP content (Nakamura et al. 1989), which was more than the 140

Table 3. Comparison between enzymes and the exerted control mechanisms as determined by the mass-action ratios in Table 2.

| **Enzyme** | **Mechanism of control** | **Mass-action ratio (MAR)** |
|---|---|---|
| PGM | Coarse | MAR ≈ *K*' |
| PGI | * Coarse | MAR ≈ *K*' |
| PFK/PFP | Coarse and fine | MAR < *K*' |
| Aldolase | **Coarse and Fine | MAR > *K*' |
| TPI | Coarse | MAR ≈ *K*' |
| GAPDH+PGK | Coarse and fine | MAR < *K*' |
| PGmu+enolase | ***Coarse | MAR ≈ *K*' |
| PK | Coarse and fine | MAR < *K*' |

*Expression data not available for PGI
**Expression data not available for aldolase
***PGmu+enolase relies on expression data of enolase

nmol $g^{-1}$ FW PEP content we observed in the current studies. Moreover, findings by ap Rees *et al*. (1977) suggest that increase in PFK activity is also accompanied by high PEP content. These reports suggest that high PEP content can be accompanied by high PFK activity under certain conditions. The current studies combined with those reported previously (Mutuku and Nose, 2010, 2012) suggest that, (a) *R. solani* infection in rice leads to increase in PEP contents, (b) 140 nmol $g^{-1}$ FW PEP content does not inhibit PFK activity in leaf sheaths of *R. solani*-infected rice plants. Therefore it could be argued that the control of PFK/PFP, aldolase, TPI, GAPDH+PGK and TK reactions in infected rice plants was closely coordinated with the demand of the cells for E-4-P and PEP both of which are utilized by DAHP synthase in the shikimate pathway. The shikimate pathway supplies substrates to the phenylpropanoid pathway where resistance products such as lignin are generated.

One of the markers for the lignification process is $H_2O_2$ (Olson and Varner, 1993). The formation of $H_2O_2$ was confirmed by the uptake and polymerization of DAB forming a dark staining (reddish-brown) reaction (Fig 3.9A). In order to rule out the possibility that the local DAB reactions were limited due to a lack of available peroxidase, we assayed for the presence of this enzyme in leaf sheaths of *R. solani*-infected rice plants of both lines. The results showed that peroxidase activity increased after *R. solani* infection. In addition, in order to examine the constitutive peroxidase activity in the control rice plants, DAB and varied concentrations of $H_2O_2$ were added. The results showed that, the dark staining reaction confirming the uptake and polymerization of DAB, changed with changing concentration of $H_2O_2$ from 'strong' to 'not visible' (Fig. 3.9B). These changes in the DAB reactions reflected increase in local $H_2O_2$. $H_2O_2$ may also serve as an immediate mechanism of disease

resistance (Apostol et al. 1989; Thordal-Christensen et al. 1997). Taken together, high PAL and peroxidase activity and generation of $H_2O_2$ indicated activation of the phenylpropanoid pathway. These increases maybe linked to increased lignification as a first line of defence in leaf sheaths of *R. solani*-infected rice plants (M. Mutuku, J. Danson, K. Wasano and A. Nose, unpublished).

Taken together, these results suggest that to control the rate of glycolysis and to enhance lignin deposition in leaf sheaths of *R. solani*-infected rice plants, one of the strategies of metabolic engineering could be to modulate the regulatory reactions of PFK/PFP, aldolase, GAPDH+PGK and PK using fine control mechanisms to enhance glycolysis during disease stress acclimation. The reactions catalysed by GAPDH+PGK and PK where coarse and fine controls overlapped might require a combination of strategies used for both metabolic controls. PGM, PGI, TPI and enolase could be manipulated by coarse control where for example, TPI is modulated to favour generation of GAP during acclimation to stress conditions. This approach targets multiple steps of glycolysis in leaf sheaths of *R. solani*-infected rice plants. This is because flux through a metabolic pathway is not determined by kinetic constants of single steps (Capell and Christou, 2004). Moreover, at branch points like those between GAP to E-4-P, and E-4-P and PEP to secondary metabolism, overexpression of transketolase, and DAHP synthase, respectively, to out-compete other enzymes using the same substrate could divert flux into appropriate pathways like those leading to lignin generation (Fig. 3.11).

Although PGM, TPI, GAPDH, enolase and PK activity increased in leaf sheaths of *R. solani*-infected rice plants, we suspect that coarse metabolic regulation of these

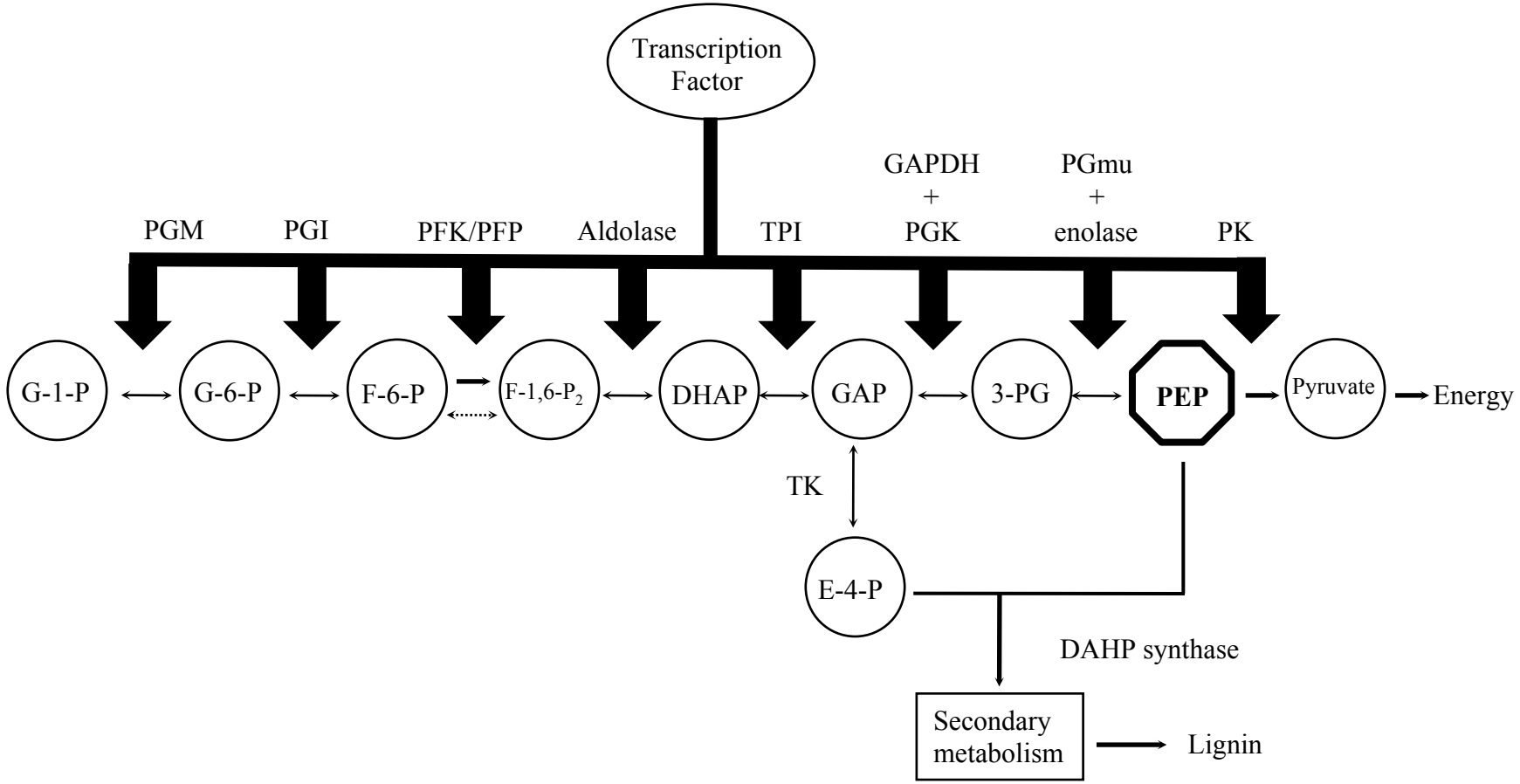


Figure 3.11. A strategy to metabolic engineering in rice plants. Instead of modulating individual steps, metabolic engineering would probably involve approaches in which multiple steps are targeted simultaneously. In this Figure, in the pathway to the desired compound PEP (phosphoenolpyruvate) during resistance response against disease stress, a transcription factor is being used to coordinately upregulate several enzymes, PGM, PGI, PFK/PFP, aldolase, TPI, GAPDH+PGK and PGmu+enolase. Increase in the flux through the glycolytic pathway can be achieved by modifying the reversible reaction catalysed by PGM, PGI, aldolase and PGmu+enolase to favour unidirectional catalysis. The TPI reaction can be modulated to favour generation of GAP during the resistance response. At branch points like those between GAP to E-4-P, E-4-P and PEP to secondary metabolism, and PEP topyruvate over expression of the key enzymes, TK, DAHP synthase and PK to out-compete other enzymes using the same substrate can divert flux into appropriate pathways. PGM, phosphoglucomutase; PGI, phosphoglucoisomerase PFP, pyrophosphate-fructose-6-phosphate-phosphotransferase; PFK, phoshofructokinase; TPI, triose phosphate isomerase; GAPDH+PGK, glyceraldehyde-3-phosphate dehydrogenase +phosphoglycerate kinase; PGmu+enolase, phosphoglycerate mutase +enolase; PK, pyruvate kinase; TK, transketolase; DAHP synthase, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase.

enzymes is not the key mechanism through which glycolysis is controlled. Regulation by a fine metabolic control system influencing the catalytic activities of pre-existing PFK/PFP, aldolase, GAPDH+PGK and PK may be predominant. This is because, (a) metabolic regulation of plant glycolysis is controlled by enzymes that catalyse regulatory reactions and the mechanisms whereby their activities are controlled *in vivo* (Blakeley and Dennis, 1992; ap Rees and Hill, 1994; Plaxton, 1996), (b) whereas coarse control is achieved through varying the total population of enzyme molecules via alterations in rates of enzyme biosynthesis or proteolysis, fine controls are generally fast and energetically inexpensive regulatory devices that sense the momentary requirements of the cells and adjust the rate of metabolic flux through the pathway accordingly (Plaxton, 1996).

The increase in enzymatic activities and metabolite contents suggested that establishment of alternative glycolysis in the cytosol occurred after infection. Changes in metabolites were utilised to determine control points of glycolysis because they represent the results of actual regulation resulting from the interaction between coarse and fine metabolic controls. Although various studies have examined metabolites (metabolome analysis) (Birkemeyer *et al*., 2006; Ishikawa *et al*., 2010), few have utilised the data to investigate regulation. The current studies therefore show a useful way to utilise metabolome data to study regulation.

The results of this study showed that in most of the cases, changes in metabolites content and enzyme activity after *R. solani* infection in the resistant and susceptible rice lines were observed at 1 dpi and we propose investigating these changes within shorter timelines like hours. These results also showed that the activities of aldolase,

GAPDH, PGK, PGmu and enolase in leaf sheaths of *R. solani*-infected rice plants of the resistant line at 1 dpi resembled those of the susceptible rice line at 4 dpi indicating that the amplitude of the response from the susceptible rice line was lower than that from the resistant rice line. Further investigations have shown that these differences between the resistant and susceptible rice lines is also evident in the amount of lignin deposited where higher levels were detected in the resistant line (M. Mutuku, J. Danson, K. Wasano and A. Nose, unpublished). This is evidence that susceptibility occurred because of failure by the plants to respond strongly and in a timely manner to the pathogen signals. This implies that susceptible cultivars may be manipulated to resist pathogen attack by altering timing and magnitude of the defence response.

Due to the flexible structure of plant glycolysis, and its variation due to external environment, we propose further investigations into fine control of glycolysis and how compartmentation affects it. Further investigations should also examine isozymes because recent studies where mRNA accumulation of PFP/PFK isozymes increased showed that not all of them are involved in defence response against sheath blight disease (Mutuku and Nose, 2012). In addition, to fill the metabolic gap between the results of the current study and the global analysis of biological processes that take place in rice after *R. solani* infection, we propose that further metabolome analysis of the *R. solani*-resistant line be done.

## 5. Summary

*Rhizoctonia solani* Kuhn causes sheath blight disease in rice and genetic resistance against it is the most desirable characteristic. Current improvement efforts are based on analysis of polygenic QTL but interpretation is limited by the lack of information on the changes in metabolic pathways. Our previous studies linked activation of glycolytic pathway to enhanced generation of lignin in the phenylpropanoid pathway. The current studies investigated the regulation of glycolysis by examining the time course of changes in enzymatic activities and metabolite contents. The results showed that the activities of all glycolytic enzymes as well as F-6-P, F-1,6-$P_2$, DHAP, GAP, 3-PG, PEP and pyruvate contents increased. These results combined with our previous findings that the expressions of PGM, TPI, GAPDH, enolase and PK increased after infection suggested the additional establishment of glycolysis in the cytosol compartment occurred after infection. Further evidence for this was our recent findings that the increase in expression of the PFK plastid isozyme *Os06g05860* was accompanied by increase in expressions of three cytosolic PFK isozymes i.e., *Os01g09570*, *Os01g53680* and *Os04g39420* as well as PFP isozymes *Os08g25720* ($\alpha$-subunit) and *Os06g13810* ($\beta$-subunit) in infected rice plants of the resistant line. The results also showed the reactions catalysed by PFK/PFP, aldolase, GAPDH+PGK and PK in leaf sheaths of *R. solani*-infected rice plants were non-equilibrium reactions *in vivo*. This study showed that PGM, PGI, TPI and PGmu+enolase could be regulated through coarse control whereas, PFK/PFP, aldolase, GAPDH+PGK and PK could be regulated through coarse and fine controls simultaneously.

# CHAPTER 4

## High activities and mRNA expression of pyrophosphate-fructose-6-phosphate-phosphotransferase and 6-phosphofructokinase are induced as a response to *Rhizoctonia solani* infection in rice leaf sheaths

### 1. Introduction

Rice plants have been reported to mobilize a complex network of active defence mechanisms, which include induction of pathogenesis-related genes, *PR1b* and *PBZ1* (Zhao *et al*., 2008), and lipoxygenase (*LOX*) gene (Taheri and Tarighi, 2010). More aggressive tactics used by plants against this pathogen have been reported to include synthesis of toxic antimicrobial compounds (phytoalexins) (Broglie *et al*., 1991), and production of oxidized phenolic compounds, which slow the spread of the infection (Groth and Nowick, 1992). Studies that have addressed changes in metabolism in response to sheath blight disease have shown that there is an inhibition of starch synthesis and activation of glycolysis (Danson *et al.,* 2000*a*; Nose *et al*., 2002; Mutuku and Nose, 2010, 2011). The consequence of this is that reduced starch synthesis leads to reduced grain yield because starch accumulated in rice leaf sheaths contributes 30% of the grain yield (Ishikawa *et al*., 1993; Hirose *et al*., 2006). Further studies revealed that *R. solani* infection in rice plants led to activation of phenylpropanoid pathway in secondary metabolism leading to increased $H_2O_2$ and lignin generation (Danson *et al.,* 2000; Mutuku and Nose, 2010; Taheri and Tarighi, 2010). Increased lignification leads to strengthening of the plant cell wall thereby restricting access of the pathogen to the plant cell (Danson, 2000*b*). Considering that

the substrates for secondary metabolism are generated from the glycolytic pathway, it is possible to argue that this pathway might be a target to identify more specific defence-related enzymes whose genes can be manipulated using methods such as metabolic engineering to enhance resistance response against *R. solani* infection (Mutuku and Nose, 2010).

One of the key regulatory reactions in the glycolytic pathway is the conversion of fructose-6-phosphate (F-6-P) to fructose-1,6-bisphosphate (F-1,6-$P_2$) by PFK (Plaxton, 1996). Previously, while investigating changes in metabolic enzymes in rice responding to *R. solani* infection, mRNA expression of pyrophosphate- fructose-6-phosphate-phosphotransferase/ 6-phosphofructokinase (PFP/PFK) common regions was reported to increase (Mutuku and Nose, 2010). Whereas most glycolytic enzymes are conserved between different organisms, various types of PFK exist. These are the ATP-dependent 6-phosphofructokinase (PFK, EC 2.7.1.11) and a second type of PFK that uses pyrophosphate (PPi) as the phosphoryl donor (pyrophosphate-fructose-6-phosphate-phosphotransferase PFP, EC 2.7.1.90) (Botha and Botha, 1991). Phosphorylation of F-6-P catalyzed by PFK is irreversible *in vivo*, while PFP reacts near equilibrium and catalyzes the reaction in both directions (Plaxton, 1996) (Fig. 4.1A). In rice plants, there are 13 PFP/PFK isozymes (Mustroph *et al*., 2007). Although our previous studies showed that mRNA expression of PFP/PFK common regions in rice plants responding to *R. solani* infection increased, isozymes were not investigated. Since isozymes often show different subcellular localisation, it might be possible to speculate that not all 13 PFP/PFK isozymes are related to mechanisms of disease resistance after *R. solani* infection. Since PFP and PFK are at the carbon crossroad between sucrose and starch synthesis, it is necessary to identify the

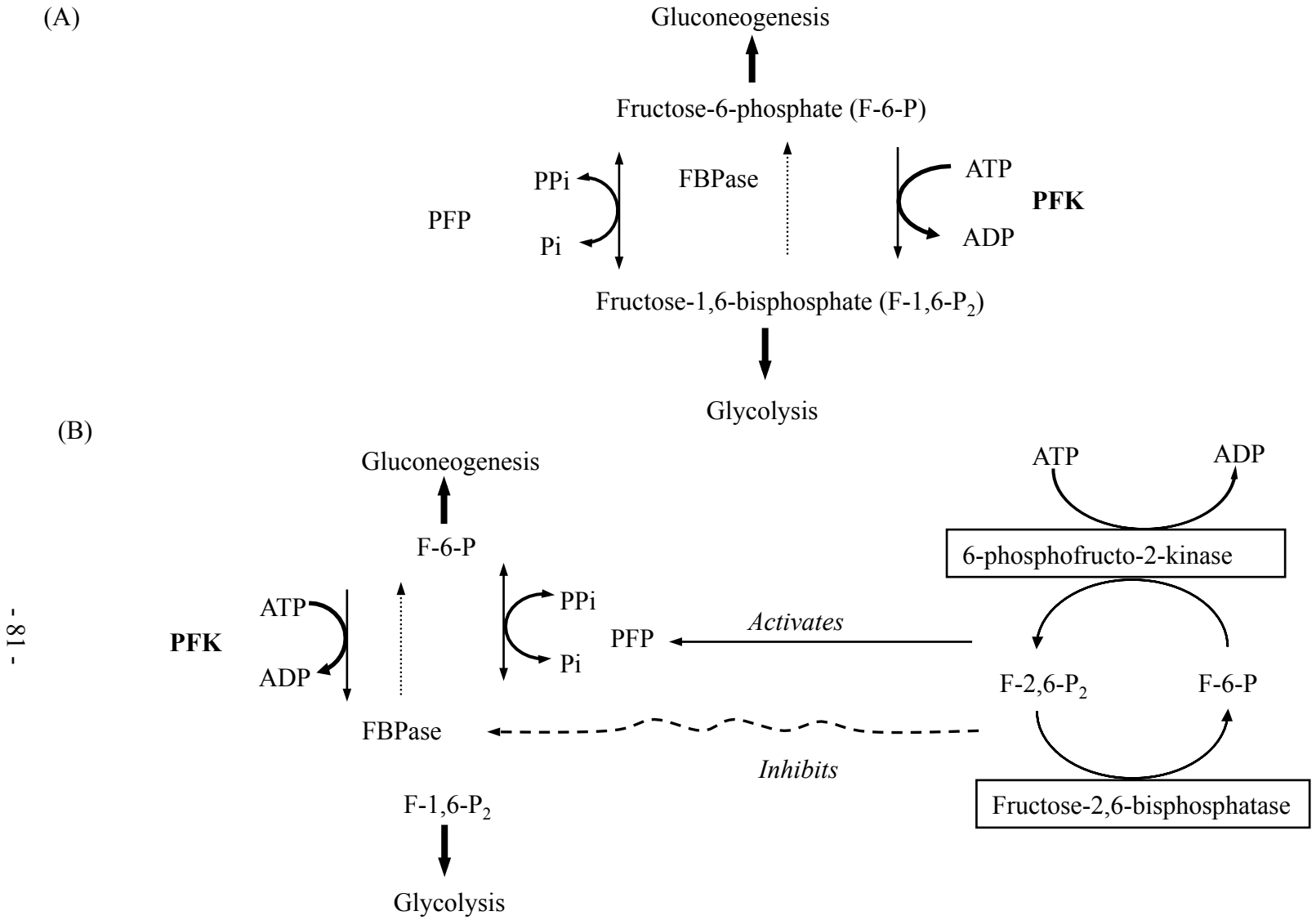


Figure 4.1 (A) Interconversion of fructose-6-phosphate (F-6-P) and fructose-1,6-bisphosphate (F-1,6-$P_2$). While ATP-dependent phosphofructokinase (PFK) and fructose-1,6-bisphosphatase (FBPase) catalyze irreversible reactions in the glycolytic and gluconeogenic directions respectively, pyrophosphate-fructose-6-phosphate-phosphotransferase (PFP) catalyzes a freely reversible reaction. (B) Regulation of the interconversion of F-6-P and F-1,6-$P_2$ in plants responding to stress conditions like those caused by *R. solani* infection. Fructose-2,6-bisphosphate (F-2,6-$P_2$) stimulates PFP and inhibits FBPase.

isozymes that are related to defence response in *R. solani*-infected rice plants (Mutuku and Nose, 2010). This is important following recent findings that showed PFK is one of the enzymes that can be targeted for manipulation by metabolic engineering to enhance the glycolytic pathway in *R. solani*-infected plants (Mutuku and Nose, 2010).

Plants PFPs are reported to be activated when plants are subjected to stress causing suboptimal conditions like anoxia and phosphate (Pi) starvation (Theodorou and Plaxton, 1996). In addition, to coarse control by regulation of the enzyme concentration, fine metabolic control of PFP is exerted by fructose-2,6-bisphosphate (F-2,6-$P_2$), pyrophosphate (PPi) (Botha and Botha, 1991; Trevanion, 2000) and pH (Ahmed *et al*., 2003). F-2,6-$P_2$ is a cellular signalling molecule found in all eukaryotes regulating a single step in cytosolic metabolism: the inter-conversion of F-6-P and F-1,6-$P_2$ (Banzai *et al*., 2003; Nielsen *et al*., 2004). This is a key junction in the pathways of cellular carbon flow. In plants, it activates PFP and inhibits cytosolic fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11) (Okar and Lange, 1999) (Fig. 4.1B). F-2,6-$P_2$ is reported to occur in plants in total concentrations ranging from 0.05-1.00 nmol $g^{-1}$ FW (Nielsen *et al*., 2004). Various reports have suggested that by using PPi as a phosphoryl donor, PFP provides an alternative route to PFK (Smyth and Black, 1984; Weiner *et al*., 1987; Blakeley and Dennis, 1993). Indeed, in phosphate (Pi)-starved *Brassica nigra* suspension cells, PFP functioned as a metabolic by-pass to PFK (Theodorou and Plaxton, 1996) while in hypoxic rice seedlings, both PFP activity and the level of F-2,6-$P_2$ increased, while PFK activity declined (Mertens *et al*., 1990). These and similar findings led to the conclusion that PFK was a maintenance enzyme while PFP was adaptive (Plaxton, 1996). But in contrast to these findings, Danson *et al*. (2000) reported that in rice plants responding *R. solani*

infection the activities of both PFP and PFK increased. Most plant PFPs typically display pH optima ranging from pH 7.2 to 7.8 in the forward direction (Nielsen, 1994; Theodorou and Plaxton, 1996). In tomato, PFP showed maximum activity at pH range 7.5-8.0 (Wong *et al*., 1990) while in wheat, maximum activity was at pH 7.5 (Mahajan and Singh, 1989).

These studies show that the regulation of the step catalyzed by PFP/PFK has significant implications for the control of the glycolytic pathway in plants responding to various stress conditions. However, there are questions that need to be answered in relation to rice plants responding to *R. solani* infection. These include (a) confirmation of the changes in the activities of PFP and PFK, (b) how the infection affects mRNA expression of PFP/PFK isozymes, and (c) how the infection affects the cofactor concentrations, metabolite effectors and pH, which are fine control mechanisms that modulate the activity of these enzymes. To answer these questions, the rice lines previously developed by Wasano *et al*. (1985) were used and changes were observed one day, two days and four days post inoculation (dpi) as previously described by Danson (2000*b*).

## 2. Materials and Methods

### 2-1. Sample preparation

Sample and fungus preparation were done as previously described (Mutuku and Nose, 2010). Briefly, the two rice lines namely, ($2F_{18}$-7-32) *R. solani*-resistant and ($2F_{21}$-21-29) *R. solani*-susceptible improved by Wasano *et al*. (1985) were grown in pots under

16 h of light and 8 h of darkness at 25 $^{o}$C. Sheath blight fungus isolate C154 from National Institute of Agrobiological Sciences Gene Bank, Tsukuba, Japan, was grown on potato-sucrose-agar medium at 28 °C for about 4 days. Then 0.25 mL of the fungal suspension was injected into the interstices between the second and the third leaf sheaths from the flag leaf of the plants at the 7-leaf growth stage. Control plants were mock treated with distilled water. Lesions and their border areas (5 cm) were excised from at least 50 plants of both treated and control plants at 10 o'clock in the morning one day, two days and four days post- inoculation (dpi). Total RNA extraction, semi-quantitative RT-PCR, PCR, agarose gel electrophoresis and gel analysis were done as previously described (Mutuku and Nose, 2010).

**2-2. Rice leaf sheath PFP and PFK enzyme extraction**

All buffers were adjusted to their respective pH values at 25 $^{o}$C, and cooled to 4 °C prior to use. In liquid $N_2$ chilled pestles and mortars, rice leaf sheaths (0.5 g) and 0.3 g of sand were pulverized with 7 mL of ice-cold extraction medium containing; 100 mM Hepes-NaOH pH 8.0, 150 mM potassium acetate, 30 mM $\beta$-mercaptoethanol, 0.5 mM monoiodoacetic acid (MIA), 2 mM phenylmethylsulfonylflouride (PMSF), 5 mM $MgCl_2$, 1 mM EGTA-NaOH pH 8.2, 20 % (w/v) glycerol, 1 % (w/v) PVP-40, 10 $\mu$g mL $^{-1}$ chymostatin, and 10 $\mu$g mL $^{-1}$ leupeptin. The brei was filtered through one layer of Miracloth (Calbiochem-Novabiochem, La Jolla, CA, USA) and centrifuged at 38,000 *g* for 10 min at 4$^{o}$C. The supernatant was desalted by passage through Sephadex G-25 (PD-10 column; Pharmacia, Uppsala, Sweden) previously equilibrated with the extraction buffer.

**2-2-1. Rice leaf sheath PFP and PFK activity assays**.

Rice leaf sheath PFP and PFK were assayed as described by Carnal and Black (1983) at 30°C in a 1.0 mL reaction mixture containing; 100 mM Hepes-NaOH pH 8.0, 2.5 mM $MgCl_2$, 0.08 mM NADH, 10 mM F-6-P, 5 U $mL^{-1}$ aldolase, 1 U $mL^{-1}$ triose-phosphate isomerase, 5 U $mL^{-1}$ $\alpha$-glycerol-3-phosphate dehydrogenase, plant extract, and either 1 mM ATP or 1 mM PPi. F-6-P was acid-treated as described by Theodorou *et al*. (1996). F-6-P was titrated to pH 3.0 with HCl, incubated for 1 h, and then neutralized with NaOH to hydrolyse contaminating traces of F-2,6-$P_2$. Enzymes supplied as $NH_4(SO_4)_2$ precipitates used for these assays were treated as described by Trevanion (2000). Enzymes were resuspended in buffer (100 mM Tris- HCl pH 8.0, 1 mM $MgCl_2$) and desalted using NAP$^{TM}$ 5 columns from Amersham Pharmacia Biotech (Uppsala, Sweden). Reactions were initiated with either ATP or PPi after allowing the reactions to run for 2 min. Reaction progress was monitored at 340 nm with a UV-visible spectrophotometer (JASCO V-550UV/VIS, Japan). The assays were done in the presence (by adding of 500 $\mu$L of F-2,6-$P_2$ crude extract) and absence of F-2,6-$P_2$. Activities are given as $\mu$mol $min^{-1}$ mg $protein^{-1}$.

**2-3. Fructose-1,6-bisphosphatase (FBPase) activity assays**

FBPase activities were measured in extracts as described by Entwistle and ap Rees (1988). The samples were 0.5 g and the buffer was 50 mM Tris-HCl, pH 7.1.

## 2-4. PFP, PFK and FBPase mRNA expression

Phosphofructokinase genes from rice were selected based on their sequence homology from public databases http://rice.plantbiology.msu.edu/ and http://rapdb.dna.affrc.go.jp/. The protein sequences used in this alignment can be found in Appendix 1.

Total RNA extraction, optimisation of semi-quantitative RT-PCR, PCR, agarose gel electrophoresis and gel analysis were done as previously described (Mutuku and Nose, 2010). The number of PCR cycles and annealing temperature were chosen separately for each primer pair; 27 cycles/ 58 $^{o}$C, actin; 35 cycles/ 60 $^{o}$C, PFK (1, 3, 5, 6, 8); 35 cycles/ 58 $^{o}$C, PFK (2, 4, 7), PFP (1, 2, 3, 4, 5) and FBPase (1, 2). Table 4.1 shows the genes, locus names, description, predicted subcellular localisation and sequences of the primers used. The sequences for Actin were obtained from Mutuku and Nose (2010) and those of FBPase were obtained from Lee *et al*. (2008).

## 2-5. Extraction of fructose-2,6-bisphosphate (F-2,6-$P_2$) from rice leaf sheaths

Extraction of F-2,6-$P_2$ was done according to Banzai *et al*. (2003) with some modifications. Rice leaf sheath samples were ground to a fine powder with a mortar and pestle in liquid nitrogen and of each, 0.5 g was suspended in 20 volumes of 50 mM KOH. The homogenate was incubated at 80 $^{o}$C for 60 min and vortexed every 10 min. Then 50 mg mL $^{-1}$ of powdered activated charcoal was added to the homogenate and incubated for 15 min on ice. The homogenate was centrifuged at 16, 000 *g* for 10

Table 4.1. Primer sequences of genes that encode pyrophosphate-fructose-6-phosphate-phosphotransferase (PFP), ATP- dependent phosphofructokinase (PFK) and fructose-1,6-bisphosphatase (FBPase)

| Gene | Locus name | Description | Biological process | Localisation | Primer sequences | |
|---|---|---|---|---|---|---|
| **Genes that encode 6-phosphfructokinase** | | | | | | |
| PFK 1 | Os01g09570 | PFK, putative, expressed | generation of precursor metabolites and energy | Cytosol | Left | TGAGGCTGAGAGTGCAGAGA |
| | | | | | Right | TTCATGCTTTTCGCAATGAG |
| PFK 2 | Os05g10650 | PFK, putative, expressed | generation of precursor metabolites and energy | Cytosol | Left | AACGTCTACTGCTCGCTGCT |
| | | | | | Right | TTTCGCACTACCAAACATGG |
| PFK 3 | Os01g53680 | PFK, putative, expressed | generation of precursor metabolites and energy | Cytosol | Left | TGGCGTCTCACATCATTCTG |
| | | | | | Right | GCACTGCATACTGCAATTTTT |
| PFK 4 | Os04g39420 | PFK-2, putative, expressed | generation of precursor metabolites and energy | Cytosol | Left | GCCGTGGTTTGAACATTTCT |
| | | | | | Right | CGCTTGGGTGTTCTCTCTTC |
| PFK 5 | Os06g05860 | PFK, putative, expressed | generation of precursor metabolites and energy | Chloroplast | Left | TTAGCATCCCAAGCTTTGCT |
| | | | | | Right | ATCTTTGCGTCCTCCACATC |
| PFK 6 | Os09g24910* | PFK, putative, expressed | generation of precursor metabolites and energy | [1] Chloroplast | Left | ATTGCCATGCAAGCTTCTCT |
| | | | | | Right | TTGCATGTGGACACCAAAGT |
| PFK 7 | Os10g26570* | PFK, putative, expressed | generation of precursor metabolites and energy | [2] Chloroplast | Left | GCAGTTGAAGCAGCACAGAG |
| | | | | | Right | CGGCTACACAAATCAGAGCA |
| PFK 8 | Os08g34050* | PFK, putative, expressed | generation of precursor metabolites and energy | [3] Chloroplast | Left | CTTTGAGGGAGGCTGAACTG |
| | | | | | Right | AGCCGTATCGAAACCAAATG |
| **Genes that encode PFP** | | | | | | |
| PFP 1 | Os02g48360 | PFP α, putative, expressed | generation of precursor metabolites and energy | Cytosol | Left | CCTCTCACTGCCTGCACATA |
| | | | | | Right | GCAAAGTTCGGTTGAAGAGC |
| PFP 2 | Os08g25720 | PFP α, putative, expressed | generation of precursor metabolites and energy | Cytosol | Left | ACAACGTGATCTGGGGACTC |
| | | | | | Right | ACCGAGCAAATCAAAACCAC |
| PFP 3 | Os06g22060 | PFP α, putative, expressed | generation of precursor metabolites and energy | Cytosol | Left | AGGTGCCCTACCCTCAAAGT |
| | | | | | Right | ACCATCGCTTTACGGTCATC |
| PFP 4 | Os09g12650 | PFP α, putative, expressed | generation of precursor metabolites and energy | Cytosol | Left | TGCAGCAAGAAGAGCAAGAA |
| | | | | | Right | GGGGTTTAGCACCATTCTGA |
| PFP 5 | Os06g13810 | PFP β, putative, expressed | generation of precursor metabolites and energy | Cytosol | Left | GCTGGAGTCATGAAGTGCAA |
| | | | | | Right | TTGCACATAAAAGCGCAAAG |
| **Genes that encode FBPase** | | | | | | |
| FBPase | Os01g64660 | FBPase 1 | | Cytosol | Left | CATCAAGGCGTGCGAATGG |
| | | | | | Right | GCCTGACGAATCATGCATGT |
| FBPase | Os05g36270 | FBPase 2 | | Cytosol | Left | GTTGACCGGTGTTACTGAAGAGT |
| | | | | | Right | AGCAAAAAAAAAATGTCTATGTTCACAAGGT |
| **Actin** | | | | | Left | TCGTCGTACTCAGCCTTGGCAAT |
| | | | | | Right | CTCTCTGTATGCCAGTGGTCGTA |

For easier handling, genes that encode **PFK** were arbitrary assigned to PFK 1 to PFK 8 and those that encode **PFP** were assigned numbers PFP 1 to PFP 5. Actin primers were taken from Mutuku and Nose (2010). PFP $\alpha$ and PFP $\beta$ represent genes that encode the PFP subunit alpha and beta respectively. The locus names and gene descriptions were obtained from rice genome annotation project (http://rice.plantbiology.msu.edu/). The subcellular localisation was predicted by analysing the sequences using the prediction program TargetP (Nielsen *et al*., 1997; Emanuelsson *et al.,* 2000) * mRNA expression was too low to be detected. [1,] Membrane bound chloroplast where chloroplast transit peptide 1-3 is found, [2] Membrane bound chloroplast where chloroplast transit peptide 1-7 is found, and [3] Membrane bound chloroplast where chloroplast transit peptide 1-9 is found, respectively.

min at 4 $^{o}$C to remove insoluble materials. Centrifugation was repeated twice. The supernatant was recovered and used for quantification of F-2,6-$P_2$.

### 2-5-1. Fructose-2,6-bisphosphate (F-2,6-$P_2$) assays

F-2,6-$P_2$ was assayed according to Van Schaftingen and Hers (1983) with some modifications. The reaction mixture, 1 mL, contained; 50 mM Tris/acetate (pH 8.0), 2 mM $MgCl_2$, 1 mM F-6-P, 0.15 mM NADH, 0.5 mM PPi, 0.002 U mL $^{-1}$ PFP, 0.1 U mL $^{-1}$ aldolase, 1 U mL $^{-1}$ triosephosphate isomerase, 0.1 U mL $^{-1}$ $\alpha$-glycerol-3-phosphate dehydrogenase, and up to 100 $\mu$l extract. The assay was started by adding PPi after 5 min pre-incubation. F-6-P used in this assay was treated to avoid contamination by F-2,6-$P_2$ as described above. The specificity of the assay for F-2,6-$P_2$ was tested as described by Claassen *et al*. (1991) by acidifying the extract with 0.1 N HCI to pH 2 and incubation for 30 min. No recovery experiments were done due to unavailability of commercial F-2,6-$P_2$. The specificity of the assay for F-2,6-$P_2$ was tested as described and no F-2,6-$P_2$ was detected after neutralization of the extract with KOH.

## 2-6. Generation of fructose-2,6-bisphosphate (F-2,6-$P_2$)

F-2,6-$P_2$ was generated by assays of 6-phosphofructo-2-kinase (F6P2K) activity in rice leaf sheaths, which were done according to Banzai *et al*. (2003). The frozen rice leaf sheaths were mixed with 20 volumes of Buffer A; 50 mM MOPS-KOH pH 7.3, 10 % (v/v) ethylene glycol, 5 mM $MgCl_2$, 1 mM EDTA, 0.1 % (v/v) $\beta$-mercaptoethanol, 0.1 % (v/v) Triton X-100, 5 mM benzamidine, 2 mM

phenylmethylsulfonylflouride (PMSF), 3 mg $L^{-1}$ leupeptin, 3 mg $L^{-1}$ antipain. The mixture was centrifuged at 5,000 *g* at 4 $^{o}$C for 10 min. The supernatant was recovered and desalted through a Sephadex G-25 column equilibrated with reaction buffer. F6P2K assay was as described by Banzai *et al*. (2003) except the reaction mixture was 500 $\mu$L and that at various times, aliquots of the reaction mixture were removed and immediately used in PFP and PFK assays as described above to measure effects of F-2,6-$P_2$ on activities of PFP and PFK.

**2-7. Extraction of pyrophosphate (PPi) from rice leaf sheaths**

Pyrophosphate (PPi) was extracted as described by Chen and Nose (2001) with modifications. About 0.5 g of ground rice leaf sheaths were pulverised in liquid nitrogen in the presence of 6 mL of ice-cold 5 % (w/v) trichloroacetic acid (TCA) containing 10 mM *N',N',N',N'*-tertraacetic acid (EGTA) and 0.35 g polyvinylpyrrolidone (PVP). The mixture was kept on ice for 30 min and centrifuged for 10 min at 20,000 *g* at 4 $^{o}$C. Then 60 mg of activated charcoal was added to the supernatant, kept in ice for 20 min and centrifuged as above. The supernatant was immediately used for measurement of PPi.

**2-7-1. Pyrophosphate (PPi) assays**

PPi content was measured according to Chen and Nose (2001) with some modifications. The reaction mixture contained in 1 mL; 0.3 mL extract, 200 mM Hepes-KOH pH 8.2, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM F-6-P, 0.15 mM NADH, 0.5 U $mL^{-1}$ aldolase (EC 4.1.2.13), 1.7 U $mL^{-1}$ $\alpha$-glycerol-3-phosphate dehydrogenase

(GDH, EC 1.1.8) and 5 U mL $^{-1}$ triosephosphate isomerase (TPI, EC 5.3.1.1). The reaction was started by the addition of 0.1 U mL $^{-1}$ of PPi-PFK. The blank contained the extract and all the other reagents except PPi-PFK, which was replaced with distilled-deionised water. Oxidation of NADH was monitored by the decrease in absorbance at 340 nm using a spectrophotometer (JASCO V-550UV/VIS, Japan) at 30 $^{o}$C until no further decrease was observed (approximately, after about 20 min). The reliability of the extraction procedure and assay protocol was confirmed using recovery experiments (Edward *et al*., 1984); in the case of PPi the recovery was found to be above 94 %. As shown in Table 4.2 when sodium pyrophosphate (100 nmol per assay) was added to the samples before they were pulverised in TCA, 94 % to 100.2 % of the added PPi was recovered suggesting that there were no serious losses of PPi during extraction. The specificity of the reaction was verified as described by Claassen *et al*. (1991) by addition of 0.2 U mL $^{-1}$ pyrophosphatase to the extract. After 60 min incubation, no PPi was detected.

**2-8. The effects of pH on the activities of rice leaf sheath PFP and PFK**

The effects of pH on the activities of rice leaf sheath PFP and PFK were examined as described above except that the buffers were substituted with the following buffers: Mes-NaOH from pH 5.0 to 6.8 and Hepes-NaOH from pH 7.0 to 8.4.

**2-9. Statistical Analysis of Data**

Student's *t*-tests were performed using the algorithm integrated into Microsoft Excel (Microsoft, Seattle). The word "significant" is used in the text only when the change

Table 4.2. Pyrophosphate (PPi) recoveries in leaf sheaths of *R. solani* infected and control plants of resistant and susceptible rice lines at 1, 2 and 4 dpi. Values are means ± SD of three separate experiments.

| | Recovery of added PPi (%) | | |
|---|---|---|---|
| Sample | 1 dpi | 2 dpi | 4 dpi |
| *R. solani* infected (resistant line) | 97 ± 5 | 94 ± 3 | 94 ± 1 |
| Control (resistant line) | 94 ± 1 | 97 ± 2 | 99 ± 1 |
| *R. solani* infected (susceptible line) | 97 ± 2 | 97 ± 5 | 97 ± 3 |
| Control (susceptible line) | 95 ± 4 | 96 ± 3 | 100 ± 2 |

in question has been confirmed to be statistically significant ($P < 0.05$) with the Student's *t*-test.

## 3. Results

### 3-1. Changes in PFP and PFK activity in rice plants responding to *R. solani* infection

Figure 4.2A shows that PFP activity in rice plants responding to *R. solani* infection increased. When both rice lines were compared, PFP activity in the resistant line was found to be significantly higher ($P < 0.05$) than that in the susceptible line. Nonetheless, PFP activities in the *R. solani*-infected rice plants of both the resistant and susceptible lines were significantly higher ($P < 0.05$) than those of control plants. Furthermore, PFP activities in the control plants of the resistant and susceptible rice lines were similar.

Figure 4.2B shows that PFK activity in the rice plants responding to *R. solani* infection increased. In the *R. solani*-infected rice plants of the resistant line, PFK activity increased at 2 dpi and did not change at 4 dpi. In addition, the activity in the resistant line was significantly higher ($P < 0.05$) than that in the susceptible line. Furthermore, when the control plants of both rice lines were compared, PFK activity in the plants of the resistant line was higher than that of the susceptible line. In the *R. solani*-infected plants of the susceptible rice line, PFK activity was similar at 1 and 2 dpi but increased at 4 dpi.

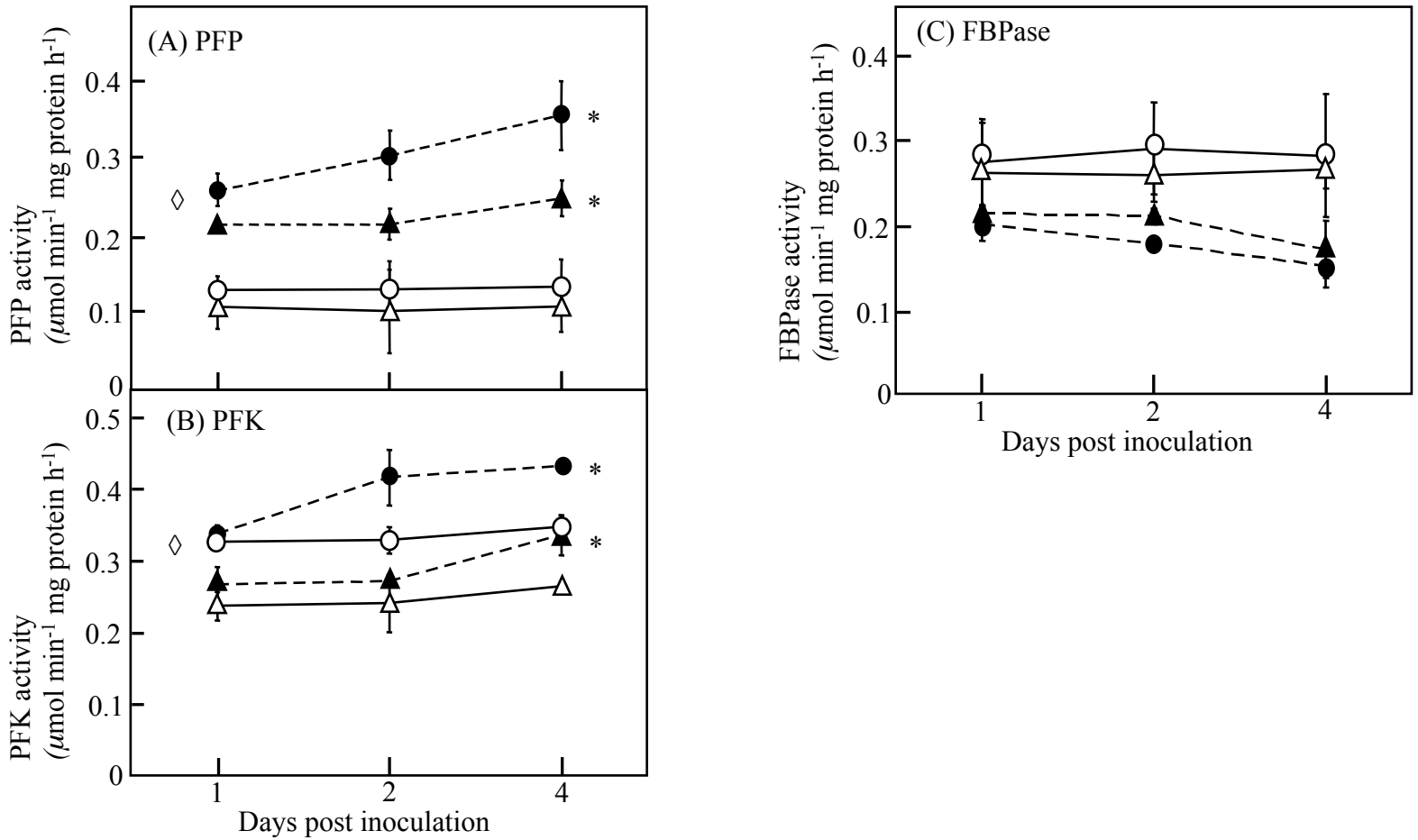


Figure 4.2. (A) PFP, (B) PFK activity in the absence of fructose-2,6-bisphophate, and (C) cytosolic FBPase activity ($\mu$mol min$^{-1}$ mg protein h$^{-1}$) in the *R. solani*-infected plants of the resistant and susceptible rice lines (closed circles, closed triangles) respectively, and those of control plants (open circles, open triangles) respectively, at 1, 2 and 4 dpi. * Values were significantly different ($P < 0.05$, Student's *t*-test) between the *R. solani*-infected plants and those of control plants. ◊ Values were significantly different between *R. solani*-infected plants of the resistant rice line and those of the susceptible rice line. Data were means ± SD of three separate experiments.

Figure 4.2C shows that FBPase activity in the rice plants responding to *R. solani* infection decreased at 1 to 4 dpi. This reduction in FBPase activity was found to be similar in infected rice plants of both resistant and susceptible lines.

Figure 4.3A shows that F-2,6-$P_2$ content in the *R. solani*-infected plants of the resistant line increased, and at 4 dpi, was significantly higher ($P < 0.05$) than that of the susceptible rice line. Although F-2,6-$P_2$ content in the *R. solani*-infected plants of the susceptible rice line did not change at 1 to 4 dpi, it was significantly higher ($P < 0.05$) than that of the control plants. The content of F-2,6-$P_2$ was measured in untreated plants at the seven-leaf stage of growth and the results showed that the content in the untreated plants of the resistant rice line was 0.13 ± 0.004 nmol $g^{-1}$ FW and that of the susceptible rice line was 0.12 ± 0.005 nmol $g^{-1}$ FW (mean ± SD of three separate experiments). When these data were compared with those of the control plants, which had been mock inoculated with distilled-deionised water, no differences were detected.

Figure 4.3B shows that PPi content in the *R. solani*-infected rice plants of the resistant line was higher than that of the susceptible line at 1 to 4 dpi. When PPi content in the *R. solani*-infected rice plants was compared with that of the control plants, significantly higher levels ($P < 0.05$) were detected in the infected plants. However, PPi content in *R. solani*- infected plants decreased especially in the resistant rice line at 4 dpi.

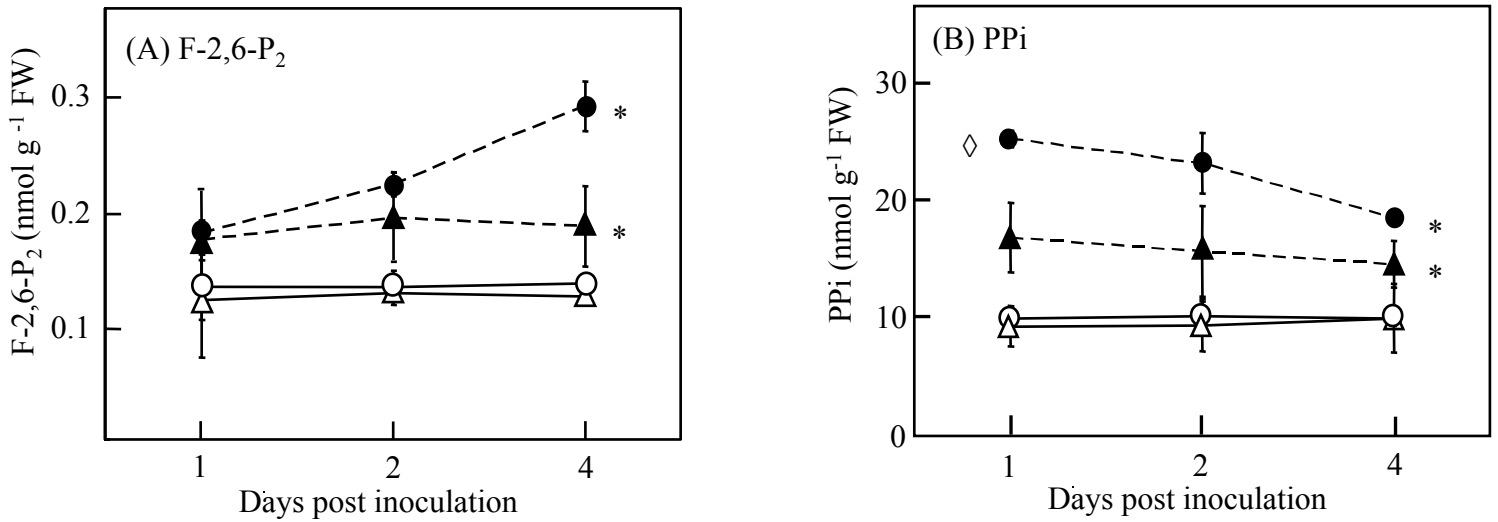


Figure 4.3. (A) fructose-2,6-bisphosphate (F-2,6-$P_2$) content and (B) pyrophosphate (PPi) content (nmol $g^{-1}$ FW) in the *R. solani*-infected plants of the resistant and susceptible rice lines (closed circles, closed triangles) respectively, and those of control plants (open circles, open triangles) respectively, at 1, 2 and 4 dpi. * Values were significantly different ($P < 0.05$, Student's *t*-test) between the *R. solani*-infected plants and those of control plants. ◊ Values were significantly different between *R. solani*-infected plants of the resistant rice line and those of the susceptible rice line. Data were means ± SD of three separate experiments.

### 3-2. PFP and PFK activity after addition of crude F-2,6-$P_2$ extract

Table 4.3 shows that when crude F-2,6-$P_2$ extract was added into the PFP assays, PFP activity was higher than that of assays without the crude extract. However, PFK activity did not change after the addition of crude F-2,6-$P_2$ into the assays.

Table 4.3. Activities of rice leaf sheath PFP and PFK ($\mu$mol min$^{-1}$ mg protein h$^{-1}$) in *R. solani*-infected rice plants of the resistant and susceptible lines in the absence and presence of F-2,6-$P_2$ at 1, 2 and 4 dpi. Values were means ± SD of three separate experiments.

| | | | Days post inoculation | | |
|---|---|---|---|---|---|
| | Rice plants | | 1 | 2 | 4 |
| PFP | Resistant | F-2,6-$P_2$ absence | 0.3 ± 0.014 | 0.3 ± 0.017 | 0.4 ± 0.044 |
| | | F-2,6-$P_2$ presence | 0.5 ± 0.001 | 0.7 ± 0.032 | 0.8 ± 0.023 |
| | Susceptible | F-2,6-$P_2$ absence | 0.2 ± 0.008 | 0.2 ± 0.008 | 0.3 ± 0.015 |
| | | F-2,6-$P_2$ presence | 0.4 ± 0.029 | 0.4 ± 0.034 | 0.3 ± 0.017 |
| PFK | Resistant | F-2,6-$P_2$ absence | 0.3 ± 0.013 | 0.4 ± 0.039 | 0.4 ± 0.007 |
| | | F-2,6-$P_2$ presence | 0.3 ± 0.019 | 0.4 ± 0.033 | 0.4 ± 0.004 |
| | Susceptible | F-2,6-$P_2$ absence | 0.3 ± 0.025 | 0.3 ± 0.001 | 0.3 ± 0.028 |
| | | F-2,6-$P_2$ presence | 0.3 ± 0.004 | 0.3 ± 0.007 | 0.3 ± 0.024 |

### 3-3. Effects of pH on PFP and PFK enzymatic activity

One of the factors that strongly affect the activities of plant PFPs and PFK is pH (Hirose *et al*., 2006). Figure 4.4 shows that rice leaf sheath PFP extracted from *R. solani*-infected rice had maximum activity at pH 7.4 and 8.0 in the resistant and susceptible line respectively. Maximum activity of PFK in the *R. solani*-infected rice plants was at pH 7.8 in both lines. Below pH 5.8, no rice leaf sheath PFP or PFK activity was detected.

### 3-4. PFP and PFK mRNA expression in rice plants responding to *R. solani* infection

As shown in Table 4.1, genes PFK 1 to 4 that encode PFK, PFP 1 to 5 that encode PFP in rice plants and FBPase 1 and FBPase 2 were cytosolic while genes PFK 5 to 8 that encode PFK in rice plants were plastidial isozymes as predicted by TargetP (http://www.cbs.dtu.dk/services/TargetP/). Gene description was obtained from the rice genome annotation project (http://rice.plantbiology.msu.edu/).

In order to analyse the mRNA expression of PFK/PFP isozymes, semi-quantitative RT-PCR analysis was performed. Figure 4.5, 4.6 and 4.7 show the time course of mRNA expression of genes that encode PFP, PFK and FBPase in rice leaf sheaths. The figure shows that in rice plants responding to *R. solani* infection, mRNA expression of PFK 1 in the resistant line was high at 1 to 4 dpi. In the susceptible line, the expression increased at 2 dpi but did not change at 4 dpi. The mRNA expression

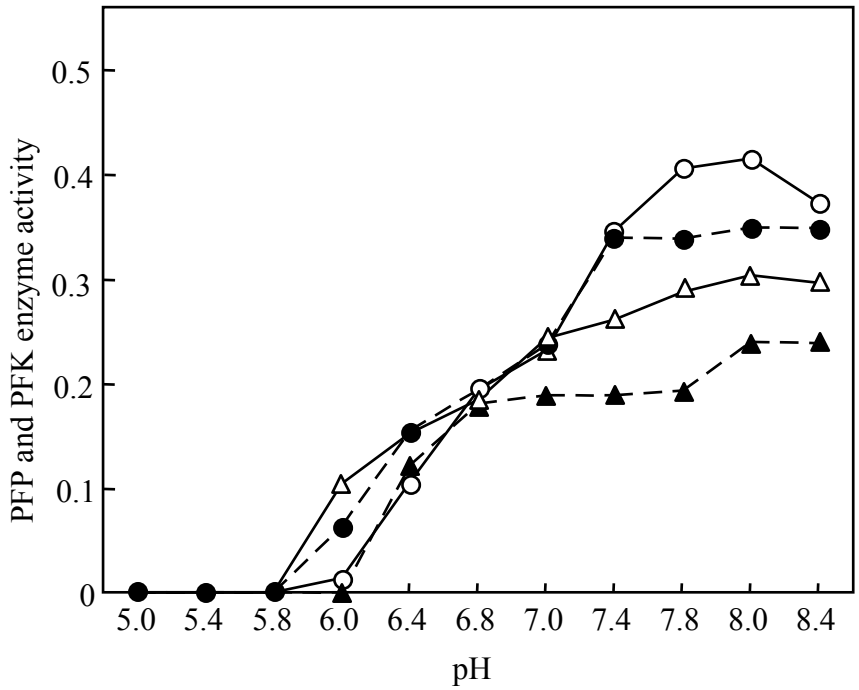


Figure 4.4. Effects of changes in pH to enzymatic activities of rice leaf sheath PFP in the absence of fructose-2,6-bisphosphate in *R. solani* infected rice plants of the resistant and susceptible line (closed circles, closed triangles), respectively, and those of PFK in resistant and susceptible lines (open circles, open triangles ), respectively. Data was obtained from *R. solani* infected rice plants at 4 dpi when highest enzyme activity was expected. The solid line represents PFK activity and the dashed line represents PFP activity. The following buffers were used as described in Material and Methods; Mes-NaOH from pH 5.0 to 6.8 and Hepes-NaOH from pH 7.0 to 8.4.

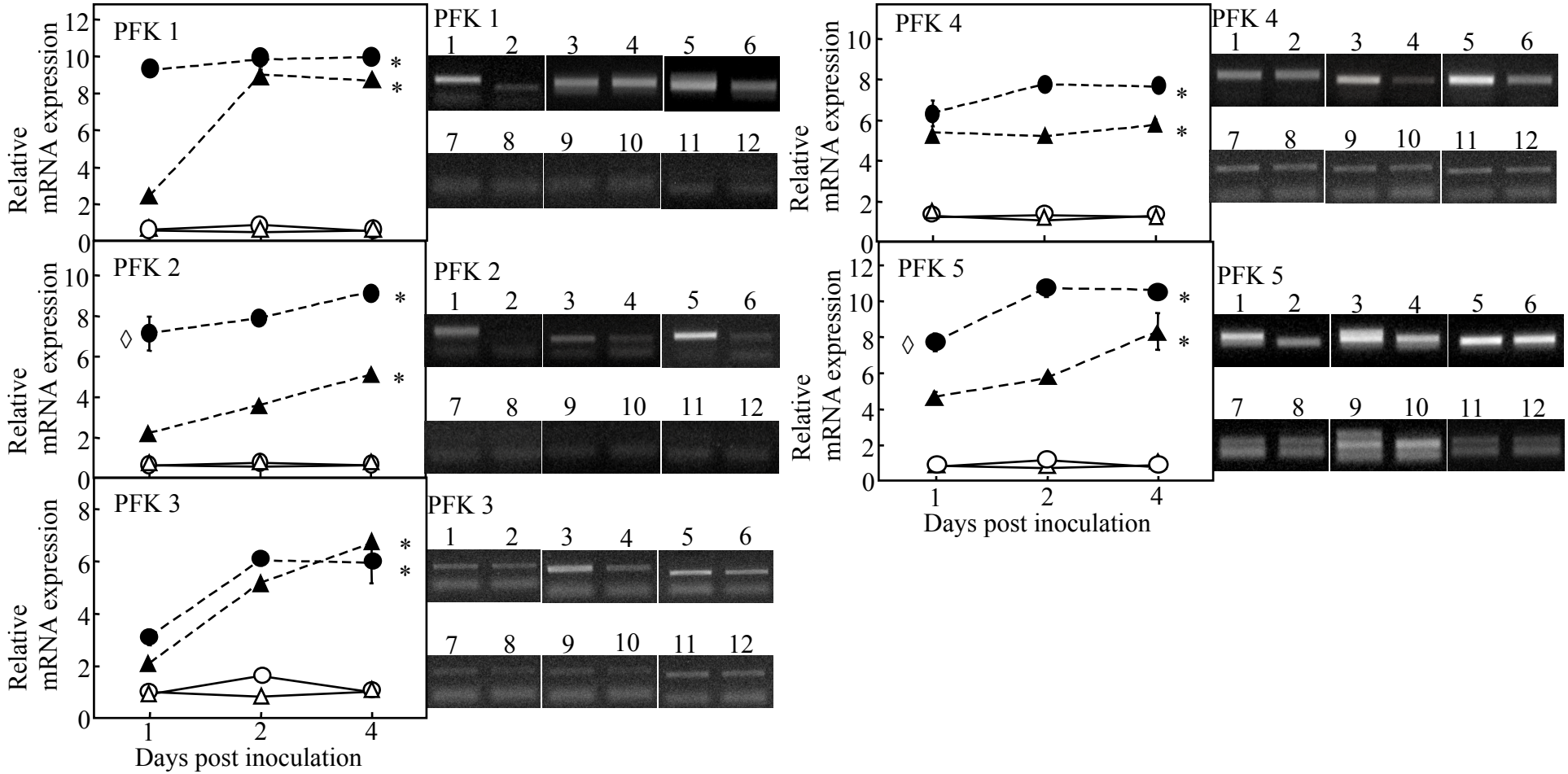


Figure 4.5. The time course of changes in the mRNA expression of gene families encoding PFP and PFK in *R. solani*-infected plants of the resistant and susceptible rice lines (closed circles, closed triangles), respectively, and those of the healthy control plants (open circles, open triangles), respectively, at 1, 2 and 4 dpi. PFK 1 to 5 represent genes that encode PFK as indicated in Table 1. The gel images show one representative result out of three independent determinations. * mRNA expression was significantly different between the *R. solani*-infected rice plants, compared to that of the control plants. ◊ mRNA expression was significantly different between the *R. solani*-infected rice plants of the resistant line, compared to that of the susceptible line. Numbers 1 to 12 represent mRNA expression of 32T-1, 29T-1, 32T-2, 29T-2, 32T-4, 29T-4, 32C-1, 29C-1, 32C-2, 29C-2, 32C-4, 29C-4, where; 32T-1, 2 and 4 are *R. solani*-infected rice plants of the resistant line; 29T-1, 2 and 4 are *R. solani*-infected rice plants of the susceptible line; 32C-1, 2 and 4 are control plants of the resistant line; 29C-1, 2, and 4 are control plants of the susceptible line at 1, 2 and 4 dpi respectively. For analysis, where there are two bands, the top band was considered.

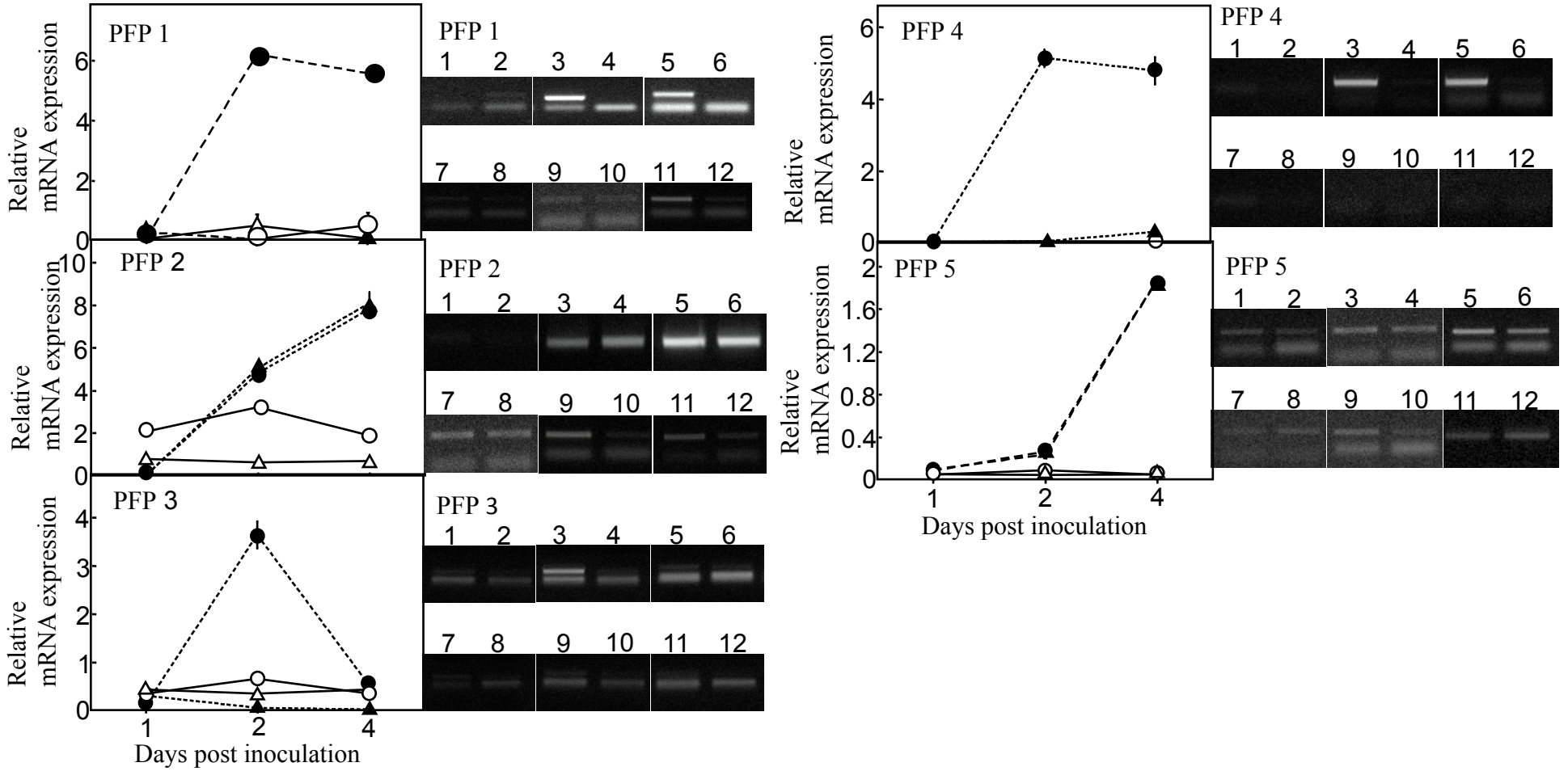


Figure 4.6. The time course of changes in the mRNA expression of gene families encoding PFP and PFK in *R. solani*-infected plants of the resistant and susceptible rice lines (closed circles, closed triangles), respectively, and those of the healthy control plants ( open circles, open triangles), respectively, at 1, 2 and 4 dpi. PFP 1 to 5 represent genes that encode PFP as indicated in Table 4.1. The gel images show one representative result out of three independent determinations. * mRNA expression was significantly different between the *R. solani*-infected rice plants, compared to that of the control plants. ◊ mRNA expression was significantly different between the *R. solani*-infected rice plants of the resistant line, compared to that of the susceptible line. Numbers 1 to 12 represent mRNA expression of 32T-1, 29T-1, 32T-2, 29T-2, 32T-4, 29T-4, 32C-1, 29C-1, 32C-2, 29C-2, 32C-4, 29C-4, where; 32T-1, 2 and 4 are *R. solani*-infected rice plants of the resistant line; 29T-1, 2 and 4 are *R. solani*-infected rice plants of the susceptible line; 32C-1, 2 and 4 are control plants of the resistant line; 29C-1, 2, and 4 are control plants of the susceptible line at 1, 2 and 4 dpi respectively. For analysis, where there are two bands, the top band was considered.

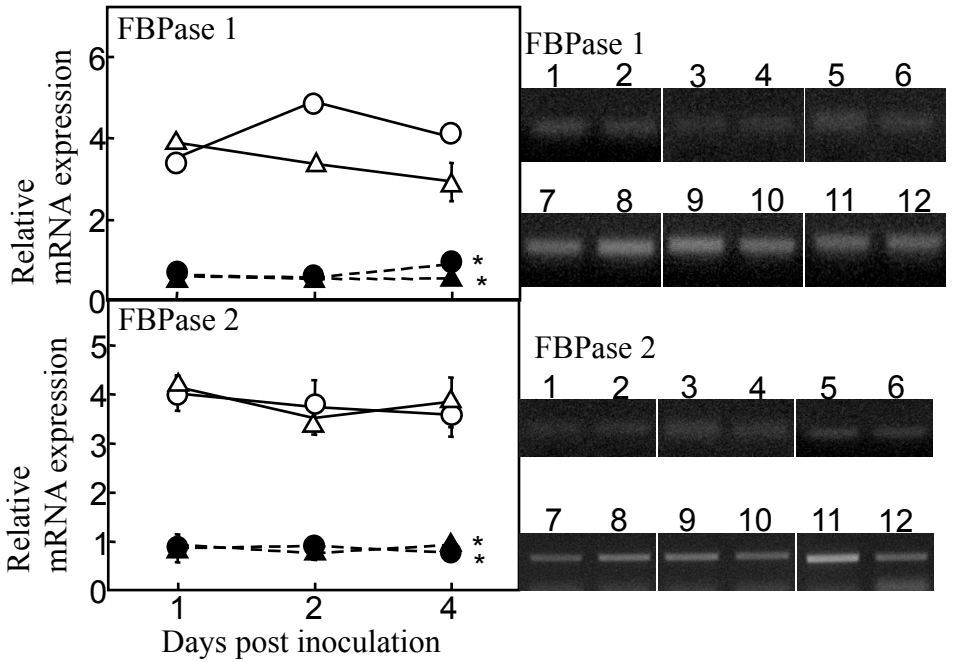


Figure 4.7. The time course of changes in the mRNA expression of gene families encoding FBPase in *R. solani*-infected plants of the resistant and susceptible rice lines (closed circles, closed triangles), respectively, and those of the healthy control plants (open circles, open triangles), respectively, at 1, 2 and 4 dpi. FBPase 1 and FBPase 2 represent genes that encode FBPase as indicated in Table 4.1. The gel images show one representative result out of three independent determinations. * mRNA expression was significantly different between the *R. solani*-infected rice plants, compared to that of the control plants. ◊ mRNA expression was significantly different between the *R. solani*-infected rice plants of the resistant line, compared to that of the susceptible line. Numbers 1 to 12 represent mRNA expression of 32T-1, 29T-1, 32T-2, 29T-2, 32T-4, 29T-4, 32C-1, 29C-1, 32C-2, 29C-2, 32C-4, 29C-4, where; 32T-1, 2 and 4 are *R. solani*-infected rice plants of the resistant line; 29T-1, 2 and 4 are *R. solani*-infected rice plants of the susceptible line; 32C-1, 2 and 4 are control plants of the resistant line; 29C-1, 2, and 4 are control plants of the susceptible line at 1, 2 and 4 dpi respectively. For analysis, where there are two bands, the top band was considered.

of PFK 2 in the rice plants responding to *R. solani* infection increased with the resistant line having significantly ($P < 0.05$) higher levels, compared to the susceptible line. The time course of mRNA expression of PFK 3 in rice plants of both lines responding to *R. solani* infection was similar. The mRNA expression in the *R. solani*-infected rice plants of both lines significantly increased at 2 dpi. The mRNA expression of PFK 4 in rice plants responding to *R. solani* infection was high although the changes in the time course of mRNA expression were different. In the *R. solani*-infected rice plants of the resistant line, expression gradually increased at 2 dpi while no changes in expression were observed in the susceptible line at 1 to 4 dpi. The mRNA expression of PFK 5 in the *R. solani*-infected plants of the resistant line was significantly higher, compared to plants of the susceptible line at 1 to 4 dpi. The mRNA expression in plants of both rice lines was significantly higher than that of control plants all sampling days. The mRNA expression of genes numbered PFK 6 to 8 was too low to be detected even after 35 PCR cycles. The mRNA expression of PFP 1 and PFP 4, both of which encode the $\alpha$-subunit of PFP in the cytosol significantly increased at 2 dpi and did not change at 4 dpi. When the expression in the *R. solani*-infected plants of the resistant line was compared to that of the susceptible line, higher levels were detected in the resistant line at 2 and 4 dpi. The mRNA expression of PFP 2 and PFP 5 in the *R. solani*-infected plants of both rice lines increased at 2 and 4 dpi. PFP 2 and PFP 5 encode the $\alpha$- and $\beta$-subunits of PFP, respectively. In addition, the changes in expression appeared similar in both rice lines. The mRNA expression of PFP 3 in the *R. solani*-infected plants of the resistant line increased at 2 dpi and decreased at 4 dpi. However, the expression level was higher in the resistant line, compared to the susceptible line. All PFK/PFPs were found to be expressed as shown in Fig. 4.3, except PFK 6, 7 and 8 whose signals were too weak to be detected.

However, differences in the time course of expressions of the PFP/PFK isozymes were observed. The expressions of PFK 1, 2, 4, and 5 in *R. solani*-infected rice plants of the resistant line at 1 dpi were high while those of PFP 1, 2, 3, and 4 increased at 2 dpi. Moreover, differences in expression were observed between the genes that encode $\alpha$- and $\beta$-subunits. In the *R. solani*-infected rice plants of the resistant line, the expression of PFP 1, 2, 3, and 4, which encode PFP $\alpha$-subunits increased at 2 dpi while that of PFP 5 that encodes $\beta$-subunits increased at 2 to 4 dpi. The changes in the time course of mRNA expression of FBPase 1 and 2 in rice plants responding to *R. solani* infection were similar. The changes showed that mRNA expression in the *R. solani*-infected rice plants was lower than that in the control plants. In addition, no significant differences in expression were observed between the *R. solani*-infected rice plants of the two rice lines.

**4. Discussion**

Our prior studies showed that activation of the glycolytic pathway to provide substrates used in the secondary metabolism to generate resistance products was important for *R. solani* resistance (Mutuku and Nose, 2010). A key regulatory point in the activation of the glycolytic pathway is the phosphorylation of F-6-P to F-1,6-$P_2$ catalyzed by PFP and PFK (Plaxton, 1996) (Fig. 4.1). In a previous study, that examined PFP/PFK common regions in rice plants responding to *R. solani* infection, the time course of changes showed that mRNA expression in the resistant line increased (Mutuku and Nose, 2010). The current study investigated this reaction further and showed that PFP and PFK activity increased especially in the resistant rice line (Fig. 4.2). The results also showed that the content of F-2,6-$P_2$ in the *R. solani*-

infected rice plants of the resistant line increased while PPi content reduced and that the maximum PFP and PFK activity was at pH range 7.4-7.8 (Fig. 4.4). Moreover, high mRNA expression was detected mostly in the cytosolic isozymes of PFP and PFK.

Since the expression of most PFP/PFK isozymes changed, one of the issues considered was whether all of them were involved in the resistance against *R. solani* infection. This is because, when a plant detects a pathogen, it does not tailor its response to the pathogen at hand but instead, it turns on many of the defence mechanisms it has, among which some may be effective against the particular pathogen (Katagiri, 2004). Therefore, it is necessary to define the genes that are adaptive or part of the defence response after infection and those that play other roles like housekeeping. Although most of the isozymes whose expression was high were cytosolic, PFK 5, which was predicted to be a chloroplast isozyme was the only exception (Fig. 4.5). Two reasons led us to speculate that PFK 5 might not be a chloroplast isozyme. (a) PFK 5, unlike the other chloroplast isozymes i.e., PFK 6, 7 and 8, did not have chloroplast transit peptides in its sequences, and (b) in a previous study where mRNA expression of chloroplast reductive pentose phosphate genes in leaf sheaths of rice plants infected with sheath blight disease was examined, the expressions of Rubisco, phosphoribulokinase, ribulose-phosphate-3-epimerase and ribose-5-phosphate isomerase were found to be low. One of the reasons for the low mRNA expression was that plastids in rice leaf sheaths are mainly etioplasts (Mutuku and Nose, 2010). This might also be the reason we could not detect mRNA expression of PFK 6, 7 and 8. Therefore, the high mRNA expression of PFK 5 especially in the resistant line, the lack of chloroplast transit peptides in its sequences and the findings

that chloroplast genes have low expression in leaf sheaths of rice plants infected sheath blight disease were taken as evidence that PFK 5 might be an etioplast isozyme of PFK.

Comparing our results with data from gene expression databases like genevestigator (www.genevestigator.ethz.ch) revealed that PFK 1 and 5, PFP 2 and 5 were induced in leaf sheaths of rice plants responding to diseases (see scatter plots in Appendix 2-4). In addition, when the mRNA expressions of these PFP/PFK isozymes in the resistant rice line were correlated with the enzymatic activity, very strong correlation coefficients, $r > 0.92$ were observed (Fig. 4.8). Further correlation with the expression of PFP/PFK common regions reported previously (Mutuku and Nose, 2010) also revealed strong positive correlations, $r > 0.91$, $r > 0.8$, $r > 0.98$, and $r > 0.93$ for PFK 1, PFK 5, PFP 2, and PFP 5, respectively. Although database searches could not show whether PFK 3 and PFK 4 were expressed in leaf sheaths of rice plants responding to diseases, the strong positive correlation between the expression of these isozymes and enzymatic activity, $r > 0.97$ and that of PFP/PFK common regions, $r > 0.8$ were taken as evidence that their expression was related to the defence response against *R. solani* infection.

Further search in the genevestigator database revealed that PFK 2, PFP 1, 3 and 4 were expressed in rice plant tissues under other stress conditions. For example, PFK 2 was highly expressed in coleoptiles of rice seedlings while, PFP 4 was highly expressed in flag leaf of rice responding to *Xanthomonas oryzae* pv. *oryzae* infection. On the other hand, PFP 1 was highly expressed in leaves of rice seedlings responding

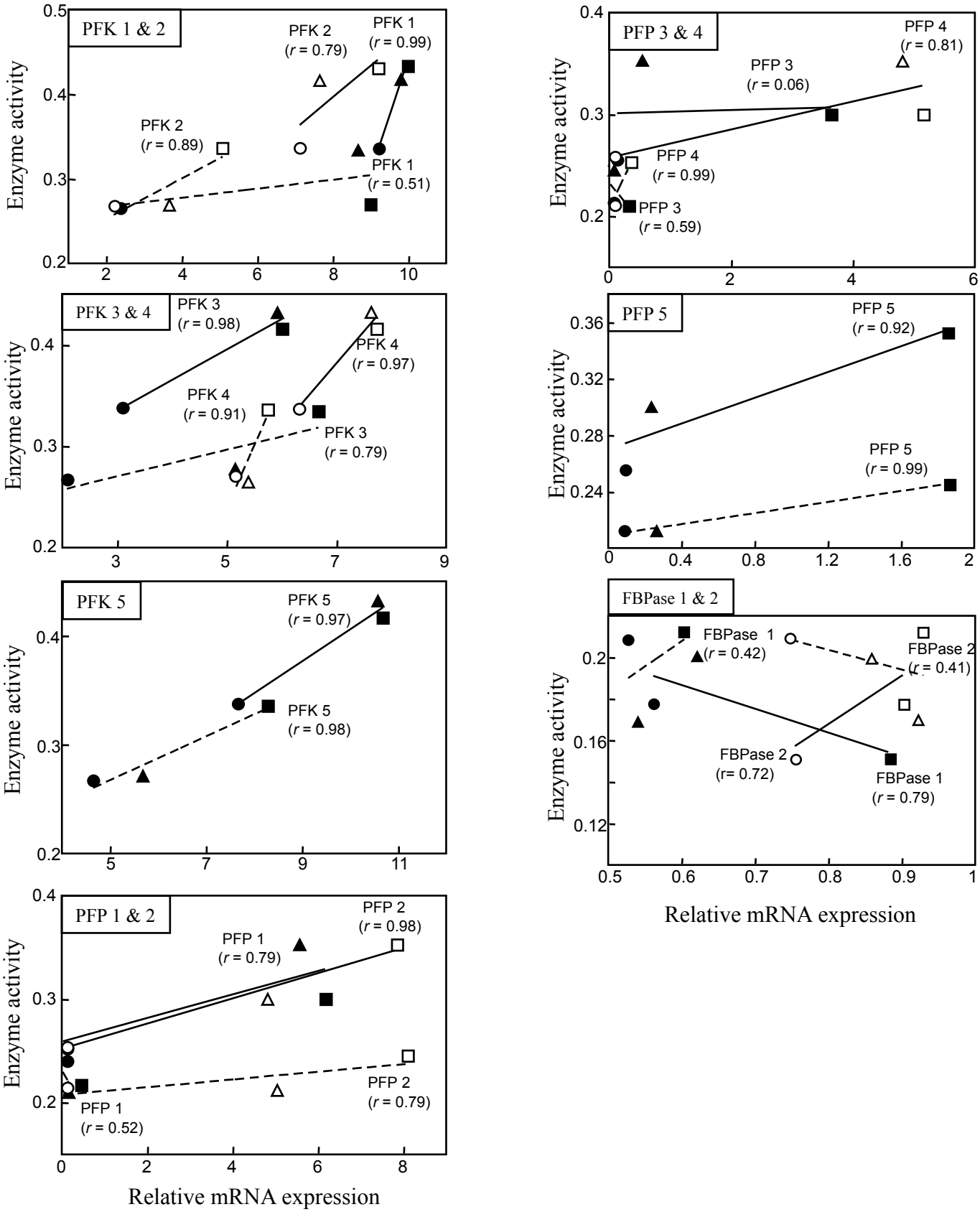


Figure 4.8. The correlation between mRNA expression of the different isozymes that encode PFP/PFK and their respective enzymatic activity in the *R. solani*-infected plants of the resistant (solid lines) and susceptible (broken lines) rice lines. The closed symbols (closed circles, closed triangles, closed squares) represent correlation between odd numbered genes and the enzymatic activity while the open symbols (open circles, open triangles, open squares) represent even numbered genes and enzymatic activity at 1, 2 and 4 dpi respectively. The (*r* ) was Pearson's correlation coefficient. Genes are named as indicated in Table 4.1.

to abiotic stress while, PFP 3 was highly expressed in rice leaf treated with *Magnaporthe grisea*. When their expression in leaf sheaths of *R. solani*-infected rice plants was compared to enzymatic activity, although significant positive correlation ($r < 0.81$) was observed, it was lower than that of the other isozymes i.e., PFK 1, PFK 5, PFP 2, and PFP 5. These results suggested that the expression of PFK 2, PFP 1, 3 and 4 was stimulated not only by *R. solani* infection but also by other biotic and abiotic stresses.

PFK was reported to be a maintenance enzyme while PFP was adaptive in plants exposed to stress conditions (Plaxton, 1996). If that were the case in rice plants responding to *R. solani* infection, no changes in PFK activity would be observed as was reported in rice plants responding to anoxia stress where, PFP activity increased while that of PFK remained unchanged (Mertens *et al*., 1990). However, the results of this current study showed that the activity of both PFP and PFK increased. This increase in the activity of both PFP and PFK after *R. solani* infection agreed with the findings of Danson *et al*. (2000*a*). In addition, the current study showed that changes in the expressions of six PFP and PFK isozymes; Os01g09570 (PFK 1), Os01g53680 (PFK 3), Os04g39420 (PFK 4), Os06g05860 (PFK 5), Os08g25720 (PFP 2) and Os06g13810 (PFP 5) were adaptive to sheath blight disease infection and linked to defense response while the expressions of four other isozymes; Os05g10650 (PFK 2), Os02g48360 (PFP 1), Os06g22060 (PFP 3) and Os09g12650 (PFP 4) although adaptive to the disease stress, were not specific to *R. solani* infection. However, there were differences in the time course of expression of PFP and PFK isozymes in the *R. solani*-infected plants of the resistant line. The results showed that the expression of PFK 1, 2, 4 and 5 in the *R. solani*-infected rice plants of the resistant line at 1 dpi were

high as opposed to the gradual increase observed in the plants of the susceptible line, and in the time course of expression of all PFP isozymes. Two hypotheses that might help to explain this are; that at the time of sampling i.e., 1 dpi, the expression of these PFK isozymes was already established, and that the expression of these PFK isozymes in the resistant line is always high. Therefore, both PFP and PFK had isozymes that played adaptive roles in rice plants responding to *R. solani* infection but PFK isozymes were expressed at higher levels within a short time after infection while those of PFP were expressed gradually. This led us to propose that future studies are necessary to investigate the expression of PFK isozymes within shorter time scales like within hours after infection.

Four of the five PFP isozymes examined in this study were genes that encode the PFP $\alpha$-subunit while one encodes its $\beta$-subunit. The activity and regulation of PFP *in vivo* are potentially modulated by differential expression of the enzymes $\alpha$- and $\beta$-subunits (Theodorou *et al*., 1992). The $\alpha$-subunit may be involved in the regulation of catalytic activity by F-2,6-$P_2$ where as the $\beta$-subunit contains the catalytic site (Botha and Botha, 1991; Weiner *et al*., 1987). If we consider PFP 2 ($\alpha$-subunit) and PFP 5 ($\beta$-subunit), the findings of the present investigation indicate that the expression of both $\alpha$- and $\beta$-subunits of PFP is responsive to *R. solani* infection. In addition, the strong positive correlation between enzymatic activity and expression of both PFP 2 and PFP 5 indicated that as the enzymatic activity increased, the expression also increased. Thus the induction of PFP activity by *R. solani* infection of rice plants appears to be based upon the paired expression of both $\alpha$- and $\beta$-subunits leading to enhancement in sensitivity of the enzyme and its regulation by F-2,6-$P_2$. These findings contrast what was reported in phosphate (Pi)-starved *Brassica nigra* suspension cells where

expression of the $\alpha$-subunit was responsive to stress conditions i.e., cellular Pi status while the $\beta$-subunit was expressed irrespective of nutrient regime (Theodorou *et al*., 1992). We also observed that the expression of all genes that encode the $\alpha$-subunit increased at 2 dpi from very low levels at 1 dpi and this was interpreted as suggesting that perhaps there was a *de novo* synthesis of the $\alpha$-subunit in *R. solani*-infected rice plants.

The concentration of F-2,6-$P_2$ in plants was reported to be responsive to environmental disturbances known to stimulate glycolytic activity like wounding Van (Schaftingen and Hers, 1983) and anoxia stress (Mertens *et al*., 1990). In non-stress conditions and in the absence of F-2,6-$P_2$, plant PFP is reported to be nearly inactive while stress conditions and its presence, cause PFP to be activated (Mertens *et al*., 1990). Indeed the enzymatic activities of PFK and FBPase in the control plants of both rice lines were higher than those of PFP but the situation promptly changed after infection (Fig. 4.2). To rule out the possibility that mock inoculation affected F-2,6-$P_2$ content in the control plants, the content of this metabolite in the control plants was compared to that in untreated plants and the results showed that there were no differences. Furthermore, F-2,6-$P_2$ concentration in the rice plants responding to *R. solani* infection increased from 0.18 to 0.30 $\mu$M and 0.17 to 0.19 $\mu$M in the resistant and susceptible line, respectively. These contents compared well with the 0.05-1.0 $\mu$M total F-2,6-$P_2$ concentration (assuming 1 g fresh weight of tissue is equal to 1 mL) reported to occur in plant tissues (Nielsen *et al*., 2004). Additionally, F-2,6-$P_2$ content in *R. solani*-infected rice plants was more than 2.3- and 1.5-fold higher than that of the respective control plants, suggesting that the increase was as a response to *R. solani* infection. One of the ways to explain this increase in F-2,6-$P_2$ content is that, 6-

phosphofructo-2-kinase (F6P2K) and F-2,6-bisphosphatase (Fig. 4.1B) were activated and inhibited, respectively, by F-6-P (Stitt, 1987) which was shown to increase during resistance response after *R. solani* infection in rice (Mutuku and Nose, 2010).

PPi concentration in the *R. solani*-infected rice plants of the resistant line reduced from 25.2 to 18 $\mu$M at 4 dpi (Fig. 4.3). This estimated concentration was lower than the 1 mM PPi that was reported to inhibit completely the glycolytic activity of PFP (Cseke *et al*., 1982). However, in comparison to the control rice plants, the *R. solani*-infected plants had higher PPi content and one possible explanation for this was that if PPi was limited, it would hamper conversion of F-6-P to F-1,6-$P_2$ therefore diverting F-6-P away from glycolysis to, possibly, sucrose synthesis (Claassen *et al*., 1991). Therefore, we interpreted these results as suggesting that the decrease in PPi content was caused by consumption by PFP in the phosphorylation of F-6-P to F-1,6-$P_2$. This was consistent with the changes in PFP activity and F-2,6-$P_2$ content towards stimulation of glycolytic activity.

The results revealed that maximum PFP activity was at pH range 7.4 and 8.0 in the resistant and susceptible line respectively, while that of PFK was at pH 7.8 in both rice lines (Fig. 4.4). The optimum pH of rice leaf sheath PFP was similar to that reported in other C3 plants like tomato, pH range 7.5-8.0 (Wong *et al*., 1990), castor beans 7.3-7.7 (Kombrink *et al*., 1984) and wheat 7.5 (Mahajan and Singh, 1989). But even though similar responses to change in pH were observed, enzymatic activities varied. These differences in the enzymatic activities in both rice lines might be linked to isozymes of PFP and PFK.

The PFP and PFK enzyme activities, the mRNA expression of PFK 1, 2, 4 and 5, PFP 1 and 4 and F-2,6-$P_2$ contents in the susceptible rice line at 4 dpi resembled those of the resistant rice line at 1 dpi indicating that the amplitude of the response from the susceptible rice line was lower than that from the resistant rice line. These results suggested that susceptibility occurred because of the failure by the plants of the susceptible rice line to respond strongly and in a timely manner to the pathogen signals (see Appendix 5 for time-course pictures of sheath blight disease progression at 1 to 4 dpi).

Previous studies showed that activation of the secondary metabolism was reliant on primary metabolism and suggested that modulation of the activation of glycolytic activity could result in increased generation of resistance products in the phenylpropanoid pathway (Danson, 2000*b*; Mutuku and Nose, 2010). Strategies to achieve this goal include engineering of single steps in a pathway to increase metabolic flux to target compounds (Plaxton, 2004). As shown in this current study, modulating PFP and PFK by increasing their activity and mRNA expression would enhance the glycolytic pathway. While the time course in mRNA expression of PFP 5 that encodes putative expressed PFP $\beta$-subunit in the *R. solani*-infected plants was similar, those of PFP 1, 3 and 4 that encode putative expressed PFP $\alpha$-subunit were different in the resistant and susceptible plants. In the resistant rice line, expressions were very low at 1 dpi but significantly increased at 2 dpi. Therefore, we propose that future studies are required to evaluate the physical and kinetic properties of PFP subunits purified from rice leaf sheaths of *R. solani*-infected rice plants and to relate these properties to the findings of the present study.

## 5. Summary

Rice sheath blight disease caused by *Rhizoctonia solani* Kuhn results in significant yield and quality losses in rice growing areas worldwide. The glycolytic pathway is important in the resistance response to *R. solani* infection in rice. This study examined one of the regulatory steps in this pathway catalyzed by pyrophosphate- fructose-6-phosphate-phosphotransferase (PFP) and 6-phosphofructokinase (PFK). PFP and PFK activity in *R. solani*-infected rice plants increased. The mRNA expression of PFP/PFK isozymes showed that PFK 1, 2, 4 and 5 in the resistant line at 1 dpi were high as compared to the gradual increase observed in the expression of all PFP isozymes. Also PFK 1, PFK 3, PFK 4, PFK 5, PFP 2 and PFP 5 were adaptive to sheath blight disease infection and linked to defense response while, the expressions of PFK 2, PFP 1, PFP 3 and PFP 4 although adaptive, were not specific to *R. solani* infection. These observations provide evidence that (a) both PFP and PFK have isozymes that play an adaptive role after *R. solani* infection but while those of PFK are expressed at higher levels within a short time after infection those of PFP are expressed gradually, (b) the adaptive activation of PFP in *R. solani*-infected rice plants is correlated with the paired expression of its $\alpha$- and $\beta$-subunits as shown by PFP 2 and PFP 5, and (c) the expression of some $\alpha$-subunits is not specific to *R. solani* infection as shown by PFP 1, PFP 3 and PFP 4.

# CHAPTER 5

## Activation of phenylpropanoid pathway leading to lignin deposition is one of the mechanisms of defence response after *Rhizoctonia solani* Kuhn infection in rice (*Oryza sativa* L.)

### 1. Introduction

Some of the proposed methods for use in development of resistant cultivars include metabolic engineering to enhance the production of specific desired compounds that enhance the resistance response (Verpoorte *et al*., 2000; Bolton, 2009).

Although little is known about the interaction between rice and *R. solani* at the molecular level, the studies that have conducted show that glycolytic pathway is activated and that it is linked to activation of the phenylpropanoid pathway (Danson *et al*., 2000*a*; Mutuku and Nose, 2010, 2012). Since it is now possible to manipulate gene expression, an important goal towards improvement for *R. solani*-resistance is to modify the output of specific biosynthetic pathways that lead to desired products via the process of metabolic engineering. From our previous studies, it was proposed that lignin was one of the desirable products in *R. solani*-infected rice plants because increased lignification protects the cells against pathogen invasion (Danson, 2000*b*; Mutuku and Nose, 2010). Reports on plant resistance response have shown that lignin and other oxidized phenolics slow infection for example in transgenic tobacco seedlings challenged with *R. solani* (Broglie *et al*., 1991), and in tobacco cells challenged with *Phytophthora nicotianae* (Oelofse and Dubery, 1996). It has been

postulated that, by slowing the penetration of the fungus, the plant gains the time necessary to activate its second line of defence, including the production of phytoalexins in large quantities. However, there have been controversies as to whether lignification occurs fast enough after infection to constitute a 'first line of defense' (Matern and Kneusel, 1988). Indeed it has been shown to represent a delayed resistance response after investigations done on infected Japanese radish (see Matern and Kneusel, 1988). Lignification as a mechanism of disease resistance and an important plant defense response specifically elicited by fungi has been studied in a variety of host-pathogen interaction including alfalfa (Dixon *et al*., 1996), carrot (Heale and Sharman, 1977), cucumber (Redman *et al*., 1999) and wheat (Ride, 1980). However, in rice, lignin as an important barrier to sheath blight fungus and its implications have not been investigated.

Our previous studies showed metabolic changes in the glycolytic pathway occurred at 1 dpi in *R. solani*-infected rice plants especially those of the resistant line (Mutuku and Nose, 2010, 2011, 2012). In addition, it was shown that activation of the glycolytic pathway was linked to activation of phenylpropanoid pathway where lignin is generated (Mutuku and Nose, 2010) (Fig. 2.1). It was therefore proposed that perhaps these changes in metabolism could be linked to lignification and if so, it could be argued that lignin detected in *R. solani*-infected rice plants at 1 dpi forms part of the 'first line of defense'. This current study also examined the pattern of lignification at 1, 2, 4, 7 and 10 dpi and also elucidated differences in the amount of lignin deposited in the resistant and susceptible rice lines in both field and greenhouse grown rice plants.

Although little is known about the host–fungus interaction at the molecular level, studies have shown that in *R. solani*-infected rice plants, glucose and fructose concentrations increase in leaf blades (Danson *et al*., 1999), glucose-6-phosphate dehydrogenase (G6PDH) is activated (Danson *et al*., 2000), PFP and PFK activity increases and this increase is accompanied by rises in expressions of PFP/PFK isozymes in both cytosolic and plastid compartments (Mutuku and Nose, 2012). Since it is now possible to manipulate gene expression, an important goal towards improvement for *R. solani*-resistance is to modify the output of specific biosynthetic pathways that lead to desired products via the process of metabolic engineering. The first step in this methodology is to identify metabolic enzymes whose genes could be targeted for manipulation. Towards this end, our previous studies that investigated metabolic changes that occur in rice plants after *R. solani* infection showed that gene expressions of phosphofructokinase (PFK), triosephosphate isomerase (TPI), phosphoglycerate kinase (PGK), enolase and pyruvate kinase (PK) in the resistant rice line increased. These increases were prerequisite for the activation of the glycolytic pathway, which was linked to activation of the phenylpropanoid pathway (Mutuku and Nose, 2010) (Fig. 2.1). When the connection between activation of the glycolytic pathway and that of phenylpropanoid pathway was examined, the findings showed that the control of the reactions catalysed by PFK, aldolase, TPI, glyceraldehyde-3-phosphate dehydrogenase+PGK would enhance the glycolytic pathway thereby increasing the generation of PEP and erythrose-4-phopshate (E-4-P), the substrates of secondary metabolism. In the phenylpropanoid pathway, PAL and peroxidase activity increased, and $H_2O_2$ generation occurred leading to the conclusion that the increase in PEP and E-4-P in the glycolytic pathway was linked to activation of phenylpropanoid pathway (Mutuku and Nose, 2011).

These findings showed that the mechanisms of disease resistance in response to sheath blight disease led to activation of the glycolytic and phenylpropanoid pathways. This led us to postulate that lignin, the product of the phenylpropanoid pathway was one of the desirable compounds in the resistance response against *R. solani* infection because increased lignification may protect the cells against pathogen invasion. The current study detected lignin in *R. solani*-infected rice plants of the resistant and susceptible lines at 1, 2 and 4 dpi and showed that there were differences in the amount of lignin deposited in the resistant and susceptible rice lines.

## 2. Materials and Methods

Two rice lines from a $17^{th}$ generation hybrid population cross of Tetep x CN4-4-2 were selected for resistance and susceptibility to sheath blight disease. The seeds were first grown in nursery and then transplanted after 28 days in the field. Two fertilizer applications were given as basal NPK (14:16:14) and top-dressed with NPK (16:0:16). Water application was done as per requirement. The plants were inoculated at 50 % flowering and sampling was done 7 days after inoculation.

Another batch of plants were grown in the green house. Their preparation was done as previously described (Mutuku and Nose, 2010). Briefly, plants were grown in a greenhouse in pots under 16 h of light and 8 h of darkness at 25 $^{o}$C. The pots contained 1:1 ratio of peat moss to vermiculite. Sampling was done when the plants reached the 7-leaf growth stage.

The use of potato-sucrose-agar medium (PSA), inoculum preparation and inoculation

followed the description of Wasano *et al*. (1983). Briefly, sheath blight fungus isolate C154 from National Institute of Agrobiological Sciences Gene Bank, Tsukuba, Japan, was grown on PSA at 28 °C for about 4 days. It was then chopped into small pieces, introduced into a syringe and 0.25 mL of the fungal suspension was injected into the interstices between the second and the third leaf sheaths from the flag leaf. Control plants were treated under the same conditions except that the pathogen preparation was replaced with distilled water.

**2.1 Lignin deposition**

Lignin deposition assays were performed using a phloroglucinol-HCl (Pg-HCl) method modified from Redman *et al.* (1999). Rice leaf sheath of about 5 cm in length (including the infection site) were washed with distilled water and decolorized in 70 % (v/v) ethanol for 24 h, washed again with distilled water and exposed to 1 % phloroglucinol for 2 h. The rice leaf sheaths were then exposed to 6 M HCl until the red colour denoting lignin deposition developed. After cross-sections were obtained, photographs were taken using an Olympus CX41LF (Olympus, Japan) microscope equipped with a camera.

**3. Results**

The phloroglucinol-HCl (Pg-HCl) caused staining in the leaf sheaths of both the resistant and the susceptible rice plants and showed that lignin deposition (detected as a reddish colouration) occurred after *R. solani* infection in rice plants of both lines planted in under field and greenhouse conditions. Similar changes in lignification

were observed in *R. solani*-infected rice plants in both field and greenhouse conditions and therefore, this discussion is about these changes and does not aim to make distinction between the two conditions. Lignification was not detected in control plants (Fig. 5.1). In this study, we detected lignin around the vascular bundles in all leaf sheaths from both resistant and susceptible rice lines and because there was no evidence that it was caused by resistance response mechanisms against *R. solani* infection, it was not considered in this discussion. Examination of the patterns of lignin deposition revealed that it occurred in both the outer surfaces of the leaf sheath as well as the inner surfaces that were in contact with other leaf sheaths (flag leaf sheath, in case inoculation was done on the second leaf sheath or second leaf sheath in case inoculation was done on the third leaf sheath). Lignin deposition was detected in the *R. solani*-infected rice plants at all sampling days i.e., 1, 2, 4, 7 and 10 dpi.

Although lignin was detected at 1 dpi in *R. solani*-infected rice plants of both lines, the amounts started to differ at 2 dpi with higher deposition occurring in the resistant line. Indeed these differences in the amount of lignin deposited were even higher at 7 dpi. Lignin deposition was detected in uninfected regions of the *R. solani*-infected rice leaf sheaths of both resistant and susceptible lines with higher uniform deposition occurring in the resistant line (Fig. 5.2). In addition, this figure also shows that lesion development occurred on the second leaf sheaths of *R. solani*-infected rice plants of both rice lines at 7 dpi but was more severe in the susceptible line. Further examinations revealed that fungal colonization occurred in the third leaf sheaths of *R. solani*-infected rice plants of both rice lines but as shown in Figure 5.3, fungal colonization was accompanied by concomitant lignin deposition in the resistant line.

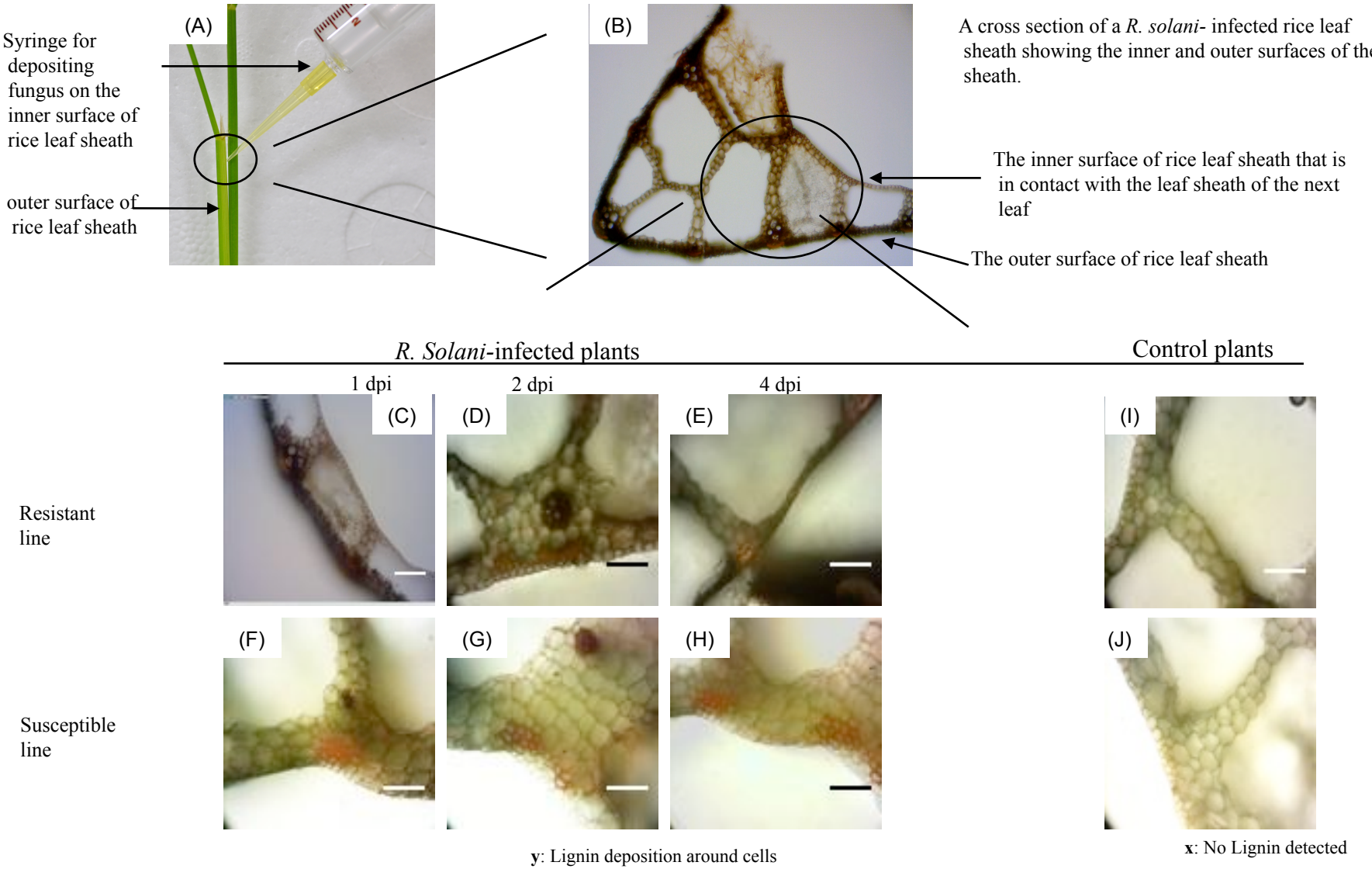


Figure 5.1. (A) Position on the leaf sheath where inoculation was done. (B) A cross section of a *R. solani*-infected rice leaf sheath showing the outer and the inner leaf sheath surfaces. (C-E) Lignin deposition in rice leaf sheaths of the *R. solani*-infected plants of the resistant rice lines at 1, 2 and 4 dpi. (F-H) Lignin deposition in rice leaf sheaths of the *R. solani*-infected plants of the susceptible rice lines at 1, 2 and 4 dpi. Lignin deposition was detected as the reddish colouration around the cell and in the case of the resistant line, the deposition appeared to increase and become more uniform at 2 dpi and 4 dpi. On the other hand, although lignin was detected in the susceptible line, the amount was low even at 4 dpi. No lignin deposition was detected in the control plants treated under similar conditions as the *R. solani*-infected plants (I) and (J). A minimum of six rice leaf sheaths was assayed for each treatment and similar trends were observed. This figure shows one representative from each treatment. Scale bar represents 4 pixel/ mm. Magnification is X100.

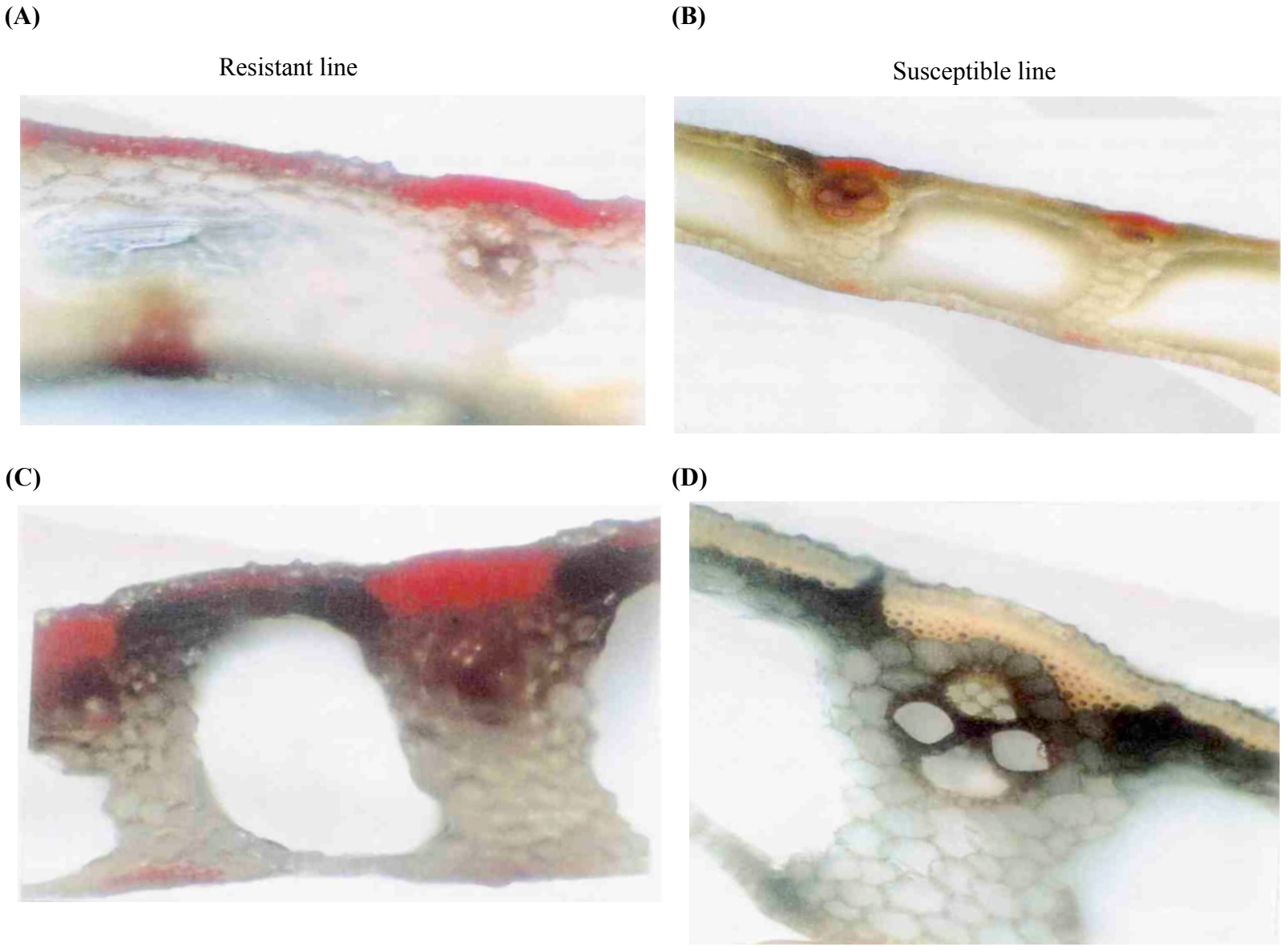


Figure 5.2. (**A**) High and uniform lignin deposition on uninfected first rice leaf sheaths of resistant line (**B**) Non-uniform lignin deposition in the susceptible line. (**C**) High and uniform lignin deposition in the *R. solani*-infected second leaf sheaths of the resistant line at 7 dpi (**D**) Low lignin deposition in lignin deposition in the *R. solani*-infected second leaf sheaths of the susceptible line at 7 dpi.

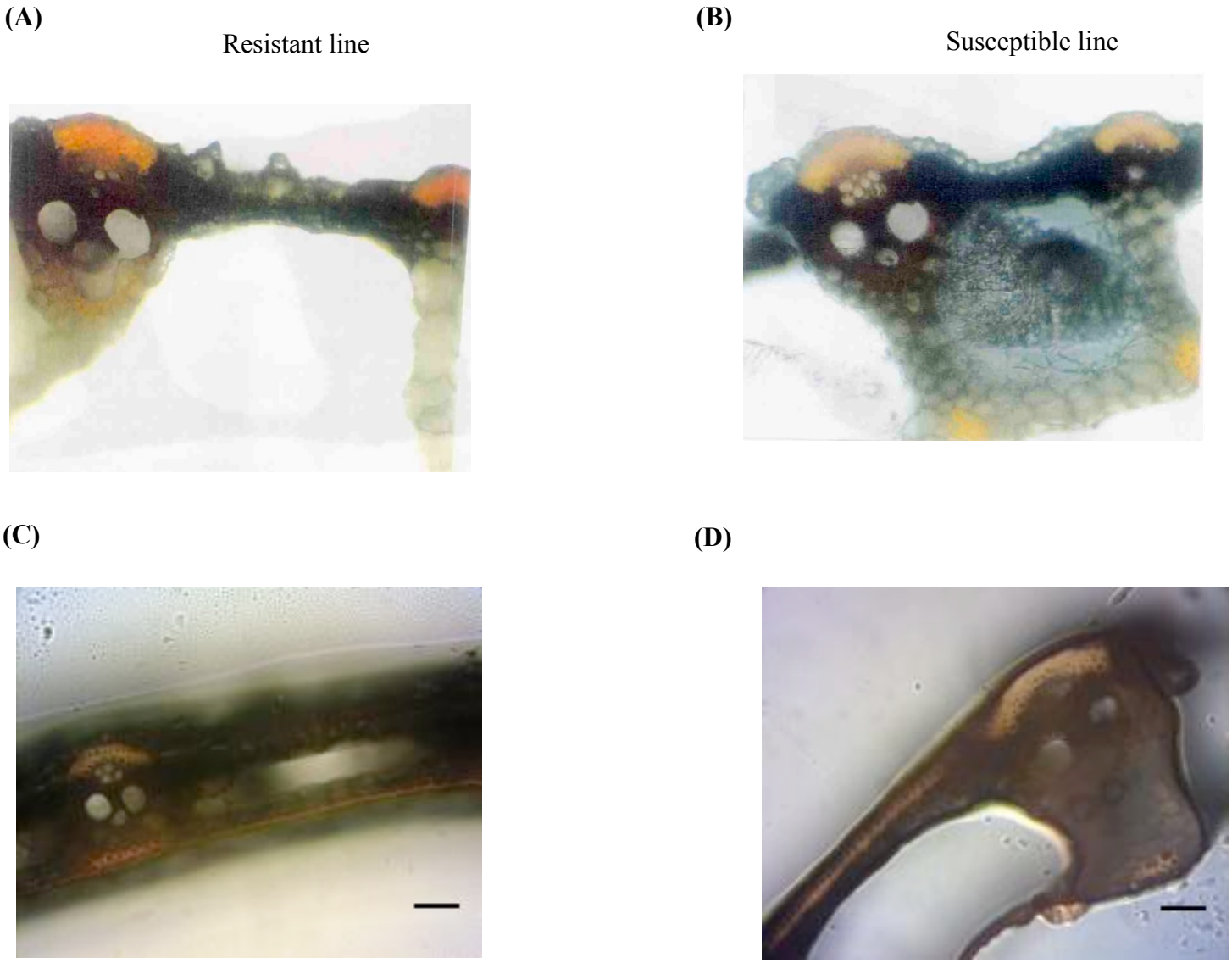


Figure 5.3. (**A**) *R. solani*-infected third leaf sheaths of resistant line and (**B**) of the susceptible line at 7 dpi (**C**) *R. solani*-infected third leaf sheaths of resistant line and (**D**) of the susceptible line at 10 dpi. Notice the uniform and high lignin deposition in the resistant line and collapsing cells of the susceptible line at 10 dpi.

Moreover Figure 5.3 also shows that uniform and high lignin deposition was also detected in *R. solani*-infected leaf sheaths of the resistant line at 10 dpi as compared to low deposition in the susceptible line. It was observed that the cell of the susceptible line appeared to collapse. When photos of the infected area were observed, a process similar to hypersensitive cell death was detected in the *R. solani*-infected leaf sheaths of the resistant line (Fig. 5.4) where what appeared to be the initial and final stages of the hypersensitive response were detected. Furthermore, Figure 5.4 also shows that lignin deposition on both the outer and inner surfaces of leaf sheaths appeared to restrict the spread of the infection to a small region resulting in a small infection scar in the resistant rice line as compared to the spreading infection scar of the susceptible line perhaps due to inadequate lignin deposition.

**4. Discussion**

In our previous studies we showed that glycolysis was at the core of carbon resource allocation for shikimate and phenylpropanoid pathways (Danson *et al*., 2000) suggesting that its regulation affected carbon allocation for these pathways. When the regulation of glycolysis was investigated, it was found to be regulated at five reactions i.e., PFK/PFP, aldolase, TPI, GAPDH+PGK and PK (Mutuku and Nose, 2011). The study suggested that the control of these five reactions led to enhancement of glycolysis and consequently phenylpropanoid pathway. Indeed high PAL and peroxidase activities combined with increased $H_2O_2$ generation were interpreted as suggesting activation of phenylpropanoid pathway occurred. This current study investigated resistance response such as lignin deposition resulting from regulation of glycolysis at five reactions. The results showed that although lignin was detected at 1

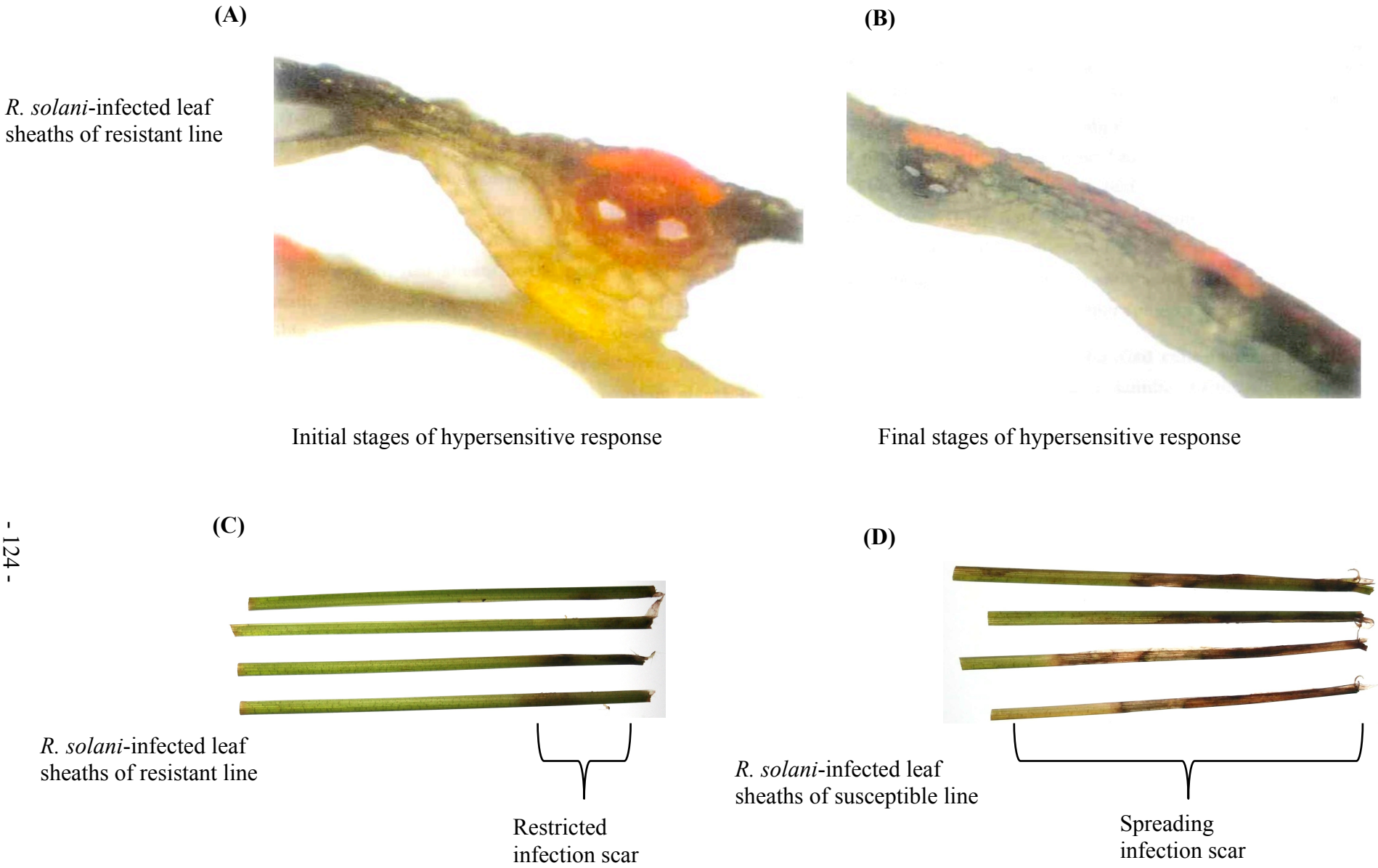




Figure 5.4. (**A**) The initial and (**B**) final stages of hypersensitive response in *R. solani*-infected leaf sheaths of the resistant line. (**C**) *R. solani* -infected leaf sheaths of the resistant line showing the infection scar that was restricted to a small region on the sheath. (**D**) *R. solani*-infected leaf sheaths of the susceptible line showing the spreading infection scar perhaps due to low lignin deposition.

dpi in leaf sheaths of *R. solani*-infected rice plants of both lines, stark differences were detected at 2 to 10 dpi with higher deposition occurring in the resistant line. Lignin deposition was found to occur on both the outer and inner surfaces of leaf sheaths and appeared to restrict the spread of the infection to a small region resulting in a small infection scar in the resistant rice line. This indicated that perhaps the initial lignin deposition occurred at the attachment zones between the epidermal cell and the fungus, before spreading to the other cells. Therefore, inhibition of penetration by the *R. solani* fungus into protected tissue was associated with the ability of the epidermis of the resistant line to lignify in response to challenge and it indicates that lignification acts as a mechanical barrier to penetration into the tissue. On the other hand, lack of adequate lignin deposition might have led to the increased spread of the infection resulting in increased disease manifestations in the susceptible rice line. Lignification in leaf sheaths of *R. solani*-infected rice plants compared to lack of lignification in control plants was taken as evidence that it was triggered by resistance response mechanisms in the infected plants.

Fungal colonization took place in infected leaf sheaths of rice plants of both lines although resistance response mechanisms in the resistant line restricted the fungal spread to small regions around the infection site as compared to the susceptible line. There was no evidence that the fungus spread from the point of infection along the sheath to the next sheath rather, infection appeared to spread along the culm especially in the susceptible line. The evidence of what appeared to be hypersensitive cell death in leaf sheaths of *R. solani*-infected plants of the resistant line must be distinguished from the collapsing cells that were detected in leaf sheaths of *R. solani*-infected plants of the susceptible line at 10 dpi. Because *R. solani* is an obligate

pathogen which requires living host tissues (strange, 1998), hypersensitive cell death and adequate lignification in the resistant line might have led to the restriction of the infection to a small region resulting in a small infection scar. Locally induced defence responses, which restrict pathogen infections to the site of attempted ingress, are characterized by a hypersensitive response, a complex, early defence response that causes necrosis and cell death in order to restrict the growth of a pathogen (Lattanzio *et al*., 2006). However, this was different in the susceptible line where large infection scars and inadequate lignification were detected. The fungus appeared to overwhelm the infected cells in the susceptible rice line, leaving them to collapse as it spread to neighbouring cells.

These results corroborate findings from earlier studies conducted on leaf sheaths of *R. solani*-infected rice plants that suggested that resistance to sheath blight disease was strongly related to activation of phenylpropanoid pathway (Mutuku and Nose, 2010, 2011). In the phenylpropanoid pathway lignification occurs through a series of enzymatic steps, starting with a PAL catalyzed reaction to produce lignin precursors, and terminating with a process that requires $H_2O_2$ and a cell-wall-bound peroxidase to bring about polymerization of $C_6$-$C_3$ units into lignin (Olson and Varner, 1993). When both PAL and peroxidae activities were investigated, the results showed that both enzymes were activated in leaf sheaths of *R. solani*-infected rice plants but higher levels were sustained in the resistant line (Mutuku and Nose, 2011). Peroxidase is of the lignin-forming type and could be involved in the strengthening of the plant cell wall. Subsequently, the hypersensitive response triggers a general resistance mechanism rendering uninfected parts of the plant less sensitive to further attack by pathogens, a phenomenon described by Lattanzio *et al*. (2006) as systemic acquired

resistance. Indeed when the uninfected regions of leaf sheaths of *R. solani*-infected rice sheaths of the resistant line were examined, high and uniform lignin deposition was detected in contrast to the very low and non-uniform deposition that occurred in the susceptible line. Therefore, it appears that the potential of plants of the resistant rice line to react to *R. solani* depends upon a signal released from the infected tissue and translocated to other parts of the plant where it induces defence reactions. One of the key signalling molecules that is considered to activate plant defence responses against invading pathogens is salicylic acid. We propose that salicylic acid be investigated in leaf sheaths of *R. solani*-infected rice plants and the results be related with the findings of this current study to fully understand the signaling mechanisms surrounding lignification as a resistance response mechanism.

The results of the current study showed that lignin is one of the desirable products of phenylpropanoid pathway in leaf sheaths of *R. solani*-infected rice plants and that increased lignification is one of the mechanisms that are prerequisite for resistance response against rice sheath blight disease. There are reports that in plants responding to pathogen attack, mechanisms that constitute the 'first line of defence' are composed of non-lignin phenolics. For example, non-lignin phenolic compounds were induced in cultured soybean cells 10 min after addition of chitosan to the cells (Kauss, 1987). However, there is still doubt as to whether these non-lignin phenolics are not a pre-formed pool rather than synthesized immediately after infection (Matern and Kneusel, 1988). In addition, it appears that different stress conditions cause different responses such that it has been difficult to define what mechanisms constitute a first line of defence.

In leaf sheaths of *R. solani*-infected rice plants, detection of lignification at 1 dpi and its continual deposition at 10 dpi was taken as evidence that lignification did not only constitute a first line of inducible defences but also a mechanism that helped to restrict the infection to small regions especially in the resistant line. This shows that resistance did not involve inhibition of pathogen penetration but rather, it reflects the inhibition of further pathogen development by activation of resistance responses like accumulation of phenylpropanoid pathway products like lignin (Fig. 5.5). The proposal shown in Figure 5.5 argues for lignin as a one of the mechanisms involved in first line of defence and shows a proposal of what might have led to the successful resistance response in the resistant line immediately after *R. solani* infection. The results of this current study showed fungal colonization occurred but timely activation of resistance response mechanisms like activation of appropriate pathways as previously reported (Danson *et al*., 2000*a*; Nose, 2002; Mutuku and Nose, 2010, 2011, 2012), and signalling led to enhanced lignification and hypersensitive response in *R. solani*-infected cells as well as cells neighbouring the infected ones where the fungus had not yet reached. This appears to have restricted the infection to small regions in the rice leaf sheaths. Lignification of the cells farther away from the initial inoculation sites (for example lignification of cells in the first leaf sheaths) might have been to prevent the fungus from spreading to new sites and forming new infection cushions. The number of infection cushions were shown to be different between resistant and susceptible cultivars with few infection cushions representing resistance (Groth and Nowick, 1992). Similar response was detected in the susceptible line, but perhaps the magnitude of the response was low leading to spread of the disease. Therefore, these findings show that the susceptible line lacked timely response to the

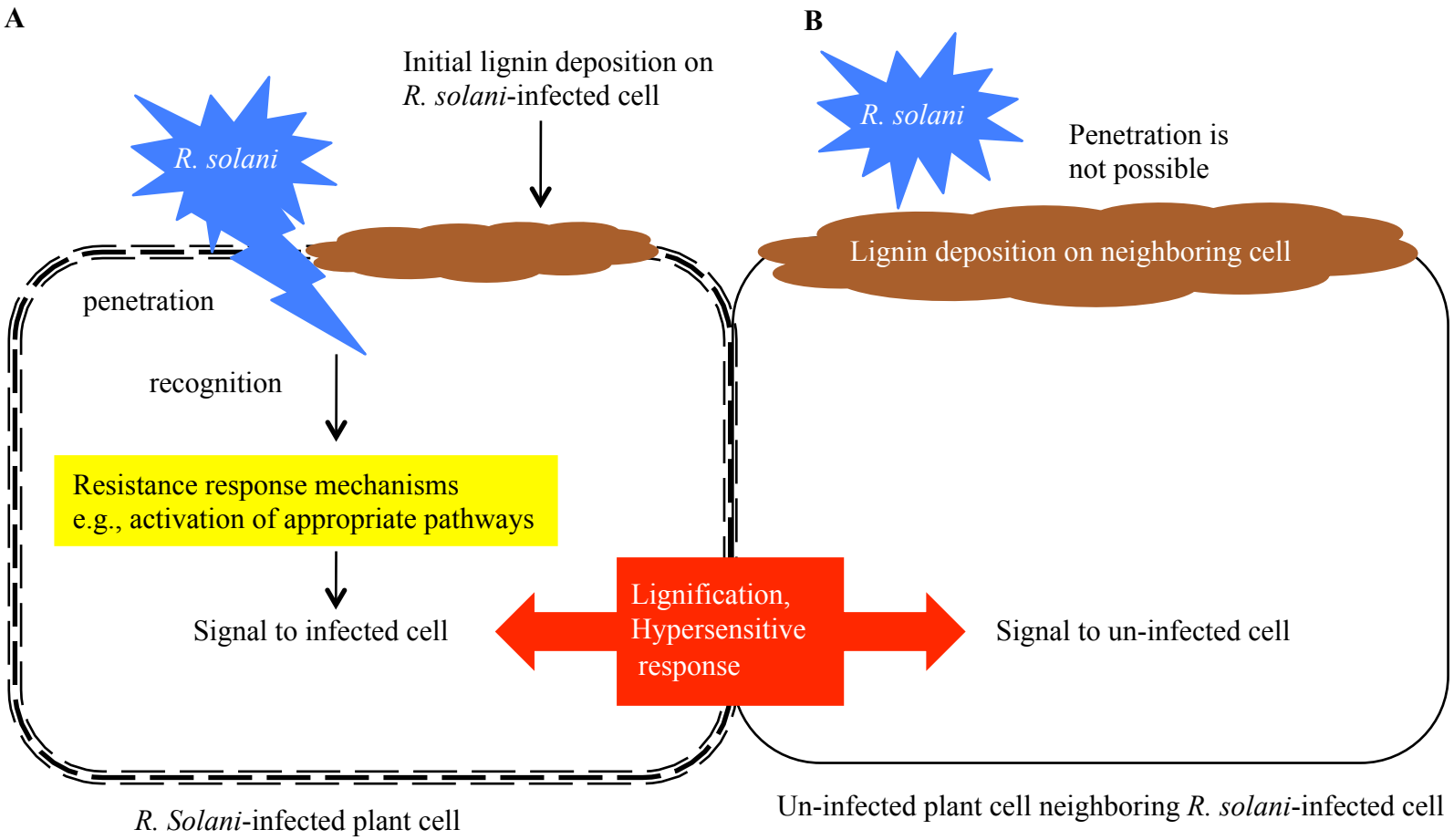


Figure 5.5. (**A**) Inoculation of *R. solani* into rice leaf sheaths led to colonization in both resistant and susceptible lines indicating the pathogen was able to penetrate the cell. In the resistant line, the penetration triggered resistance response mechanisms leading to hypersensitive response and lignification of the cells. Lignification and hypersensitive response was linked to slowing of the infection (**B**) Lignification was not only detected in *R. solani*-infected cells, it was also detected in cells that were not yet infected indicating that the resistance response mechanisms not only slowed the infection but prevented it from spreading further. Lignification of cells farher away from initial inoculation sites (for example lignification in the cells of firt leaf sheath) was perhaps to prevent new infection cushions. This was not the case in the susceptible line where the infection spread to wide regions of the rice leaf sheaths.

pathogen signals and it could be manipulated to resist *R. solani* by among other things, altering the timing and magnitude of the resistance response.

## 5. Summary

Rice sheath blight disease caused by *Rhizoctonia solani* Kuhn results in significant yield and quality losses in all rice growing areas worldwide. Activation of the glycolytic and phenylpropanoid pathways is important in the resistance response to *R. solani* infection in rice. This study examined lignin deposition because lignin was proposed as one of the desirable products in the defence response against sheath blight disease. The results showed that lignification occurred in *R. solani*-infected rice plants at 1 to 4 dpi with higher levels detected in the resistant line. In addition, there were differences in the amounts of lignin deposited in the resistant and susceptible rice lines. These results led to the conclusion that lignin is one of the desirable products of phenylpropanoid pathway in *R. solani*-infected rice plants and that increased lignification is one of the mechanisms that are prerequisite for defence response against rice sheath blight disease.

# CHAPTER 6

## General Discussion

As shown in the current studies, *R. solani* infection causes changes in carbon allocation that lead to generation of products associated with resistance mechanisms. The results shown in Chapter 2 combined with previous studies in our laboratory (Danson *et al*., 2000*a*; Nose *et al*., 2002*a*,*b*) suggested that glycolysis was at the core of carbon allocation in leaf sheaths of *R. solani*-infected rice plants. The current studies investigated the regulation of carbon allocation in leaf sheaths of *R. solani*-infected rice plants and linked it to other resistance response mechanisms like activation of phenylpropanoid pathway where resistance products like lignin are generated. This was accomplished by investigating changes in gene expression in glycolysis, together with those of other pathways that utilize carbon resources obtained from glycolysis such as OPPP, shikimate, TCA and phenylpropanoid pathways. Because studying regulation of glycolysis is complicated by existence of alternative reactions in the cytosol, studies designed to investigate its regulation must examine all enzymes together with their alternative enzymes in cytosol compartment. As an example of such studies, we examined the reaction catalyzed by PFK/PFP. As regulation of cellular carbon allocation is discussed, we expect that the resistance response mechanisms against sheath blight disease caused by *R. solani* infection in rice will become clear. In addition, this General Discussion will propose a strategy to modulate the glycolysis of infected plants using metabolic engineering to enhance their resistance response.

The studies in Chapters 2 to 5 show that resistance response relies on changes that occur in multiple metabolic pathways. They include alteration in carbohydrates metabolism, which has been shown to play an important role during plant-pathogen interactions because carbohydrates are the basic building blocks for the synthesis of various defence chemicals such as lignin (Danson *et al*., 2000; Mutuku and Nose, 2010, 2012). Gene expression studies in Chapter 2 have shown that substantial changes in rice gene expression in both primary and secondary metabolic pathway could be associated with regulation of carbon allocation to enhance *R. solani* resistance response. In the interaction between rice and *R. solani*, activation of glycolytic pathway was accompanied by the activation of OPPP, TCA, shikimate and phenylpropanoid pathways. One way to interpret this was that glycolysis was at the core of carbon allocation for these pathways after *R. solani* infection. This suggested that perhaps the regulation of glycolytic pathway in leaf sheaths of *R. solani*-infected rice plants affected carbon allocation for OPPP, TCA, shikimate and phenylpropanoid pathways.

In Chapter 2 we also showed that RPPC was not activated in leaf sheaths of *R. solani*-infected rice plants. We interpreted this as suggesting that RPPC genes did not play a role in the resistance response against *R. solani* infection because rice leaf sheaths are mainly etioplasts. The localized reduction in expression of RPPC photosynthetic genes in conjunction with increased cellular demands during the resistance response is reported to initiate the transition from source status to sink status in infected tissue (Bolton *et al*., 2008). This transition is accompanied by an increase in cell-wall invertase activity in rice plants after *R. solani* infection (Danson *et al*., 1999*c*) and this is accompanied by increase in hexose phosphates utilized in glycolysis. Invertase

functions to cleave sucrose into glucose and fructose. These can be utilised in glycolysis or pentose phosphate pathway. The findings in Chapter 2 that the expression of OPPP enzymes increased while the gene expression of those of the non-oxidative PPP were low after infection can be used to argue that the PPP was not activated. However, this interpretation combined with the findings that shikimate pathway that utilizes PEP from glycolysis and E-4-P from PPP was activated raised questions regarding the source of E-4-P. This question was resolved later when glycolytic regulation was examined.

In Chapter 2, the expressions of PGM, TPI, GAPDH, enolase and PK in infected plants of the resistant line were shown to have increased. Additionally, Chapter 3 shows the activities of all enzymes and metabolite contents in leaf sheaths of *R. solani*-infected rice plants of the resistant line increased. These increases in gene expressions, enzymatic activities and metabolite contents might have occurred because in addition to the maintenance glycolysis in the plastid, establishment of alternative glycolysis in the cytosol compartment occurred as a response to infection. Further evidence for this were the results in Chapter 4 that increase in expression of the PFK plastid isozyme i.e., *Os06g05860* was accompanied by increase in expressions of three cytosolic PFK isozymes i.e., *Os01g09570*, *Os01g53680* and *Os04g39420* in infected rice plants of the resistant line. Furthermore, the increase in expressions of these PFK isozymes localised in plastid and cytosol compartments was strongly correlated with increase in PFK activity in the same plants. It has been reported that glycolysis is a network of reactions with possible sites for substrate entry into and out of different compartments (Sung *et al*., 1988; Kammerer *et al*., 1998; Givan, 1999) and that induction of glycolysis in the cytosol compartment occurs to

facilitate plant acclimation to environmental stress (Merten, 1991; Plaxton, 1996). Moreover, induction of PFP in the cytosol was accompanied by an increase in expressions of two PFP isozymes i.e., *Os08g25720* ($\alpha$-subunit) and *Os06g13810* ($\beta$-subunit). This was interpreted as showing that activation of PFP in the cytosol compartment was accompanied by enhancement of its sensitivity to regulation by F-2,6-$P_2$ as indicated by the increase in the expressions of both $\alpha$- and $\beta$-subunits.

To determine *in vivo* control points of glycolysis in leaf sheaths of *R. solani*-infected rice plants, mass-action ratios were estimated and compared with apparent equilibrium constants (*K´*). The results provided strong evidence that reactions catalysed by PGM, PGI, TPI and PGmu+enolase in infected rice plants were near equilibrium *in vivo* (Chapter 3). Although it is not an invariable rule, enzymes catalysing near-equilibrium reactions are unlikely to produce large effects on metabolites concentration (Fell, 2005). This might explain why for example, contents of G-1-P, G-6-P and F-6-P, which are involved in the reactions catalysed by PGM and PGI appeared similar in infected rice plants of the resistant line. The TPI reaction was different because significant changes in the DHAP/GAP ratios occurred. The equilibrium point of the TPI reaction is at DHAP:GAP= 24:1 (Rozovsky and McDermott, 2007) but in leaf sheaths of *R. solani*-infected rice plants it was DHAP:GAP= 1:1.8 in favour of GAP. GAP can be utilised in the generation of E-4-P by transketolase. E-4-P can also be generated in the non-oxidative PPP by transaldolase. We could not detect transaldolase activity in leaf sheaths of *R. solani*-infected rice plants. This failure to detect transaldolase while at the same time detecting transketolase and E-4-P was taken as showing that E-4-P was obtained from

GAP by action of transketolase. This helped to clarify the question of the source of E-4-P that we raised in Chapter 2.

As shown in Chapter 3, reactions catalysed by PFK, aldolase, GAPDH+PGK and PK in leaf sheaths of *R. solani*-infected rice plants were non-equilibrium *in vivo* and regulatory. This was a significant finding because it shows that in leaf sheaths of *R. solani*-infected rice plants, glycolysis is regulated at four reactions i.e., PFK, aldolase, GAPDH+PGK and PK. In published reports, the regulation of glycolysis occurs at three reactions i.e., hexokinase, PFK and PK (Plaxton, 1996).

To investigate the regulation of glycolysis, we examined gene expression, enzymatic activities and metabolite contents in the glycolytic pathway. This is because gene expression, enzymatic activities and metabolite contents can be used to determine metabolic control mechanisms. Metabolic controls are of two types i.e., coarse and fine controls. Whereas coarse control is achieved through varying the total population of enzyme molecules via alterations in the rates of enzyme biosynthesis or proteolysis, fine controls sense the momentary requirements of the cells and adjust the rate of metabolic flux through the pathway accordingly (Plaxton, 1996). PGM, TPI, GAPDH, enolase and PK activities in leaf sheaths of *R. solani*-infected rice plants shown in Chapter 3 were compared with the expressions of their genes in the same plants as shown in Chapter 2 and strong positive correlations (Pearson's correlation coefficient, $r > 0.8$) were observed especially in the resistant line. These increases in enzymatic activities and gene expressions suggested coarse metabolic control was exerted to PGM, TPI, GAPDH, enolase and PK. Perhaps the increase in activities of PGI, aldolase and PGmu as well as PFK and PFP (Chapter 4) in infected plants

especially those of the resistant line might also have been as a result of mechanisms of coarse control. The results showed that the reactions catalysed by PFK, GAPDH+PGK and PK were non-equilibrium *in vivo* suggesting that these reactions exerted fine metabolic control of glycolysis. Reactions that exert fine metabolic control can be recognized by the fact that they are greatly displaced from equilibrium *in vivo* (ap Rees and Hill, 1994; Plaxton and Podesta, 2006). These results show that coarse control was exerted to the reactions catalysed by PGM, PGI, TPI and PGmu+enolase whereas, a combination of both coarse and fine controls was exerted to the reactions catalysed by PFK/PFP, aldolase, GAPDH+PGK and PK.

Taken together, these results suggest that the control of the reactions catalysed by PFK, aldolase and TPI might have led to increased generation of GAP which was utilised in the generation of E-4-P by transketolase and in down-stream reactions for generation of PEP through GAPDH+PGK. Indeed the negative $\Delta G$ values of the reactions catalysed by PFK, aldolase, TPI and GAPDH+PGK were interpreted as suggesting the reactions spontaneously favoured glycolysis. This might have led to increase in GAP and subsequently E-4-P and PEP both of which are utilised by DAHP synthase in the shikimate pathway. Therefore it could be argued that the control of PFK, aldolase, TPI, TK and GAPDH+PGK reactions was closely coordinated with the demand of the cells for E-4-P and PEP both of which are substrates of the shikimate pathway. The shikimate pathway supplies substrates to the phenylpropanoid pathway where resistance products such as lignin are generated. As shown in Chapters 3 and 5 activation of the phenylpropanoid pathway led to increased $H_2O_2$ and lignin generation. These products enhanced the protection of cells from invading *R. solani* and it can be argued they were important components of the

resistance response. For better understanding of the findings a schematic representation of the biochemical reactions involved in glycolysis and the regulating reactions in rice leaf sheaths and those in other published reports are as shown in Figure 6.1.

Since it is now possible to manipulate nucleic acids and gene expression, an important goal of biotechnology is to modify the output of specific pathways via the process of metabolic engineering. One of the goals of metabolic engineering in plants is to enhance the production of more of a desired compound (Verpoorte *et al*., 2000; Bolton, 2009). Taken together, these results suggest that to control the rate of glycolysis and to enhance lignin deposition in leaf sheaths of *R. solani*-infected rice plants, one of the strategies of metabolic engineering could be to modulate the regulatory reactions of PFK/PFP, aldolase, GAPDH+PGK and PK using fine control mechanisms to enhance glycolysis during disease stress acclimation. The reactions catalysed by GAPDH+PGK and PK where coarse and fine controls overlapped might require a combination of strategies used for both metabolic controls. PGM, PGI, TPI and enolase could be manipulated by coarse control where for example, TPI is modulated to favour generation of GAP during acclimation to stress conditions. This approach targets multiple steps of glycolysis in leaf sheaths of *R. solani*-infected rice plants. This is because flux through a metabolic pathway is not determined by kinetic constants of single steps (Capell and Christou, 2004). Moreover, at branch points like those between GAP to E-4-P, and E-4-P and PEP to secondary metabolism, overexpression of transketolase, and DAHP synthase, respectively, to out-compete other enzymes using the same substrate could divert flux into appropriate pathways like those leading to lignin generation.

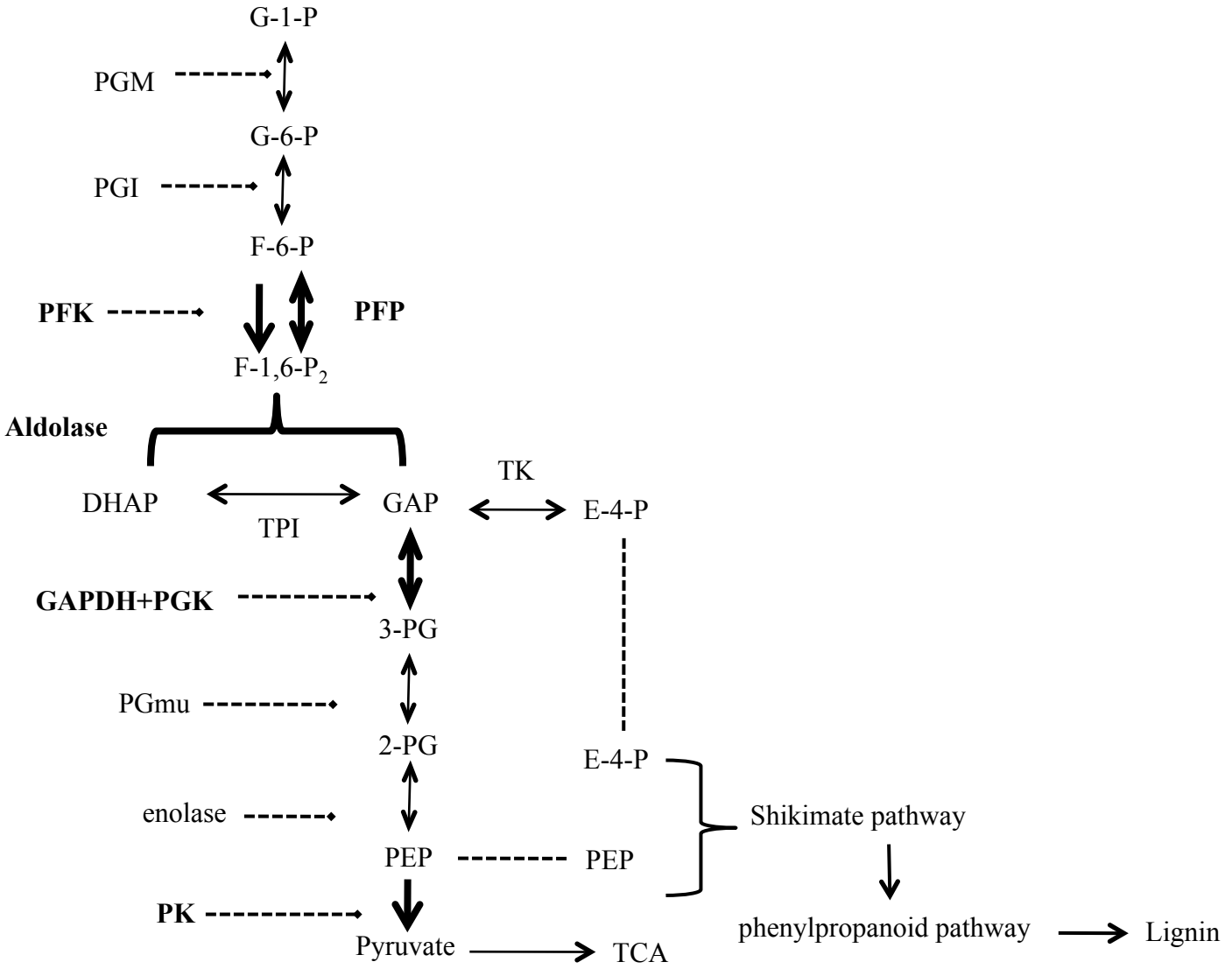


Figure 6.1. The glycolytic pathway and its link to shikimate and phenylpropanoid pathways in leaf sheath of *R. solani*-infected rice plants. It was shown that erythrose-4-phosphate (E-4-P) was obtained from GAP by the action of transketolase (TK). E-4-P and phosphoenolpyruvate (PEP) were utilised in shikimate pathway which generated substrate for the phenlypropanoid pathway where lignin was generated. Control of phosphofructokinase (PFK/PFP), aldolase, triosephosphate isomerase (TPI) and transketolase (TK) might have led to increase in glyceraldehyde-3-phosphate (GAP) which was utilised by TK to generate erythrose-4-phosphate (E-4-P) and in downstream reactions by glyceraldehyde-3-phosphate dehydrogenase+phosphoglycerate kinase (GAPDH+PGK), phosphoglycerate mutase (PGmu) and enolase in the generation of PEP. The bold arrows show the reactions that were found to be regulatory in infected plants i.e., PFK/PFP, aldolase, GAPDH+PGK and pyruvate kinase (PK)

Rice is not the only *R. solani*-host plant. Others include wheat, maize, sorghum, beans, soybean, peas, cabbage, lettuce, tomato, Japanese radish, potato, carrot, onion and tobacco and all of them representing important crops both for food and economic value. Nevertheless, various factors increase the attractiveness of rice as a model plant for metabolic engineering over the other plants. For example, the sequenced rice genome was found to be small (International Rice Genome Sequencing Project, 2005). In addition, studies discussed in this thesis represent a major step towards understanding the cellular carbon allocation as part of resistance response mechanisms against *R. solani*. Furthermore, these studies also showed that the resistance product lignin is deposited as a first line of defence and that susceptibility to *R. solani* occurs due to poor timing and weak response. To our knowledge, no such comprehensive studies have been done on any of the other host plants affected by *R. solani*. Moreover, since no resistance to *R. solani* infection has been reported in any of these host plants either, it can be argued that findings relating to the host-pathogen interactions at the molecular level between rice and *R. solani* may be closely related to what would be expected in the other plants. We can speculate that there is a similarity between carbon allocation in rice and the other host plants after *R. solani* infection because for example, lignification occurs in bean plants after *R. solani* (Guillon *et al*., 2002). Considering this one case, it could be argued that the same approach to rice metabolic engineering could be applicable in bean plants and perhaps relatively easily in other $C_3$ plants like wheat, lettuce, tomato, potato and tobacco.

## Future studies

These studies argued for modulation of all the regulatory enzymes in the glycolysis of *R. solani*-infected rice plants but the relative contribution of each to pathway flux should be estimated.

As shown in Chapter 3, and in General Discussion, additional establishment of glycolysis in the cytosol after infection suggested there was exchange of metabolites between the cytosol and plastid compartments. Such exchanges could happen depending on the environment surrounding the cell. Therefore we propose that these sites for substrate entry into and out of glycolysis in different compartments be investigated. The starting point could consider that non-photosynthetic plastids import carbon in the form of hexose phosphates via the G-6-P/ phosphate translocator (Kammerer *et al*., 1998) that is different from the triose phosphate/phosphate and plastidic PEP/phosphate translocators. This suggests that metabolites are not transported by a single transport system, but by what appears to be a set of different phosphate translocators perhaps with partially overlapping substrate specificities.

Chapter 5 showed that lignin was one of the desirable resistance products in the interaction between rice and *R. solani*. In addition, it was also shown that part of the resistance response mechanisms include activation of PAL, peroxidase and $H_2O_2$ generation. We propose that future studies examine the existence of other resistance products. We also propose that signalling pathways be investigated because as shown in these studies, prompt detection led to timely enhancement of the generation of resistance products leading to restriction of the pathogen to small areas in inoculated

leaf sheaths of the resistant line. This suggests that signalling pathways must have been activated.

The overall goal of these studies is to elucidate resistance response mechanisms in the interaction between rice and *R. solani* with the aim of generating sustainable varieties. The proposal to modulate enzymes in glycolytic, pentose phosphate and shikimate pathways if applied, may lead to generation of plants with improved resistance to *R. solani* infection. However, we appreciate one of the problems inherent in this approach because as it is the case currently, a focus on improving crops for resistance to disease infection leads to reduced yield because the pre-accumulated carbon resources maybe used for disease resistance mechanisms instead of grain development (Wasano *et al.,* 1983; Ishikawa *et al.,* 1993). However, we strongly argue that when all factors including environmental degradation due to use of agricultural chemicals are considered, generating sustainable varieties outweighs other concerns. Besides, if reduction in yield occurs, it can be considered in separate experiments or be solved by other approaches.

**Summary**

There is no problem confronting humans more fundamental than feeding its expanding populations. The greater proportion of our food is derived from relatively few plant species among which cereal crops are main. Rice (*Oryza sativa* L.) one of the 'big three' cereals, is the principal food for 60 % of the worlds' people. However, rice is subject to diseases that place major biological constraints on its production. Of these, rice sheath blight is one of the most prevalent causing great damage to rice yield and quality worldwide (Lee and Rush, 1983). Sheath blight disease is caused by the fungus *Rhizoctonia solani* Kuhn and is a serious threat to food security worldwide because it is prevalent in all rice growing areas where it causes significant yield losses. Because of the economic importance of rice sheath blight disease, research has been focused on development of resistant cultivars. This is not easy because in rice, major gene resistance to *R. solani* has not been found. This interaction relies on polygenic characters.

Chapter 2 showed that the interaction between rice and *R. solani* relied on alterations in carbohydrates metabolism. Further investigation revealed that activation of glycolytic pathway was accompanied by the activation of OPPP, TCA, shikimate and phenylpropanoid pathways. This suggested that glycolysis was at the core of carbon allocation for these pathways after infection. This implied that perhaps the regulation of glycolysis in leaf sheaths of *R. solani*-infected rice plants affected carbon allocation for OPPP, TCA, shikimate and phenylpropanoid pathways.

To investigate the regulation of glycolysis, we examined gene expression, enzymatic activities and metabolite contents in the glycolytic pathway. The results showed that the activities of all enzymes as well as the contents of metabolites in leaf sheaths of *R. solani*-infected rice plants increased. These increases combined with the increase in expressions of PGM, TPI, GAPDH, enolase and PK in infected plants of the resistant line (Chapter 2) might have occurred because in addition to the maintenance glycolysis in the plastid, establishment of alternative glycolysis in the cytosol compartment occurred as a response to infection. Further evidence for this was the results in Chapter 4 that increase in expression of the PFK plastid isozyme i.e., *Os06g05860* was accompanied by increase in expressions of three cytosolic PFK isozymes i.e., *Os01g09570*, *Os01g53680* and *Os04g39420* in infected rice plants of the resistant line.

Gene expression, enzymatic activities and metabolite contents were used to determine metabolic control mechanisms. The increase in expressions and enzymatic activities of PGM, TPI, GAPDH, enolase and PK were strongly correlated suggesting that coarse controls were exerted to the reactions catalysed by these enzymes. The reactions catalysed by PFK/PFP, aldolase, GAPDH+PGK and PK in leaf sheaths of *R. solani*-infected rice plants were non-equilibrium *in vivo* suggesting that these reactions exerted fine metabolic control of glycolysis. Taken together the results showed that coarse control was exerted to the reactions catalysed by PGM, PGI, TPI and PGmu+enolase whereas, a combination of both coarse and fine controls was exerted to the reactions catalysed by PFK/PFP, aldolase, GAPDH+PGK and PK.

Finally, these studies proposed that tools such as metabolic engineering might be used to modulate the reactions catalysed by PGM, PGI, PFK/PFP, aldolase, TPI, GAPDH+PGK, PGmu+enolase and PK to enhance glycolysis during acclimation to disease stress. Moreover, these studies argued that modulation of these reactions in leaf sheaths of *R. solani*-infected rice plants requires different strategies of engineering that consider coarse and fine metabolic controls. Where both controls are exerted, regulation through a combination of both metabolic controls could be done simultaneously.

**Abstract in Japanese**

人口増加に対応した食糧確保は、緊急に解決すべき課題である。我々の食料の大部分は、 少数の植物種に依存し、中でもイネ（*Oryza sativa* L）は「三大穀物」の一つで、世界人口の60%が主食としている。しかし、 その生産において病害は収量を制限する大きな要因である。特に、イネ紋枯病はイネの収量及び品質に大きな損害を与える病気の一つである（Lee and Rush, 1983）。イネ紋枯病は *Rhizoctonia solani* (Kuhn)と呼ばれる糸状菌によって引き起こされ、世界の食物確保に大きな脅威となっている。

本研究は、Wasano *et al.* (1985)によって開発された*R. solani* 抵抗性系統及び感受性系統を用いてイネ紋枯病に対する抵抗性メカニズムを解明することを目的として実施した。調査は、*R. solani* を接種した 抵抗性及び感受性系統の解糖系、ペントースリン酸回路、シキミサン経路、フェニルプロパノイド代謝とTCA回路の関連酵素のmRNA発現について行った。その結果、解糖系、ペントースリン酸回路、シキミサン経路、フェニルプロパノイド代謝及びTCA回路を活性化させる炭素分配の部分が、罹病後に変化した。しかし、還元的ペントースリン酸回路は活性化しなかった（Mutuku and Nose, 2010）。同時に、F-6-P、DHAP、GAP、3-PG、PEP、 ピルビン酸、E-4-P及びATPの含量が増加した。さらに、アルドラーゼ、TPI、GAPDH、PGK、エノラーゼ及びPKの酵素活性は、*R. solani*に罹病したイネで高かった。トランスケトラーゼ、PAL及びパーオキシダーゼの高い活性と$H_2O_2$の蓄積が認められ、*R. solani* に対する イネの病害抵抗性の メカニズム

に関連していることが明らかになった（Mutuku and Nose, 2011）。さらに、*R. solani*を接種したイネを用いた解糖系の*in vivo*での 制御特性については、PFK、アルドラーゼ、TPI、GAPDH+PGK及びPKによる触媒反応が平衡状態より 抑制された状態にあるこ とが観察された。

解糖系の制御部として重要なF6PとF-1,$6P_2$の相互転換部において、 *R. solani* 抵抗性系統でpyrophosphate-fructose-6-phosphatephosphotransferase (PFP)と6-phosphofructokinase(PFK)の活性が増加することが明らかになった。さらに、13種類のPFP/PFKアイソザイムのmRNA発現について解析した結果、*0s01g09570*(PFK1)、*0s01g53680*(PFK3)、*0s04g39420*(PFK4)、*0s06g05860*(PFK5)、*0s08g25720*(PFP2)及び*0s06g13810*(PFP5)というイネ紋枯病に特異的なアイソザイムが存在した。これらの結果は、(a)PFPとPFKに*R. solani*罹病対応のアイソザイムが存在し、(b)罹病に伴うPFPの活性化は、その$\alpha$-及び$\beta$-サブユニットの両方が関係している(PFP2及びPFP5)こと、 (c) 無関係な$\alpha$-サブユニットが存在することを示している（Mutuku and Nose, 2012）。また、解糖系の活性化はリグニンを生成するフェニルプロパノイド代謝の活性化と関連していた。事実、*R. solani*感受性系統と比較して抵抗性系統のイネで活発なリグニン蓄積が観察された。

以上の結果は、*R. solani*に罹病したイネがその抵抗性を高めるための解糖系制御の代謝工学的アプローチには、遺伝子発現によって直接制御される部分(

coarse　control)とタンパク合成後の生体内調整(fine　control)の両方の制御が必要であることを示している。

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## Appendices

Appendix 1

**Rice plant PFPs**

**PFP 1** ***Os02g48360***

MDSDYGVPRELSEVQKKRALYQPELPPCLQGTTVRVEYGDAAIAADPAGAH
VISHAFPHTYGQPLAHFLRKTANVPDATVISEHPVVRVGVVFCGRQSPGGHN
VIWGLHEAIKAHNPNSKLIGFLGGSDGLLAQKTLEITDEVLSSYKNQGGYDM
LGRTKDQIRTTEQVNGAMASCQALKLDALVIIGGVTSNTDAAQLAETFAEAK
CATKVVGVPVTLNGDLKNQFVETTVGFDTICKVNSQLISNVCTDALSAEKYY
YFIRMMGRKASHVALECALQSHPNMVILGEEVAASKLTIFDITKQICDAVQAR
AEKDKYHGVVLIPEGLVESIPELYALLQEIHGLHGKGVSMENISSQLSPWASA
LFEFLPPFIRKQLLLHPESDDSAQLSQIETEKLLAQLVEDEMNRRMKEGTYKG
KKFNAICHFFGYQARGSLPSKFDCDYAYVLGHVCYHILAAGLNGYMATVTN
LKSPANKWRCGAAPISSMMTVKRWSRGPAATQIGKPAVHMATVDLKGKAF
ELLRNNSTSFLIDDIYRNPGPLQFEGAGADSKPISLCVEDQDYMGRIKKLQEYL
EKVKSIVKPGCSQDVLKAALSAMSSVTETLAIMTSSSTGQGTPL

**PFP 2** ***Os08g25720***

MNADFGAPKDLAGGLQQRRSLYQPTLPPCLQGATVKVEYGDATTTIDPTCA
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GLHDALKAYNPHSVLYGFVGGTEGLFANKTIEITDDVLASYKNQGGFDLLGR
SIDQIRSTKQVNAAMTTCNNLNLDGLVIIGGVTSNSDAAQLAEALVQNNCKT
KVVGVPVSLNGDLKNQFVETTVGFDTVCKVNSQLVSNVCLDAISAGKYYYF
VRLMGRKASHVAFECALQSHPNMLILAEEVALSKLTLMEVINKICDGVQARA
ELGKFHGVLLIPEGLIESIPEMYALLQEINILHNNNVPVAEMPSQLSPWAAALF
QFLPPFIRRELLLHQESDNSAQLSQIDTEQLLAHLVEAEMIKRTKEGRYKGRK
FSSVCHFFGYQARGSVPSNFDCDYAYALGRISLHMIAAGLTGYMATVANLKD
PVDKWRCAAAPLTAMMSVKRHLRGPGAIPIGKPAIHPSPIDLKGKAYELLRE
KASSFLLDDFYRTPGGIQYEGPGCNAKPITLTIENQDYMGDIEILKDCLSKVRT
MVKPGCSREVLKAAISSMLSVTDVLTVMSHPLNAELPLYHFN

**PFP 3** ***Os06g22060***

MGSVAMDSDYGVPRELSALQKARALYRPDLPPCLQGTTVKVEYGDAAIAAD
VAGAHVISHAFPHTYGQPLAHFLRKTAAVPDATVITEHPVVRVGVVFSGRQS
PGGHNVIWGLHDAIKAHNANSKLIGFLGGTDGLFAQKTLEISDEVLSSYKNQ
GGYDLLGRTRDQIRTTEQVNAAMTACQALKLDALVIIGGVTSNTDAAQLAET
FAESKCSTKVVGVPVTLNGDLKNQFVETTVGFDTICKVNSQLISNVCTDALSA
EKYYYFVRLMGRKASHVALECALQSHPNMVILGEEVAASKLTIFDITKQICD
AVQARAEKDKNHGVVLIPEGLVESIPELYALLQEIHGLHDKGVSVENISSHLS
PWASALFEFLPPFIRKQLLLHPESDDSAQLSQIETEKLLAQLVEAEMNKRLKE
GTYKGKKFNAICHFFGYQARGALPSKFDCDYAYVLGHVCYHILAAGLNGYM
ATVTNLRSPVNKWKCGAAPITSMMTVKRWSRGPAASQIGKPAVHMASIDLK

GKPYELLRQNSSSFLMEDIYRNPGPLQFEGPGGETKPISLCVEDRDYMGRIKQ
LQEYLEKVKSIVKPGCSQDVLKAALSAMASVTEMLTIMSSPSFSGQATI

**PFP 4 *Os09g12650***

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GIYDAMKTQNLQSVLLGFIGGTEGLFANQTLEITDDVLSAYRNQGGFDFLGR
TVDQIHTTEQVNAAMSTCCDLDLDGLVIIGGVTSNSDAAQLAETFANHNCKT
KVVGVPVSLNGDLKNQFVETTVGFDTVCKVNSQLISNVCLDAISAGKYYHFV
RVMGWKASHVALECALQSQPNMVILGEEVAFSKLTLKEIISKICDGVQARAA
QEKYHGVLLISEGLIESIPEMFALIQEINILHSNKVPENNIPSQLSPWATALYNY
LPPFIRRELLLHQDSDNSAQLSQIDTEQLLAHLVEAEMNKRMKEGKYIGRKFS
SVCHFFGYQARGSLPSNFDCDYAYVLGHICMHILAAGLNGYMAFATNLKEPT
NKWRCAAVPLTAMMSVKRHSRSPGAVPTGKPVIHPSPVDLQGKAYALLREK
ASSFLLDDFYRTPGGIQFDGSGTNVKPITLTVEDQDYLGDIELLQDYLEKVRNI
VKPGCSREILKAAISSMSSVKDVLKVMSAPFYAELPLFNLN

**PFP 5 *Os06g13810***

MAAAAVAANGGGDGAQASNAPAPTRLASVYSEVQTSRLKHALPLPSVLRSP
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QAPGGHNVICGIFDYLQEYAKGSVMYGFKGGPAGVMKCKYVELTADYVYP
YRNQGGFDMICSGRDKIETPEQFKQAEDTVNKLDLDGLVVIGGDDSNTNACL
LAEYFRGKNMKTRVIGCPKTIDGDLKCKEVPTSFGFDTACKIYSEMIGNVMT
DARSTGKYYHFVRLMGRAASHITLECALQTHPNVALIGEEVAAKKETLKSVT
DYITDIVCKRAELGYNYGVILIPEGLIDFIPEVQKLIAELNEILAHDVVDEAGA
WKSKLQPESRQLFDFLPKTIQEQLLLERDPHGNVQVAKIETEKMLIAMVETEL
EKRKAEGKYPAHFRGQSHFFGYEGRCGLPTIFDSNYCYALGYGSGALLQCGK
TGLITSVGNLAAPVEEWTVGGTALTSLMDVERRHGKYKPVIKKAMVELDGA
PFKKFASLRDEWSLKNHYISPGPIQFSGPGSNDANHTLMLELGAEA

**<u>Rice plant PFKs</u>**

**PFK 1 *Os01g09570***

MEAVGVAPAPAGVPEKKLLEVKESRKAAPAAPSTSMAAKWAMKKKLVGGD
AGYVLEDVPHLTDYLPELPTYPNPLQDNPAYSVVKQYFVNTDDTVTQKIVVH
KTSARGTHFRRAGPRQRVYFQSDEVNAAIVTCGGLCPGLNTVIRELVCGLYD
MYGVTSVVGIEGGYKGFYSRNTVALTPKSVNDIHKRGGTVLGTSRGGHDTG
KIVDSIKDRGINQVYIIGGDGTQKGASVIYEEVRRRGLKCSVVGVPKTIDNDIA
VIDKSFGFDTAVEEAQRAINAAHVEAESAENGIGVVKLMGRNSGFIAMYATL
ASRDVDCCLIPESPFYLEGKGGLLEFIEKRLKDNGHMVIVVAEGAGQDLIAKS
MNFVDTQDASGNKLLLDVGLWLSQKIKDHFKKKRNFPITLKYIDPTYMIRAV
RSNASDNVYCTLLAHSALHGAMAGYTGFTVAPVNGRHAYIPFYRITEKQNK
VVITDRMWARVLCSTNQPCFLSHEDVEHLKHDDDEHHLHNTQLLEGESSPV
KDSSKCNGTAAPV

**PFK 2** ***Os05g10650***

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AKKIVVHKGSARGTHFRRAGPRQRVFFQPDEVSAAIVTCGGLCPGLNTVIREL
VCGLHDMYGVTSVVGIEGGYRGFYARNTVELTPRSVNGIHKRGGTVLGTSR
GGQDTGKIVDSIQDRGINQVYIIGGDGTQKGAATIHAEVQRRGLKCAVVGVP
KTIDNDIAVIDRSFGFDTAVEEAQRAINAAHVEAESAENGVGVVKLMGRNSG
FIAMYATLASRDVDLCLIPESPFYLEGKGGLLEFAEKRLRENGHMVIVVAEGA
GQDVIARSMRLADAHDASGNKVLLDVGLWLCAKIKDHFKKKANFPITLKYI
DPTYMIRAVPSNASDNVYCSLLAHSAIHGAMAGYTGFTVAPVNGRHAYIPFY
RITEKQNKVVITDRMWARVLCSTNQPCFLSTEDVEKAGQDDEEPIVPLVEGE
NSLVKAPPLLANAGDRAALCNGAA

**PFK 3** ***Os01g53680***

MASHIILPKEEEAALGVAVEEDHDSPAAPGYQHQQGPPVAKALPFSATCVRIS
RDSYPNLRALRNASAMSLPDDDAAYAKLEEGDYGYLLDDVPHFTDYLSDLP
TFPNPLQDHPAYSTVKQYFVNADDTVPEKVVVQKDSPRGVHFRRAGPRQRV
YFESEDVKACIVTCGGLCPGLNTVIRELVCGLSHMYNVNDIFGIQNGYKGFYS
SNYLPMTPKSVNDIHKRGGTVLGTSRGGHDTKKIVDNIQDRGINQVYIIGGDG
TQKGAYEIYKEIRRRGLKVAVAGVPKTIDNDIAVIDKSFGFDSAVEEAQRAID
AAHVEASSAENGIGLVKLMGRYSGFIAMYATLASRDVDCCLIPESPFYLEGEG
GLFEYIEKRLKENNHMVIVVAEGAGQDLIAKSIAAADQIDASGNKLLLDVGL
WLTHKIKDYCKNKKMEMTIKYIDPTYMIRAIPSNASDNVYCTLLAHSAIHGA
MAGYSFTVGMVNGRHAYIPFHRVTSTRNKVKITDRMWARLLSSTNQPSFLSQ
KDIDAAREADKLASKSPVPVNTKEHGENVKKPANGEK

**PFK 4** ***Os04g39420***

MTAESARDPRGPYMSGCHVGRPASPSTGAVDEPCRCGNAHRLRPRLQKPHQ
KPSPRSRSPLDPAPRRWRRLRRWELVVGLRELYGVRDVFGVAAGYRGFYGP
DADHARLDLAAVDDWHKKGGTVLKTTRGGFDLNKIVDGIVARGYTQCLTSN
TFYHDYILLKVYAIGGDGTMRGAVAIFNEFKRRGLNISITGIPKTVDNDIGIIDR
SFGFQTAVEIAQQAIDAAHVEAVSAVNGIGLVKLMGRSTGHIALHATLSSRD
VDCCLIPEVDFYLEGKGGLFEFLYERIKQKGHAVVVVAEGAGQELIPRTDDQ
KREQDESGNIVFLDVGPWLKSELGKWWKREHPSELFTVKYIDPTYMIRAVPA
NATDNLYCTLLAHSAIHGIMAGYTGFVPGPINGNYSYIPLEDVAVAKNPVDV
NDHKWAWVRSVTNQPDFMKPKY

**PFK 5** ***Os06g05860***

MASPPTASASASEAAESGRRSAPGPIDVPSPRDHLHHLLDRRDTPRVVHVEGT
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SNPLQDNPAYSVVKQYFVNPDDTVCQKAIVHKDGPRGNHFRRAGPRQRVFF
ESDEVHACIVTCGGLCPGLNTVIREIVCGLYDMYGVSRVLGIQGGYRGFYAC
NTIDLSPKSVNDIHKRGGTVLGTSRGGHDTMKIVDSIQDRGINQVYVIGGDGT
QRGAGVIFEEIRRRGLKVAVAGIPKTIDNDIPVIDRSFGFDTAVEEAQRAINAA
HVEAGSAENGIGLVKLMGRHSGFIAHYATLASRDVDCCLIPESPFYLEGEGGL
FRYLEKRLKENGHMVIVVAEGAGQKLINETKESMGKDASGNSILLDVGLWLS

QKIKEHFKKIKTTINLKYIDPTYMIRAIPSNASDNVYCTLLAHSVVHGAMAGY
TGFTVGQVNGRHCYIPFYRITEKQNKVSITDRMWARLLSSTNQPSFLSKKDVE
DAKMEEERASKFFDGPPPNPKVEDKVASNGKAVK

**PFK 6** ***Os09g24910***

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TDGGSLSADKWNGYVNKDDRALLKVIKYASPTSAGAECVDPDCSWVEHWI
HRAGPRKEIYYEPAEVKAAIVTCGGLCPGLNDVIRQIVFTLEIYGVKNIVGIQF
GYRGFFEKGLKEMPLSRKVVENINLSGGSFLGVSRGGAKTSEIVDSIQARRID
MLFVIGGNGSHAGANAIHEECRKRKLKVSVVAVPKTIDNDILFMDKTFGFDT
AVEEAQRAINSAYIEARSAYHGIGLVKLMGRSSGFIAMQASLSSGQIDVCLIPE
VSFTLDGEHGVMRHLEHLLEKKGFCVVCVAEGAGQDLLQKSNATDASGNVI
LSDFGVHMQQKIKSHFKDIGVPADVKYIDPTYMVRACRANASDAILCTVLGQ
NAVHGAFAGFSGITSGICNTHYAFLPITEVITKPKRVNPNSRMWHRCLTSTGQ
PDFH

**PFK 7** ***Os10g26570***

MALKSPVDFAGSITSGQKDPCCFGVPGCNPRCVRYNKKSRTCRLVTRAISVD
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GFFEDHLAEVPLNRQVVQNINLAGGSFLGVSRGGANISDIVDSIQARRLDMLF
VLGGNGTHAGANLIHEECRKRKLKVSIVGVPKTIDNDILLMDKTFGFDTAVE
AAQRAINSAYIEAHSAFHGIGLVKLMGRSSGFITMHASLSSGQVDICLIPEVPF
TLDGPNGVLQHLEHLIETKGFALICVAEGAGQEHLQQSNATDASGNMILGDIG
VHLHQKIKAHFKEIGVHSDVKYIDPTYMVRAVRANASDAILCTVLGQNAVH
GAFAGFSGITTGICNTHNVYLPISEVIKSTRFVDPNSRMWHRCLTSTGQPDFH

**PFK 8** ***Os08g34050***

MAVSLKSSGSFCSTPPQWLHSTRDRILYGYSHSNAKECTCKKTKRPAPLCVK
ATSTKVELDFNDPSWKQKFQEDWDKRFNLPRITDIYDLKPRPTTFSLKKNRSP
AGDENGTPMDKWNGYVNSDDRALLKVIKYSSPNSAGAECIDPDCSWVEQW
VHRAGPRKEIYYEPEEVKAAIVTCGGLCPGLNDVIRQIVFTLETYGVKNIVGIP
FGYRGFFEKGLKEMPLSRHLVENINLAGGSFLGVSRGGAKTSEIVDSIQARRID
MLFVLGGNGTHAGANAIHEECRKRKLKVSVVAVPKTIDNDILLMDKTFGFDT
AVEEAQRAINSAYIEARSAYHGIGLVKLMGRSSGFIAMHASLSSGQVDVCLIP
EVPFTLDGEYGVLRHLEHLLKTKGFCVVCVAEAAGQLFYVHYRSLQKSGAT
DASGNVILSDIGVHMQQKIKMHFKDIGVPADVKYIDPTYMVRACRANASDAI
LCTVLGQNAVHGAFAGFSGITSCICNTHYVYLPITEVITVPKRVNPNSRMWHR
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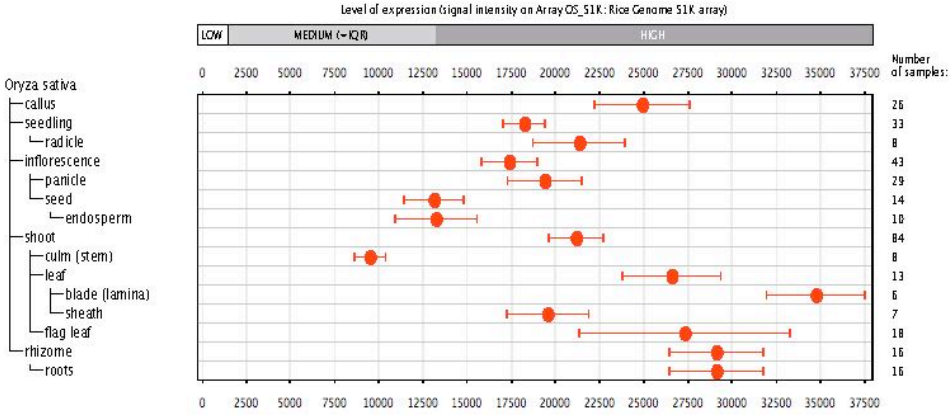


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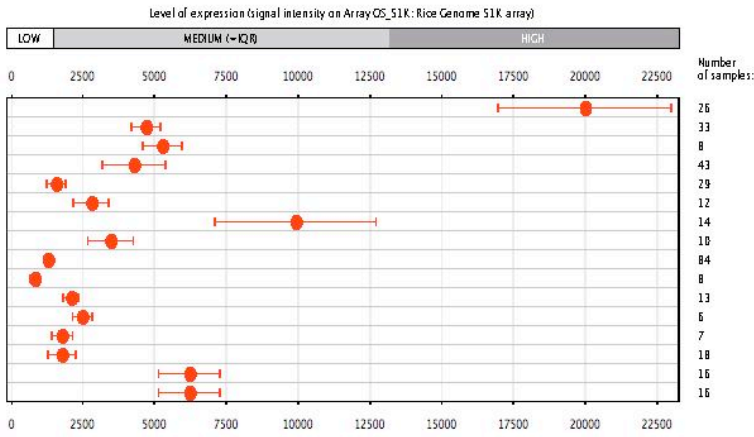
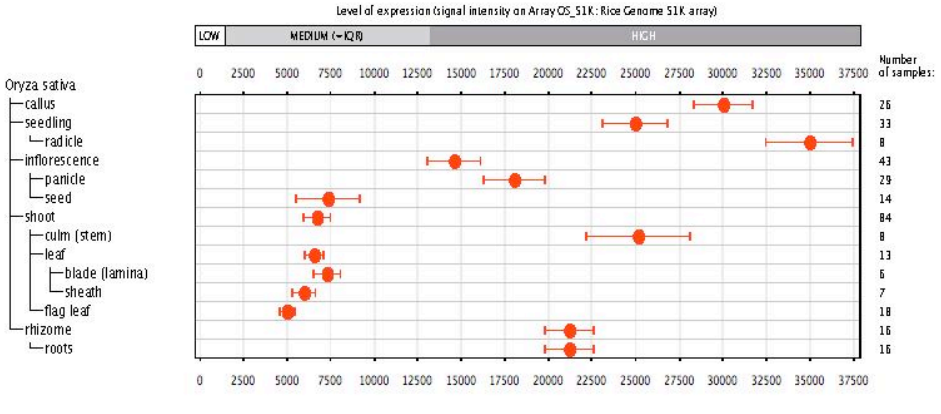



PFK 2



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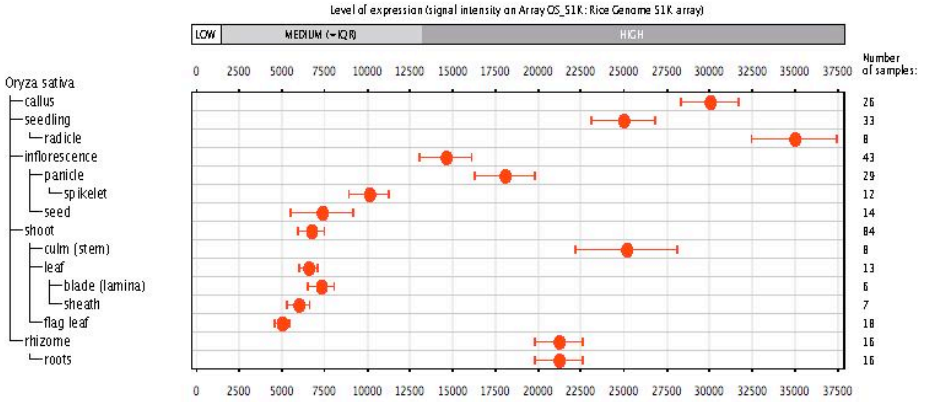
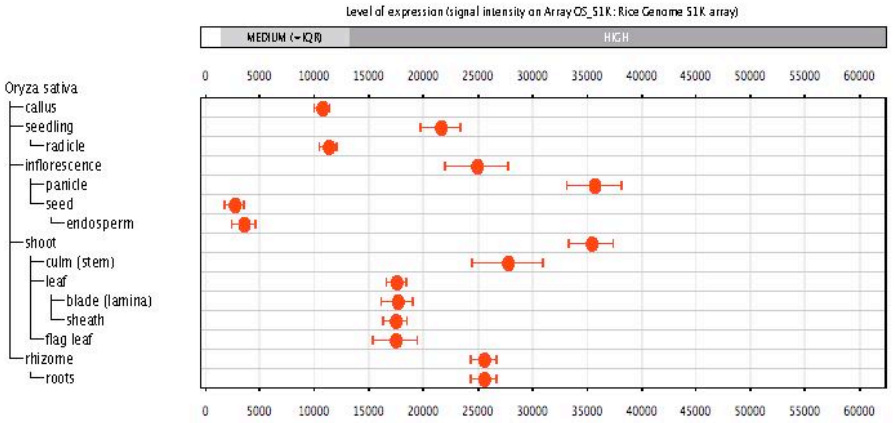
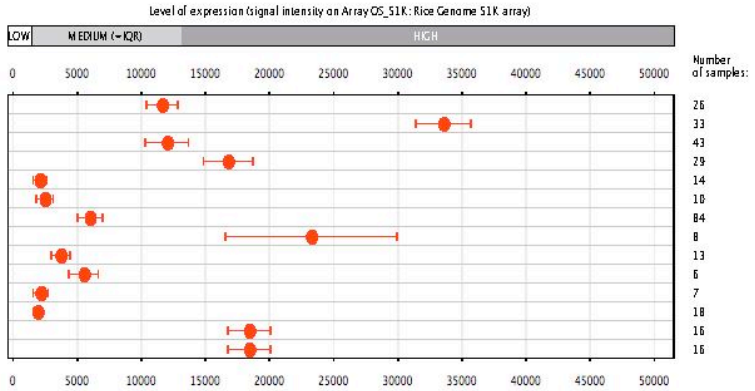



PFK 4

Appendix 2. Scatter diagrams of level of expression of PFK 1 (Os01g09570), PFK 2 (Os05g10650), PFK 3 (Os01g53680), PFK 4 (Os04g39420), in rice leaf sheaths. Scatter diagrams were obtained from https://www.genevestigator.com

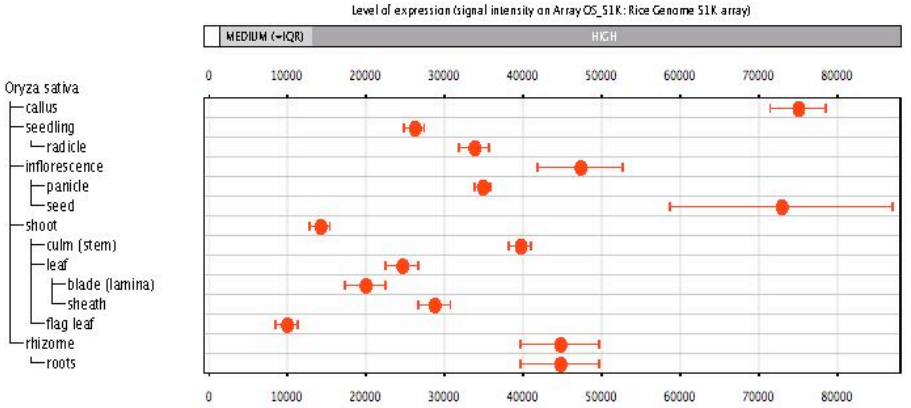



PFK 5

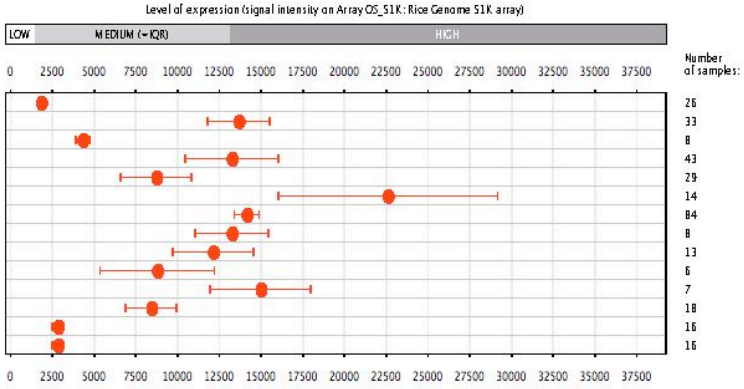



PFP 1



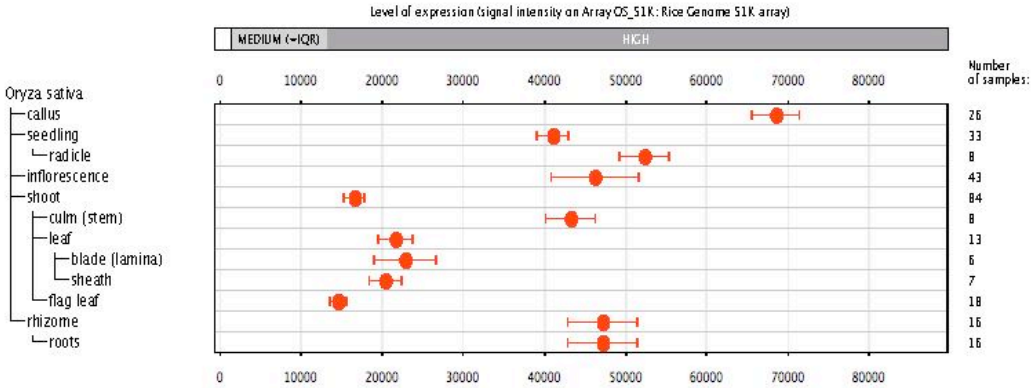



PFP 2

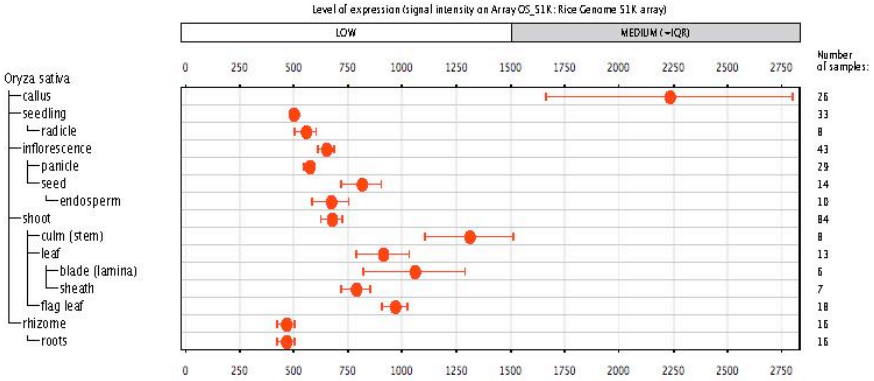



PFP 3

Appendix 3. Scatter diagrams of level of expression of PFK 5 (Os06g05860), PFP 1 (Os02g48360), PFP 2 (Os08g25720), PFP 3 (Os06g22060), in rice leaf sheaths. Scatter diagrams were obtained from https://www.genevestigator.com




PFP 5




PFP 4

Appendix 4. Scatter diagrams of level of expression of PFP 4 (Os09g12650) and PFP 5 (Os06g13810) in rice leaf sheaths. Scatter diagrams were obtained from genevestigator


Appendix 5. The time course pictures showing the disease symptoms in leaf sheaths of *R. solani*-infected rice plants. 32T-1, 32T-2, 32T-4, and 32C represents *R. solani*-infected rice plants of the resistant line at 1, 2, 4 dpi, and control plants, respectively. 29T-1, 29T-2, 29T-4 and 29C represents *R. solani*-infected rice plants of the susceptible line at 1, 2, 4 dpi, and control plants, respectively.