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Changes in the Contents of Metabolites and Enzyme Activities in Rice Plants Responding to *Rhizoctonia solani* Kuhn Infection: Activation of Glycolysis and Connection to Phenylpropanoid Pathway

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(Received October 31, 2011; Accepted March 25, 2012)

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 quantitative trait loci (QTLs), but interpretation is limited by the lack of information on the changes in metabolic pathways. Our previous studies linked activation of the 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 fructose-6-phosphate (F-6-P), fructose-1,6-bisphosphate (F-1,6-P_2), dihydroxyacetone phosphate (DHAP), glyceraldehyde-3-phosphate (GAP), 3-phosphoglycerate (3-PG), phosphoenolpyruvate (PEP) and pyruvate contents increased. These results combined with our previous findings that the expression of phosphoglucomutase (PGM), triosephosphate isomerase (TPI), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), enolase and pyruvate kinase (PK) increased after infection suggested that 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 6-phosphofructokinase (PFK) plastid isozyme *Os06g05860* was accompanied by an increase in expression of three cytosolic PFK isozymes, i.e. *Os01g09570*, *Os01g53680* and *Os04g39420*, as well as pyrophosphate-dependent phosphofrucokinase (PFP) isozymes *Os08g25720* (α-subunit) and *Os06g13810* (β-subunit) in infected rice plants of the resistant line. The results also showed that the reactions catalysed by PFK/PFP, aldolase, GAPDH + phosphoglycerate kinase (PGK) and PK in leaf sheaths of *R. solani*-infected rice plants were non-equilibrium reactions in vivo. This study showed that PGM, phosphoglucose isomerase (PGI), TPI and phosphoglycerate mutase (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.**

Keywords: Coarse and fine metabolic controls • Glycolytic pathway • Phenylpropanoid pathway • *Rhizoctonia solani* • Rice (*Oryza sativa* L.) • *R. solani*-resistant rice line.

Abbreviations: ADH, alcohol dehydrogenase; DAB, 3,3′-diaminobenzidine; DAHP synthase, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; dpi, days post-inoculation; DHAP, dihydroxyacetone phosphate; E-4-P, erythrose-4-phosphate; F-1,6-P_2, fructose-1,6-bisphosphate; F-6-P, fructose-6-phosphate; G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydroganse; GAP, glyceraldehyde-3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDH, α-glycerophosphate dehydrogenase; H_2O_2, hydrogen peroxide; HK, hexokinase; LDH, lactate dehydrogenase; OPPP, oxidative pentose phosphate pathway; PAL, phenylalanine ammonialyase; PEP, phosphoenolpyruvate; PFK, 6-phosphofructokinase; PFP, pyrophosphate-dependent phosphofrucokinase; 2-PG, 2-phosphoglycerate; 3-PG, 3-phosphoglycerate; PGK, phosphoglycerate kinase; PGM, phosphoglucomutase; PGmu, phosphoglycerate mutase; PGI, phosphoglucose isomerase; PGK, phosphoglycerate kinase; PK, pyruvate kinase; QTL, quantitative trait locus; TCA, tricarboxylic acid; TK, transketolase; TPI, triosephosphate isomerase.

Introduction

Rhizoctonia solani Kuhn is a strongly parasitic plant pathogenic fungus with a wide host range and worldwide distribution (Wasano et al, 1985, Broglie et al. 1991). In rice (*Oryza sativa* L.), it causes sheath blight disease, which is responsible for significant yield and quality loss. 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

Plant Cell Physiol. 53(6): 1017–1032 (2012) doi:10.1093/pcp/pcs047, available online at www.pcp.oxfordjournals.org

major gene resistance based on Mendelian genetics shows that a breakdown of host resistance could occur after several years owing to the 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 cross-breeding, 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 (QTLs) (Gaire et al. 2011). Resistance based on polygenic characters is desirable because it involves a self-defense mechanism and it is stable over a long time (Wasano and Dhanapala 1982). However, interpretation of polygenic QTL studies is limited by the lack of information on metabolic pathways (McMullen et al. 1998). Also, in order to utilize 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 defense mechanisms display a 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 the glycolytic pathway is activated accompanied by the activation of the phenylpropanoid pathway (Danson et al. 2000, Nose 2002, Nose et al. 2002). 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 the oxidative pentose phosphate pathway (OPPP), the tricarboxylic acid (TCA) cycle and the shikimate and phenylpropanoid pathway (Mutuku and Nose 2010). However, the low expression of genes of the non-oxidative pentose phosphate pathway raised questions about the source of erythrose-4-phosphate (E-4-P) utilized 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 (HK), PFK and PK (Faiz-ur-Rahman et al. 1974, Kubota and Ashihara, 1990).

Taken together, our previous studies (Danson et al. 2000, Nose et al. 2002, Mutuku and Nose 2010) have shown that the glycolytic pathway might be a target to identify specific defense-related enzymes for enhancement using methods such as metabolic engineering. However, since its regulation has not been investigated, it is not clear: (i) which steps might be targeted to enhance this pathway; (ii) whether regulation of this pathway may be done through coarse or fine control; and (iii) the implications of regulation of glycolysis on 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 defense response (Bolton 2009). Strategies for achieving this goal include up-regulating 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 catalyze reactions far from equilibrium can control metabolic pathways (ap Rees et al. 1977, Kubota and Ashihara 1990, Plaxton 1996). Then we examined the activation of the phenylpropanoid pathway by investigating phenylalanine ammonialyase (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, the pentose phosphate pathway, the TCA cycle, and the shikimate and phenylpropanoid pathway are as shown in **Fig. 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).

Results

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

Fig. 2 shows the time course of changes in 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), dihydroxyacetone phosphate (DHAP), glyceraldehyde-3-phosphate (GAP), 3-phosphoglycerate (3-PG), 2-phosphoglycerate (2-PG), phosphoenolpyruvate (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 days post-inoculation (dpi). This study showed that there were significant changes in metabolite 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 1–4 dpi. When the lower part of the 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.

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 with 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 1–4 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 with those of the susceptible line. E-4-P and ATP contents in the infected rice plants of both lines increased at 1–2 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 1–4 dpi while the NADH : NAD ratio and the ATP : ADP ratio increased at 1–4 dpi.

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

As shown in **Fig. 3**, the time course of changes in enzymatic activities showed that phosphoglucomutase (PGM) in the infected rice plants of the resistant line increased >1.4-fold between 1 and 4 dpi. The time course of changes in phosphoglucose isomerase (PGI), TPI and PK activities in infected rice plants of both lines appeared similar. Aldolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate mutase (PGmu) and enolase activities in the infected rice plants of the resistant line increased at 1–4 dpi and were >1.2-, 1.3-, 1.4- and 1.1-fold higher, respectively, at 4 dpi compared with 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 with 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 whereas peroxidase increased gradually at 1–4 dpi. We attempted to measure transaldolase activity in the infected rice plants, but its level 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. High aldolase, TPI, GAPDH, PGK, PGmu, enolase, TK, PAL and peroxidase activities in the infected plants of the resistant rice line were detectable 1 d after infection.

Mass–action ratios, equilibrium constant (K'), standard free energy change ($\Delta G^{0\prime}$) and free energy change (ΔG) in vivo

One of the strategies used to determine in vivo control points of metabolism is the comparison of apparent equilibrium

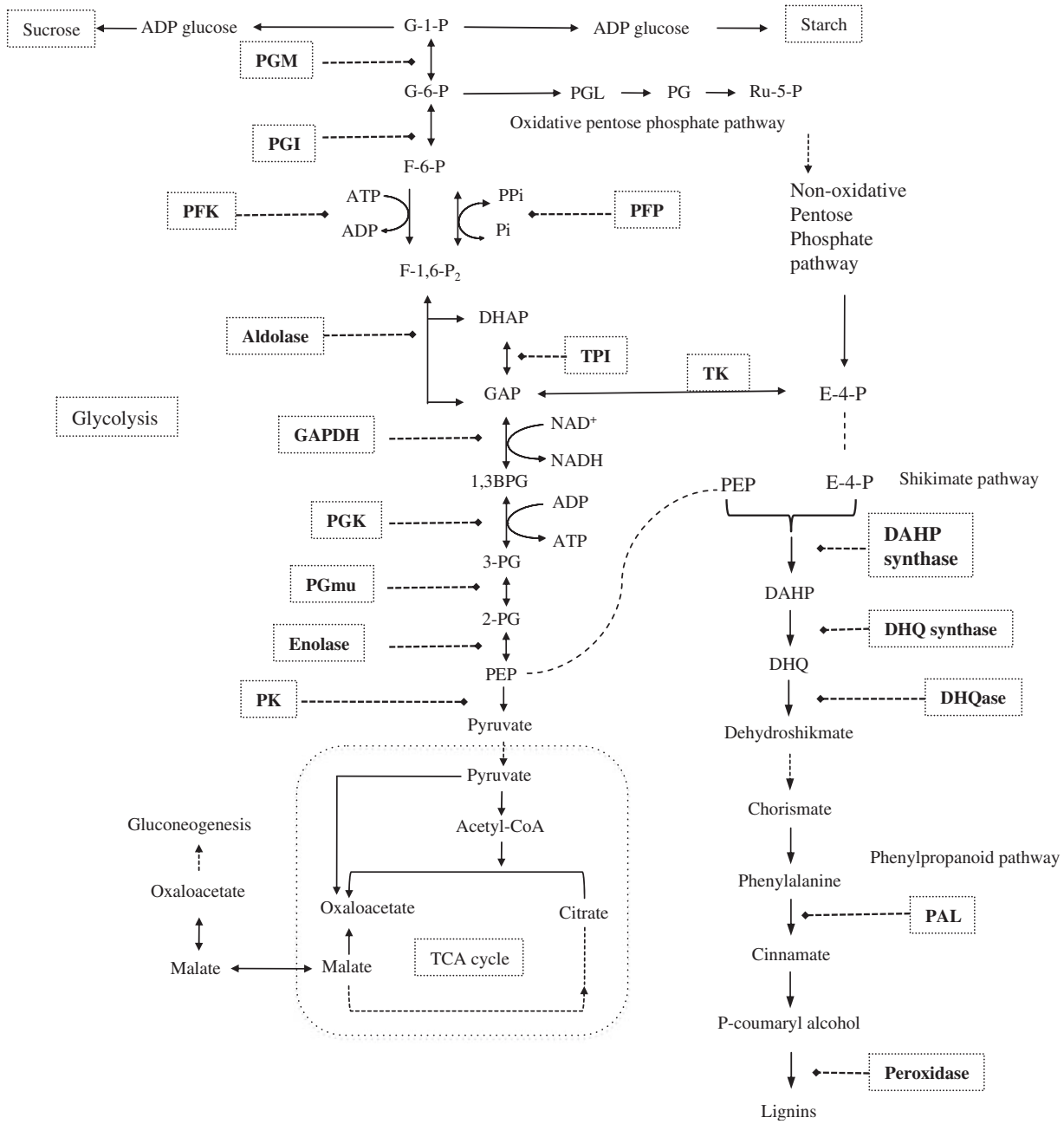

Fig. 1 Simplified diagram showing the positions of metabolites and enzymes examined in the glycolytic pathway and secondary metabolism. In rice leaf sheaths, glycolysis occurs in the plastid and it is central in the allocation of carbon resources for sucrose and starch synthesis, and the pentose phosphate, 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 of pyrophosphate-dependent phosphofrucokinase (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) and Mutuku and Nose (2010).

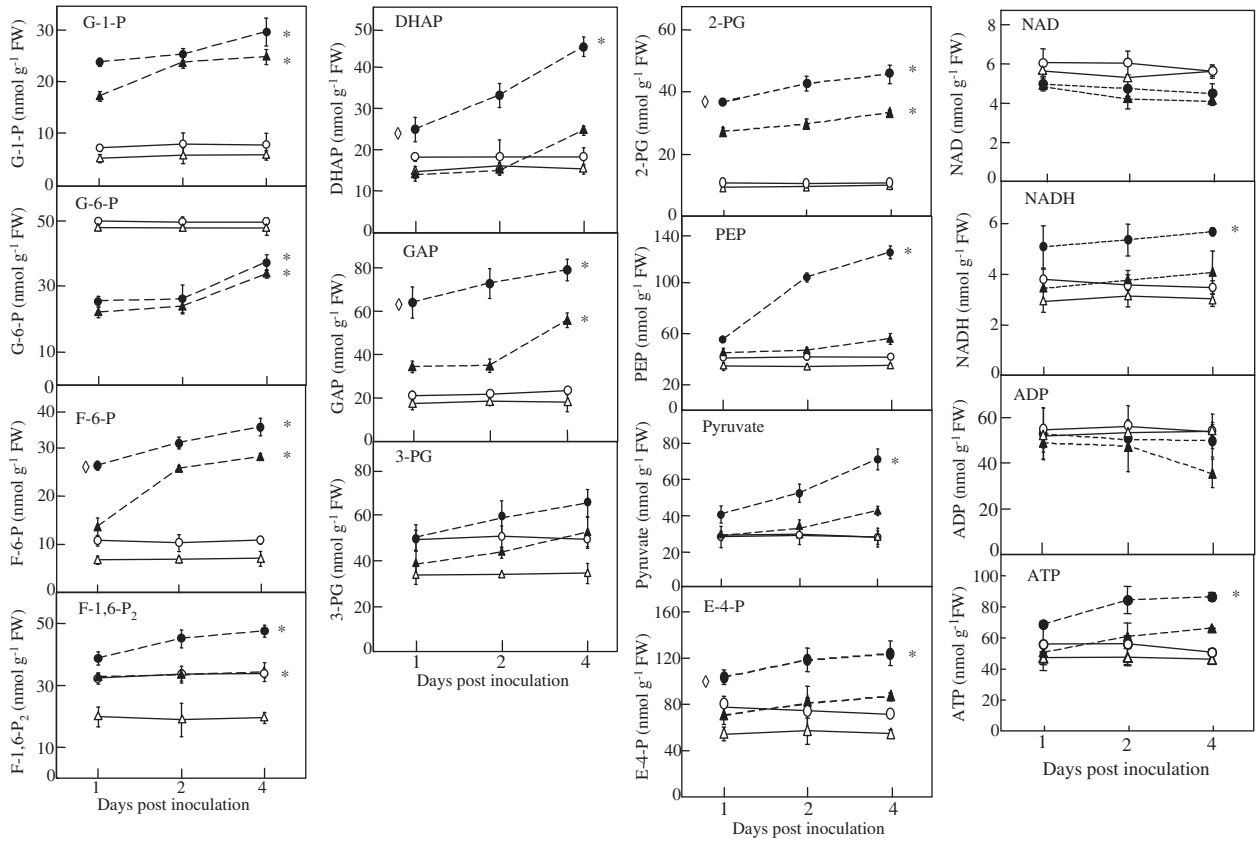

Fig. 2 Metabolite contents in leaf sheaths of *R. solani*-infected rice plants of the resistant and susceptible lines (filled circles, filled triangles) and control plants (open circles, open triangles), respectively. G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; F-1,6-P2, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; E-4-P, erythrose-4-phosphate. Values were means ± SD of four separate experiments. *The metabolite contents were significantly different ($P < 0.05$) between the infected plants and the control plants. ◇Metabolite contents were significantly different ($P < 0.05$) between the infected plants of the resistant rice line and the susceptible rice line at 1, 2 and 4 dpi.

constants (K') with mass–action ratios (Kubota and Ashihara 1990). When metabolites contents were corrected using the recovery proportions (**Table 1**), the corrected values indicated changes of between 10 and 20% in the mass–action ratios. For example, when the 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. Metabolite concentrations were obtained by assuming that 1 g FW 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 change (ΔG) 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.

Table 2 shows the mass–action ratios calculated from the estimated metabolite concentrations, $\Delta G^{0\prime}$, ΔG together with the K' cited from the references (see footnote in **Table 2**). The calculated mass–action ratios of PGM, PGI, TPI and PGmu + enolase were not far from K', while those of PFK, GAPDH + PGK and PK in the infected rice plants were >53,000-, 1,900- and 24,000-fold smaller than their respective K', indicating that the reactions were far from equilibrium in vivo (**Table 2**). In the case of the reaction catalyzed by aldolase, the calculated mass–action ratios were bigger than K' and appeared to decrease towards K' when mass–action ratios of infected plants were compared with those of control plants. The $\Delta G^{0\prime}$ values of the reactions catalyzed by PGM, PGI and aldolase were positive, whereas 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

3,3′-Diaminobenzidine (DAB) was readily taken up by the *R. solani*-infected leaf sheaths of both rice lines. Hydrogen peroxide (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 color in leaf sheaths of *R. solani*-infected rice plants of both lines (**Fig. 4A**). The use of the 'DAB-uptake method' for studying H_2O_2 in situ and in vivo requires the

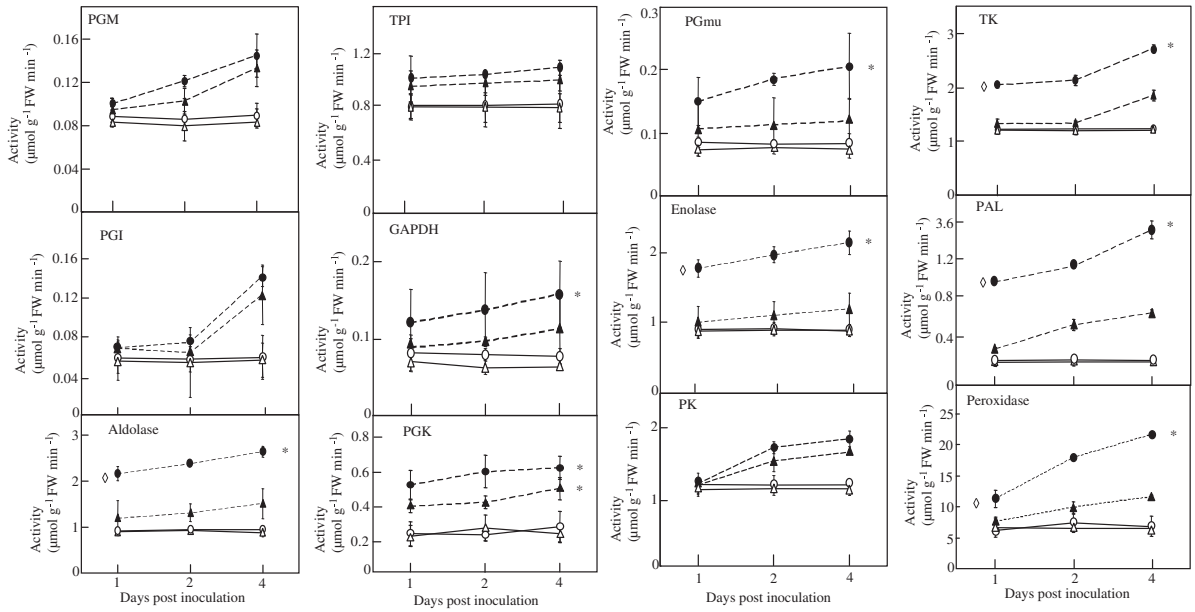

Fig. 3 Enzyme activity (μmol g^{-1} FW min^{-1}) in leaf sheaths of *R. solani*-infected plants of the resistant and the susceptible rice lines (filled circles, filled triangles) and control plants (open circles, open triangles), respectively, at 1, 2 and 4 dpi. PGM, phosphoglucomutase (EC 5.4.2.2); PGI, phosphoglucose isomerase (EC 5.3.1.9); aldolase (EC 4.1.2.13); 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); 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) and peroxidase (EC 1.11.1.14). *Activity in the infected plants was significantly different ($P < 0.05$) from that of the control plants. ◇ Activity in the infected plants of the resistant rice line was significantly different ($P < 0.05$) from that of the susceptible line. Values were means ± SD of three separate experiments.

Table 1 Recoveries of metabolites from leaf sheaths of *R. solani*-infected rice plants at 4 dpi

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

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 the Materials and Methods.

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. **Fig. 4B** 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 and 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 color faded with reducing concentration of H_2O_2.

Discussion

In our previous studies, we found out that in the interaction between rice and *R. solani*, activation of the glycolytic pathway was accompanied by the activation of the OPPP, TCA, shikimate and phenylpropanoid pathways (Danson et al. 2000, Nose et al. 2002, 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

Table 2 Mass–action ratios (MARs), calculated from estimated metabolites concentrations based on the assumption that 1 g FW of tissue is equal to 1 ml, were compared with equilibrium constants (K'), standard free energy change ($\Delta G^{0\prime}$) and free-energy change in vivo (ΔG) 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), glyceraldehyde-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 leaf sheaths of *R. solani*-infected (T) and control (C) plants of the resistant (32) and the susceptible (29) rice lines at 1, 2 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
	$\Delta G^{0\prime} = 7.42$	ΔG	−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
	$\Delta G^{0\prime} = 2.53$	ΔG	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
	$\Delta G^{0\prime} = 16.88$	ΔG	−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	$K' = 6.7 \times 10^{-5}$	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
	$\Delta G^{0\prime} = 23.81$	ΔG	−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
	$\Delta G^{0\prime} = -7.66$	ΔG	−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	$K' = 1{,}550$	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
	$\Delta G^{0\prime} = -18.20$	ΔG	−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
	$\Delta G^{0\prime} = 1.72$	ΔG	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
	$\Delta G^{0\prime} = -18.83$	ΔG	−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 **Fig. 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 ($\Delta G^{0\prime}$) was calculated from $\Delta G^{0\prime} = -RT \times \ln K'$. Free energy change in vivo was calculated from $\Delta G = \Delta G^{0\prime} + RT \times \ln I$, Where I is mass–action ratio, R is 8.314 J mol^{-1} K^{-1} and T is 298 K.

showed that the expression 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 expression 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 an increase in expression of the PFK plastid isozyme, i.e. *Os06g05860*, was accompanied by an increase in expression of three cytosolic PFK isozymes, i.e. *Os01g09570*, *Os01g53680* and *Os04g39420*, in infected rice plants of the resistant line. Furthermore, the increase in expression of these PFK isozymes localized in plastid and cytosol compartments was strongly correlated with the increase in PFK activity in the same plants. Moreover, the increase in pyrophosphate-dependent phosphofrucokinase (PFP) activity in the cytosol was strongly correlated with the increase in expression of two isozymes, i.e. *Os08g25720* (α-subunit) and *Os06g13810* (β-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) and that induction of glycolysis in the cytosol compartment occurs to facilitate plant acclimation to environmental stress (Mertens 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 catalyzed by PGM, PGI, TPI and PGmu + enolase in infected rice plants were near equilibrium in vivo (**Table 2**). Although it is not an invariable rule, enzymes catalyzing near-equilibrium reactions are unlikely to produce large effects on metabolite concentrations (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 catalyzed 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 favor of GAP.

GAP can be utilized in the generation of E-4-P by TK. E-4-P can also be generated in the non-oxidative pentose phosphate pathway 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

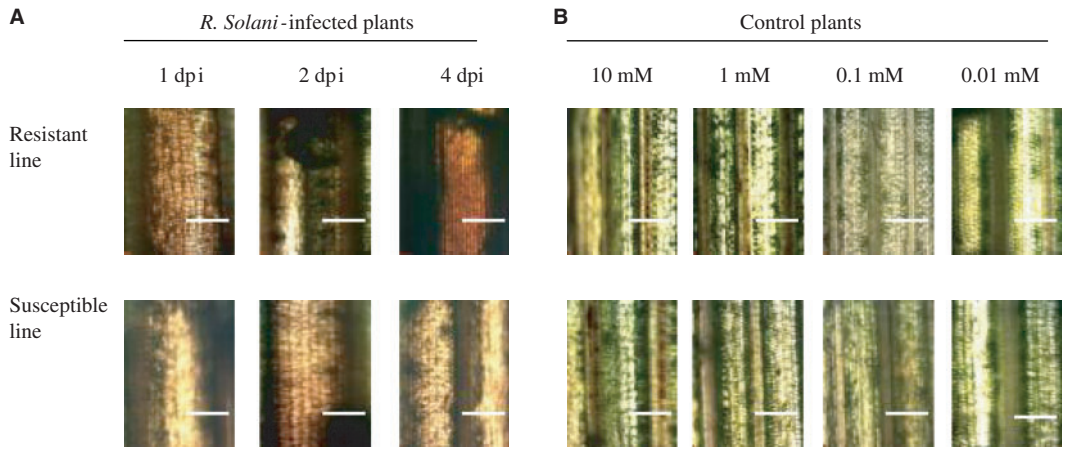

Fig. 4 (A) Take up and polymerization of DAB is confirmed by the dark staining (reddish-brown) reaction in the rice leaf sheaths of the *R. solani*-infected plants of the resistant and susceptible lines at 1, 2 and 4 dpi. (B) In vivo DAB reaction controls. Sensitivity of staining for peroxidase activity 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 X-100 (supplied with H_2O_2 to either 10, 1.0, 0.1 or 0.01 mM) for 10 min at room temperature. Notice the dark staining (reddish-brown) became lighter as the concentration of H_2O_2 was reduced from 10 to 0.01 mM. Magnification × 100 and scale bars = 5 mm.

TK and E-4-P was taken as showing that E-4-P was obtained from GAP by the action of TK. 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 the K'. The aldolase reaction was reported to have a small ΔG 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 2** showed that the ΔG 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 catalyzed 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 (**Table 2**).

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 indicating that the negative ΔG values showed that the forward reactions of PFK, GAPDH, PGK and PK were spontaneous were interpreted as showing that the reactions catalyzed by these enzymes were not restricted.

GAPDH exists in plants in three different forms, the NAD$^+$-specific, NADP$^+$-specific and non-phosphorylating forms (Blakeley and Dennis 1993). We examined the non-phosphorylating NAD$^+$-dependent GAPDH reaction whose mass–action ratios can be determined as shown in the Results. The apparent equilibrium constant of the reaction catalyzed 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) and Hirose et al. (2006) show that rice leaf sheaths have no photosynthetic capacity so NADP$^+$-GAPDH was not considered in this study. Also, in the last step of glycolysis, in addition to the PK reaction, there are two other possible reactions for the formation of pyruvate from PEP. These reactions are catalyzed by PEP phosphatase (EC 3.1.3.60) and a non-specific phosphatase (Nagano and Ashihara 1993). PK is the major enzyme for glycolysis (Sung et al. 1988), although reports by Ukaji and Ashihara (1987), Duff et al. (1989b) and Nagano and Ashihara (1993) suggest that PEP phosphatase activity might be adaptive to stress conditions such as Pi starvation. In this study, PK activity was determined by measuring the decrease in absorbance at 340 nm caused by a decrease in NADH in the following reaction;

$$\text{PEP} + \text{ADP} \rightarrow \text{Pyruvate} + \text{ATP} \text{ (by PK)};$$
$$\text{Pyruvate} + \text{NADH} \rightarrow \text{Lactate} + \text{NAD} \text{ [by lactate dehydrogenase (LDH)]}.$$

Although it is possible to speculate that this measurement of PK activity might have included PEP phosphatase activity, which can be determined as in the following reaction,

$$\text{PEP} \rightarrow \text{Pyruvate} + \text{Pi} \text{ (by PEP phosphatase)};$$
$$\text{Pyruvate} + \text{NADH} \rightarrow \text{Lactate} + \text{NAD} \text{ (by LDH)}$$

it was not possible to distinguish between them because of complications arising from lack of data on localization, kinetics and substrate specificity of PEP phosphatase and the non-specific phosphatase. For example, the activities of these enzymes appear to be located in vacuoles and the alternative reactions they catalyzed are hypothesized to be possible only if vacuoles are involved as sites of reactions involved in glycolysis, although there is no evidence of such involvement (Nagano and Ashihara 1993).

PGM, TPI, GAPDH, enolase and PK activities in leaf sheaths of *R. solani*-infected rice plants were compared with the expression 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. 5**). These increases in enzymatic activities and gene expression suggested that coarse metabolic control was exerted on 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 catalyzed 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 on the reactions catalyzed by PGM, PGI, TPI and PGmu + enolase, whereas a combination of both coarse and fine controls was exerted to the reactions catalyzed by PFK/PFP, aldolase, GAPDH + PGK and PK (**Table 3**).

The results showed that the increase in the levels of enzymatic activities, gene expression and metabolite contents suggested that additional establishment of glycolysis in the cytosol occurred after infection in the resistant line. Furthermore, coarse control was exerted on the reactions catalyzed by PGM, PGI, TPI and PGmu + enolase, whereas a combination of both coarse and fine controls was exerted on the reactions catalyzed 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 an increase in the generation of GAP, which was utilized in the generation of E-4-P by TK and in downstream 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 $>20\ \mu M$ inhibit its activity. Nevertheless, studies in developing endosperm of rice grain showed that PFK activity was not inhibited by $0.18\ \mu mol\ g^{-1}$ FW PEP content (Nakamura et al. 1989), which was more than the $140\ nmol\ g^{-1}$ FW PEP content we observed in the current study (**Fig. 2**). Moreover, findings by ap Rees et al. (1977) suggest that an increase in PFK activity is also accompanied by high PEP content. These reports suggest that a 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 (i) *R. solani* infection in rice leads to an increase in PEP contents; and (ii) $140\ nmol\ g^{-1}$ 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 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (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. 4A**). 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 varying 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 concentrations of H_2O_2 from 'strong' to 'not visible' (**Fig. 4B**). These changes in the DAB reactions reflected an 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 may be linked to increased lignification as a first line of defense 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 catalyzed 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 favor 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

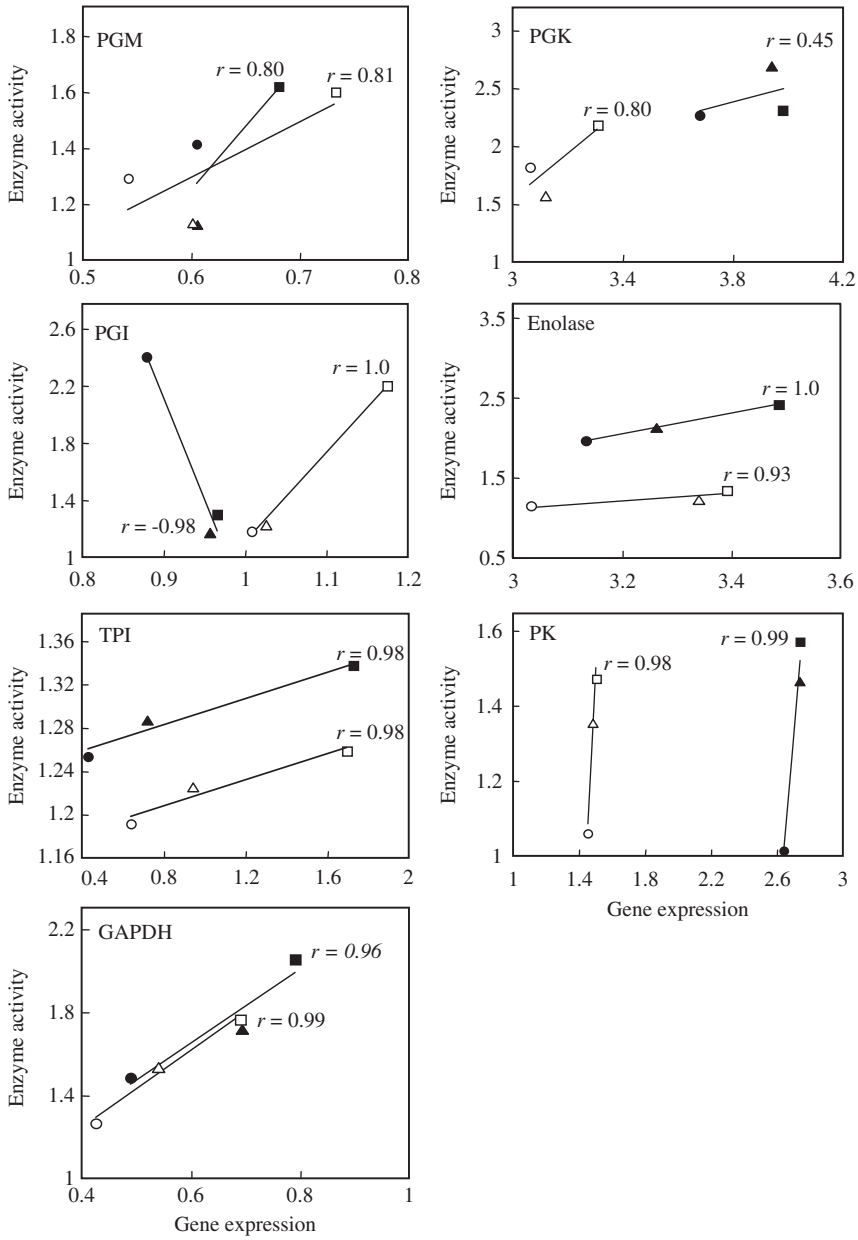

Fig. 5 The correlation between gene expression and enzyme activity in leaf sheaths of *R. solani*-infected rice plants of the resistant line at 1, 2 and 4 dpi (filled circles, filled triangles, filled squares), respectively, and the susceptible line at 1, 2 and 4 dpi (open circles, open triangles, open squares), respectively. PGM, phosphoglucomutase (EC 5.4.2.2); PGI, phosphoglucose isomerase (EC 5.3.1.9); TPI, 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); 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. Gene expression data were obtained from Mutuku and Nose (2010).

single steps (Capell and Christou 2004). Moreover, at branch points such as those between GAP and E-4-P, and E-4-P and PEP to secondary metabolism, overexpression of TK and DAHP synthase, respectively, to out-compete other enzymes using the same substrate could divert flux into appropriate pathways such as those leading to lignin generation (**Fig. 6**).

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 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 (i) metabolic regulation of plant glycolysis is controlled by enzymes that catalyze regulatory reactions and the mechanisms whereby their activities are controlled in vivo (Blakeley and Dennis 1993, ap Rees and Hill 1994, Plaxton 1996); and (ii) 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).

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 $\approx K'$
PGI	Coarse[a]	MAR $\approx K'$
PFK/PFP	Coarse and fine	MAR $< K'$
Aldolase	Coarse and fine[b]	MAR $> K'$
TPI	Coarse	MAR $\approx K'$
GAPDH + PGK	Coarse and fine	MAR $< K'$
PGmu + enolase	Coarse[c]	MAR $\approx K'$
PK	Coarse and fine	MAR $< K'$

[a]Expression data not available for PGI.
[b]Expression data not available for aldolase.
[c]PGmu + enolase relies on expression data of enolase.

The increase in enzymatic activities and metabolite contents suggested that establishment of alternative glycolysis in the cytosol occurred after infection. Changes in metabolites were utilized 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 utilized the data to investigate regulation. The current studies therefore show a useful way to utilize metabolome data to study regulation.

The results of this study showed that in most of the cases, changes in metabolite 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 time lines such as 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

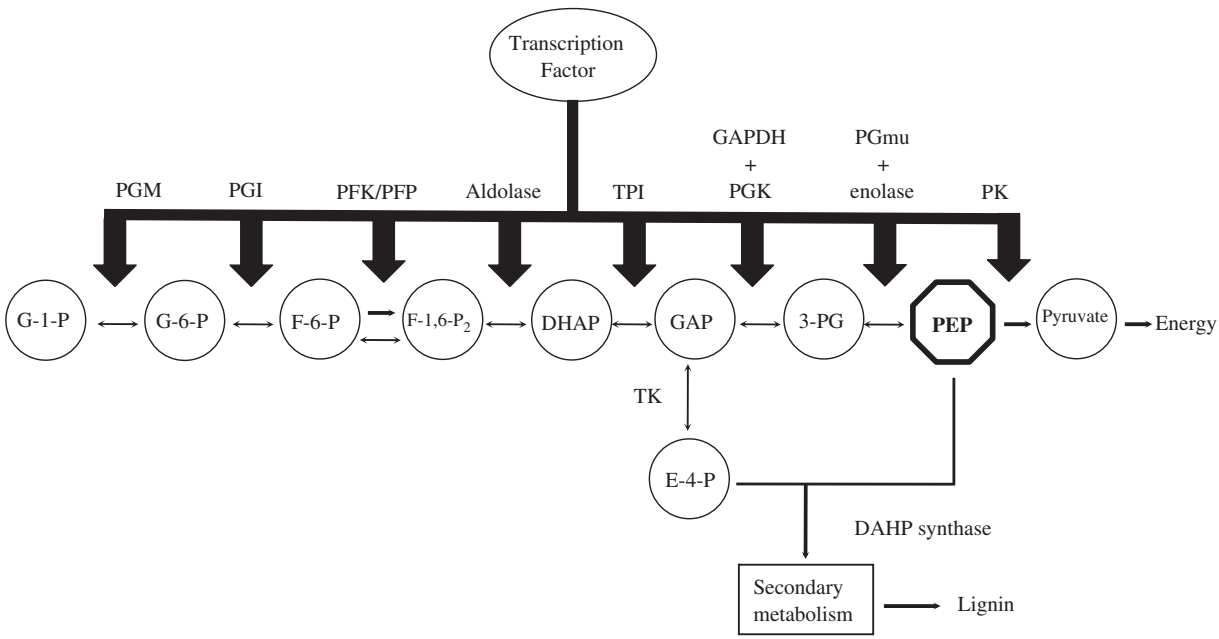

Fig. 6 A strategy for metabolic engineering in rice plants. Instead of modulating individual steps, metabolic engineering would probably involve approaches in which multiple steps are targeted simultaneously. In this figure, in the pathway to the desired compound PEP (phosphoenolpyruvate) during resistance response against disease stress, a transcription factor is being used to up-regulate several enzymes coordinately, PGM, PGI, PFK/PFP, aldolase, TPI, GAPDH + PGK and PGmu + enolase. An increase in the flux through the glycolytic pathway could be achieved by modifying the reversible reactions catalyzed by PGM, PGI, aldolase and PGmu + enolase to favor unidirectional catalysis. The TPI reaction could be modulated to favor generation of GAP during the resistance response. At branch points such as those between GAP to E-4-P, E-4-P and PEP to secondary metabolism, and PEP to pyruvate overexpression of the key enzymes, TK, DAHP synthase and PK to outcompete other enzymes using the same substrate could divert flux into appropriate pathways. PGM, phosphoglucomutase; PGI, phosphoglucoisomerase PFP, pyrophosphate-dependent phosphofrucokinase; PFK, phosphofructokinase; 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.

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 the timing and magnitude of the defense response.

Due to the flexible structure of plant glycolysis, and its variation due to the external environment, we propose that further investigations into fine control of glycolysis and how it is affected by compartmentation and alternative parallel reactions should be conducted. For example, PEP phosphatase and a non-specific phosphatase are reported to catalyze alternative parallel reactions to that of PK, although their localization, kinetics and substrate specificity are still unknown (Sung et al. 1988, Duff et al. 1989a). We propose that these be investigated especially to determine whether PEP phosphatase has an adaptive role during sheath blight disease stress acclimation similar to PFP (Mutuku and Nose 2012). Further investigations should also examine isozymes because recent studies where the expression of PFP/PFK isozymes increased showed that not all of them are involved in defense 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 conducted.

Materials and Methods

Plant samples and fungus preparations were done exactly as described by 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 10:00 h, 1, 2 and 4 dpi, ground in liquid nitrogen and stored at −80°C until use. The plant materials used in the current studies were the same as those used in our previous studies (Mutuku and Nose 2010).

Extraction and determination of metabolites

All commercially available enzymes used for determination of metabolites were obtained from Sigma, and their sources were as follows: aldolase, α-glycerophosphate dehydrogenase (GDH), LDH, TPI, PGM and PK (rabbit muscle); alcohol dehydrogenase (ADH), enolase, glucose-6-phosphate dehydroganse (G6PDH), GAPDH, HK, PGK, PGI and transaldolase (baker's yeast); myokinase (chicken muscle).

Metabolites were extracted according to 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 a 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.) at 4°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°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°C, after which it was used immediately for determinations of metabolites using a Jasco V-550 UV/VIS spectrophotometer (Jasco Corporation).

G-6-P, G-1-P and 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 U ml^{-1} of G6PDH, 1.0 U ml^{-1} PGM and 1.0 U ml^{-1} PGI 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°C.

F-1,6-P_2, DHAP and 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 GAPDH, 5.0 U ml^{-1} TPI 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°C.

3-PG

3-PG was measured in a 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 PGK and GAPDH was added. 3-PG (nmol g^{-1} FW) was determined as the difference between the values before and after addition of the enzymes.

2-PG, 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} 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} 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 20 U ml^{-1} enolase in the PEP reaction above (Lamprecht and Heinz 1984).

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 µl of extract, 25 mM glycylglycine pH 7.4, 0.12 mM NADH, 0.3 mM F-6-P. After the optical density was measured at 340 nm (A1), 23 U ml^{-1} TPI and 2 U ml^{-1} GDH were added and the reaction was let to run for 8 min to obtain A2. After 1.0 U 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.

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 to 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 ADB (final concentration, 40 µg ml^{-1}). After placing the cuvette containing the reaction mixture in a UV-visible spectrophotometer for measurement at 570 nm, 40 µl of 80% ethanol was added to start the reaction.

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 µl of 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 and 0.7 U ml^{-1} PGI. After 2 min incubation, 0.6 U ml^{-1} HK was added. ADP was measured in a volume of 1 ml containing 100 µl of extract plus 100 mM Tris–HCl (pH 8.1), 2 mM $MgCl_2$, 10 mM KCl, 60 µM NADH, 1.5 mM PEP and 2.5 U ml^{-1} LDH. After 2 min incubation, 4 U ml^{-1} PK and 15 U ml^{-1} myokinase were added.

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) were added to the pestle and mortar and pulverized with the leaf sheath tissue. **Table 1** shows that 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.

Enzyme assays

Rice leaf sheath tissues (0.5 g) was prepared as above and pulverized in 5 vols. of extraction buffer (100 mM Tris–HC1, pH 8.0; 2 mM $MgCl_2$; 1 mM EDTA; 28 mM β-mercaptoethanol; 1 mM phenylmethylsulfonyl fluoride) together with 250 mg of polyvinyl polypyrrolidone using a pestle and mortar chilled in liquid nitrogen. The mixture was then centrifuged for 10 min at 10,000 × *g* at 4°C and the supernatant was assayed at once at 25°C for 20 min in the following 1.0 ml reaction mixtures according to the accompanying references: PGM (EC 5.4.2.2), 50 mM HEPES (pH 7.6), 1 mM $MgCl_2$, 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); PGI (EC 5.3.1.9), 75 mM glycylglycine (pH 8.5), 10 mM $MgCl_2$, 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); TPI (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); GAPDH (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); PGK (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); PGmu (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); PK (EC 2.7.1.40), 50 mM 3-(*N*-morpholino) 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). Assays with a minus ADP control were not conducted. 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. TK (EC 2.2.1.1) was measured in a manner similar to transaldolase except 20 µM 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).

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 µl 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 Tris–HCl buffer pH 8, 200 µl of enzyme preparation and 6 µM 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 µl of 5 N HCl. The amount of trans-cinnamic acid formed was determined by measuring the absorbance at 290 nm.

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 µl of the supernatant extract to 999.5 µl of the reaction mixture. Activity was measured as a change in the absorbance at 470 nm.

H_2O_2 detection by the 'DAB-uptake method'

Rhizoctonia solani-infected rice leaf sheaths from both resistant and susceptible lines were cut and placed in 1 mg ml^{-1} DAB-HCl pH 3.8 (Sigma) 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) was 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).

Peroxidase detection

In situ peroxidase activity was detected in rice leaf sheaths following pre-treatment 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 and 0.01 mM and incubation was performed by mild agitation at room temperature for 10 min.

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.

Funding

This work was supported by the Japan Ministry of Education, Culture, Sports, Science and Technology [Grants-in-Aid for Scientific Research (grant No. MK 20580016) 2009–2011]; United Graduate School of Agricultural Sciences, Kagoshima University, Japan [The Rendai-Student Supporting Program 2009–2011].

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