European Journal of Plant Pathology **106:** 555–561, 2000.

Infection of rice plants with the sheath blight fungus causes an activation of pentose phosphate and glycolytic pathways

Jedidah Danson, Kikuo Wasano and Akihiro Nose*
Faculty of Agriculture, Saga University, 1 Honjo, Saga 840-8502, Japan;
**Author for correspondence (Fax: +81-952-288737; E-mail: nosea@cc.saga-u.ac.jp)*

Accepted 5 April 2000

Key words: Rhizoctonia solani, sheath blight disease, glycolysis, pentose phosphate pathway

Abstract

The response of key regulatory enzymes of the pentose phosphate and glycolytic pathways in disease development was assessed in genetically-related rice plants resistant and susceptible to the sheath blight fungus, *Rhizoctonia solani*. The plants were grown and maintained under greenhouse conditions and inoculated at 50% flowering. Uninoculated healthy plants served as controls. The activities of pentose phosphate pathway enzymes (glucose-6 phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) increased more than two-fold in both the resistant and susceptible plants. Activities of ATP- and pyrophosphate-dependent phosphofructokinase and phosphoenolpyruvate phosphatase increased in infected plants while activity of phosphoenolpyruvate carboxylase in infected plants was lower than in the healthy plants. Furthermore, for enzymes with increased activity, the levels were higher in the resistant line than in the susceptible line. The enhancement of the enzyme activities correlated well with the post infection period. These data suggest that altered carbohydrate metabolism in sheath blight infections may play an important role in modulating the rice plant's response to infection. The isolation of an infection-induced gene encoding a basic enzyme of pentose phosphate and glycolytic pathways could be used to develop plants with more resistance towards sheath blight disease.

Introduction

The breeding of pest-resistant cultivars is one of the primary objectives of many rice improvement programmmes worldwide (Khush and Virmani, 1985). Among the most widespread and important diseases of irrigated rice is sheath blight caused by the fungus *Rhizoctonia solani* (Bonman et al., 1992). Rice breeders have successfully identified the source of resistance in cultivated rice germplasm and incorporated genes for resistance through conventional hybridization. However, major gene(s) linked to high resistance of rice plants to sheath blight disease have not been found (Li et al., 1995) and only partial resistance has been reported (Bonman et al., 1992; Xie et al., 1992).

Plants have the ability to acquire an enhanced level of resistance to pathogen attack after being exposed to specific biotic stimuli (Pieterse, 1998). In plant resistance, carbohydrate metabolism plays an important role in plant gene responses (Koch, 1996). Previous results showed alterations in carbohydrate metabolism during infection of rice plants with the sheath blight fungus (Danson et al., 1999). The enzymatic interface of secondary metabolism with the carbohydrate metabolism is provided by the shikimate pathway, which condenses erythrose-4-phosphate and phosphoenolpyruvate of the pentose phosphate and glycolytic pathways, respectively, to produce substrates for the aromatic amino acids synthesis. Of these amino acids, phenylalanine and tyrosine are needed as precursors for the synthesis of lignin. Early defense responses by plants to infection have been linked to the lignification of infected cells (Bell, 1981). It follows that lignin formation may involve the interaction of

several metabolic pathways such as the pentose phosphate, glycolytic and shikimate pathways (Hrazdina, 1994).

A response in the key enzymes of pentose phosphate pathway and glycolysis during infection would reflect an increased demand for NADPH and ATP, respectively. Therefore genes encoding for enzymes within those pathways may provide a better understanding of how the pathways and resistance mechanisms interact. The aim of this work was to investigate changes in patterns of metabolism that occur during infection by looking at the key enzymes within the pentose phosphate and glycolytic pathways in relation to sheath blight fungus infection in rice plants.

Materials and methods

Two rice lines from a 16th generation hybrid population cross of Tetep × CA-4-39-21 (Wasano K, Faculty of Agriculture, Saga University, unpublished) were selected for resistance (3F16-15) and susceptibility (3F16-10) to sheath blight disease. The seeds obtained from these plants were sterilized in 70% ethanol for 2–3 min and 1% sodium hypochlorite for 10 min prior to soaking in water (24 h) and then sowed in pots containing commercially produced compost. One seedling was planted in each pot in a randomized block design with two replicates. NPK (14 : 16 : 14) 5 kg/10a served as the basal fertilizer while, NPK (16 : 0 : 16) 5 kg/10a was applied as a top dressing. The rice plants were maintained under standard greenhouse conditions throughout the growing period.

Inoculation of the rice plants with sheath blight fungus (maintained on nutrient agar at 28 °C) was conducted essentially as described by Wasano et al. (1983). Uninoculated plants served as healthy controls. The plants were inoculated at a time when the plants exhibited 50% flowering. Sampling commenced immediately after inoculation and after 24, 48, 96 and 192 h. The lesion length was determined by measuring the length of the whole infected third leaf sheath area of at least 10 sheaths from each of the two pots (total 20 sheaths) sampled per replicate and averaged.

The sheaths used in determining the infected area were preserved for enzyme assays. The samples of infected and healthy sheaths were collected at 10 a.m. Lesion *per se* and their border areas were also excised to include about 10 cm of the sheaths. The samples were immediately placed in liquid nitrogen and stored at −80 °C until used for enzyme extraction. Non-inoculated plants served as a control.

Extracts were prepared as follows: the leaf sheaths were cut into small pieces and weighed. Sea sand (500 mg) and polyvinylpolypyrrolidone (50 mg) were added to 1 g (fresh weight) of cut leaf sheaths in 5 ml of extraction buffer (100 mM Hepes pH 7.5, 1 mM EDTA, 5 mM dithiothreitol, 0.5% triton X-100, 20% glycerol and 0.5% BSA). The samples were homogenized by grinding with a pestle in an ice-chilled mortar and the homogenate was filtered through one layer of Mira-cloth (Calbiochem-Novabiochem, La Jolla, USA). The homogenate was centrifuged for 10 min at 38,000*g* at 4 °C and the supernatant desalted (25 mM Hepes pH 7.5, 2.5 mM dithiothreitol) by passage through a 5-ml Sephadex G-25 column (Hitrap Desalting, Pharmacia Biotech, Sweden). The desalted material was used as the crude enzyme source.

Assay conditions were as described in the following references except for the variations given: glucose-6-phosphate dehydrogenase (Tecsi et al.,1994); 6-phosphogluconate dehydrogenase (Tecsi et al.,1994); ATP-dependent phosphofructokinase: 50 mM Tris–HCl (pH 8.0) (Plaxton, 1990); pyrophosphate-dependent phosphofructokinase: 50 mM Tris–HCl (pH 8.0) (Plaxton, 1990); phosphoenolpyruvate phosphatase: 50 mM Hepes (pH 7.2), 2 U lactate dehydrogenase (Plaxton, 1990); Pyruvate kinase: 50 mM Hepes (pH 7.2), 10 mM $MgCl_2$, 5 mm KCl, 0.2 mM NADH, 1 mM ADP, 2 U lactate dehydrogenase and 2 mM phosphoenolpyruvate; phosphoenolpyruvate carboxylase: 100 mM Tris–HCl (pH 7.6) (Schuller et al.,1990).

All assays were conducted at 30 °C in a final volume of 1 ml. Changes in NADH or NADP levels were assessed at 340 nm using a spectrophotometer (UV 160, Shimadzu, Japan).

Results

Several key enzymes involved in carbohydrate metabolism (glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, ATP-dependent phosphofructokinase, pyrophosphate-dependent phosphofructokinase, phosphoenolpyruvate carboxylase, phosphoenolpyruvate phosphatase and pyruvate kinase) as well as development of pathogenic lesion were analyzed in the genetically related resistant and susceptible rice selection lines compatible with the sheath blight fungus. The results indicated significant

Table 1. Lesion development and relative activity of the key enzymes of glycolysis and pentose phosphate pathway in rice plants infected with the sheath blight fungus

Time (h)	Lesion size (mm)		Relative Activity											
	R	S	G6PD		6PDH		ATP-PFK		PPi-PFK		PEPase		PEPC	
			R	S	R	S	R	S	R	S	R	S	R	S
0	0	0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.96	1.00
24	13.36 ± 1.74	11.26 ± 0.34	1.39	1.38	1.52	1.41	3.64	3.17	2.33	3.07	1.56	1.25	0.47	0.89
48	14.74 ± 0.72	71.68 ± 1.29	1.69	1.30	2.06	1.83	3.33	3.47	2.67	3.26	1.71	1.45	0.28	0.52
96	21.14 ± 0.36	83.74 ± 2.16	1.99	1.27	2.38	1.90	5.35	3.78	4.21	2.89	1.55	1.97	0.06	0.18
192	25.28 ± 1.52	98.58 ± 3.13	2.14	1.39	2.76	1.97	5.24	5.03	5.23	3.29	1.56	2.17	0.05	0.07

R: Resistant rice line (3F16-15), S: susceptible rice line (3F16-10), G6PD: glucose-6-phosphate dehydrogenase, 6PDH: 6-phosphogluconate dehydrogenase, ATP-PFK: ATP-dependent phosphofructokinase, PPi-PFK: pyrophosphate-dependent phosphofructokinase, PEPase: phosphoenolpyruvate phosphatase, PEPC: phosphoenolpyruvate carboxylase.

differences during post infection. Although phosphoenolpyruvate carboxylase is not a glycolytic enzyme, Plaxton (1996) called the pathway to pyruvate via phosphoenolpyruvate carboxylase reaction as a 'alternative route of glycolysis'. Thus phosphoenolpyruvate carboxylase will be considered among the glycolytic enzymes in the results presented here.

Lesion development in infected sheaths of both the susceptible and resistant lines was initially similar at 24 h (Table 1). However, by 48 h large differences in lesion size were observed. Lesion size in the susceptible line increased 6- and 9-fold by 48 and 192 h, respectively. Overall, lesion development in the resistant line was slow and not significantly different between 24 and 192 h.

Samples used in the measurement for lesion development were further used for enzyme analysis of the pentose phosphate and glycolytic pathways. By 24 h, two enzymes of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, were induced when compared to the uninfected control plants (Figure 1). Enzyme induction at 24 h for glucose-6-phosphate dehydrogenase was similar in both lines. However, by 48 h the increase in activity seen in the resistant line was greater than the increase observed in the susceptible line. A similar observation was noted for 6-phosphogluconate dehydrogenase. The two rice lines exhibited large differences in enzyme activities in relation to infection in later stages. By 192 h, both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities in the resistant line had doubled and tripled which was slightly more than in the susceptible line. The difference in enzyme activity between the resistant and susceptible line was significant at 96 and 192 h for glucose-6-phosphate dehydrogenase and 48, 96 and 192 h for 6-phosphogluconate dehydrogenase, respectively. Infection seems to have had an effect on the gradual increase in enzyme activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase as the levels of these enzymes in the healthy control plants remained unchanged for both lines.

Among the four glycolytic enzymes assayed, ATP- and pyrophosphate-dependent phosphofructokinase and phosphoenolpyruvate phosphatase activities increased after infection. The ATP- and pyrophosphate-dependent phosphofructokinase activities more than doubled 24 h after infection (Figure 2) in both lines and were similarly enhanced after 48 h. Thereafter, the increase in the resistant line was more than the susceptible line. At the end of the sampling period, both ATP- and pyrophosphate-dependent phosphofructokinase had increased 5-fold in the resistant line, and by about 3-fold in the susceptible line. The difference in activity between the resistant line and susceptible line was significant at 96 and 192 h for ATP-dependent phosphofructokinase, while there was no significant difference for pyrophosphate-dependent phosphofructokinase at any of the sampling times. In the healthy controls of both lines, the two enzyme activities were barely detected. Omitting ADP in the pyruvate kinase assay to determine phosphoenolpyruvate phosphatase contamination indicated very low activity for pyruvate kinase, therefore only the results for phosphoenolpyruvate phosphatase are reported. The increase in phosphoenolpyruvate phosphatase occurred early after plant infection (24 h) and continued until the end of the sampling period. In contrast,

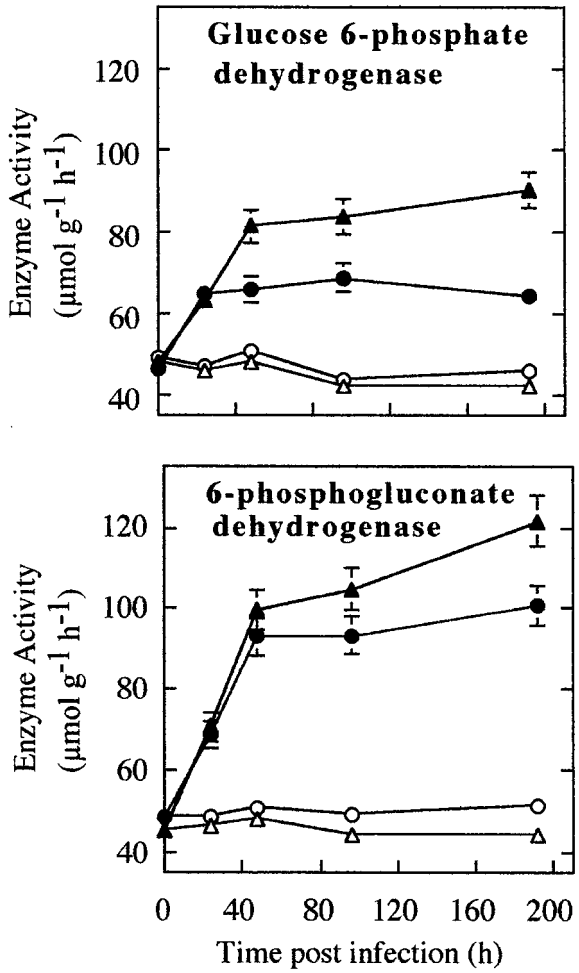

Figure 1. The effect of sheath blight infection on the activities of the pentose phosphate pathway enzymes in resistant (R) and susceptible (S) rice selection lines (infected plants: ▲ R, • S; uninfected plants: △ R, ○ S). Bars indicate (±) standard deviations.

phosphoenolpyruvate carboxylase showed a decreased activity in infected sheaths early in the infection period (24 h), but this declined steadily towards the end of the sampling period. The decrease was higher in the resistant line than the susceptible line. However this difference was only significant at 24 and 48 h respectively.

Discussion

The carbohydrate physiology of host–pathogen relationships has been a topic for extensive study, particularly for biotrophic fungal pathogens. Unfortunately, relatively limited information regarding the physiology of tissue resistant to fungal infection is available. In view of these shortcomings, the response of several key enzymes within the glycolytic and pentose phosphate pathways was investigated in relation to fungal diseases using the sheath blight fungus of rice plants as our model. Based on the results obtained from the present study we postulate that infection causes an activation of both pathways resulting in an enhancement of the key enzymes.

The use of genetically-related selection lines minimized the influence of cultivar differences on the results. Both 3F16-15 and 3F16-10 lines used as resistance and susceptible selection lines respectively are compatible with the sheath blight fungus (Wasano, unpublished). The two lines were continuously screened for sheath blight disease resistance over several years. After inoculation the development of sheath blight disease was monitored on the third leaf sheaths over the 192 h period. The response of the key enzymes of the pentose phosphate pathway, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, showed an early response to infection soon after inoculation. There were changes for both enzyme activities in the healthy plants leading to the conclusion that the activation of these enzymes was a result of infection (Sindelarova et al.,1997; Tesci et al.,1994). This increase was higher in the resistant plants. The pentose phosphate pathway furnishes NADPH to the plasma membrane NADPH-oxidase responsible for the H_2O_2 production (Pugin et al.,1997). H_2O_2 from the oxidative burst triggers the hypersensitive death of challenged cells and functions in surrounding cells as a diffusible signal for induction of defense genes encoding enzymes involved in cellular protection (Levine et al.,1994) or associated with systemic acquired resistance (Chen et al.,1993). An activation of the pentose phosphate pathway also reflects an enhanced demand for the precursors for the synthesis of amino and nucleic acids in the infected plants.

Except for pyruvate kinase and phosphoenolpyruvate carboxylase, all the glycolytic enzyme activities assayed increased in the infected plants as compared with healthy controls and more so in the resistant plants. The activities of ATP- and pyrophosphate-dependent phosphofructokinase and phosphoenolpyruvate phosphatase started to increase at early infection period. By the end of the experiment all the enzyme

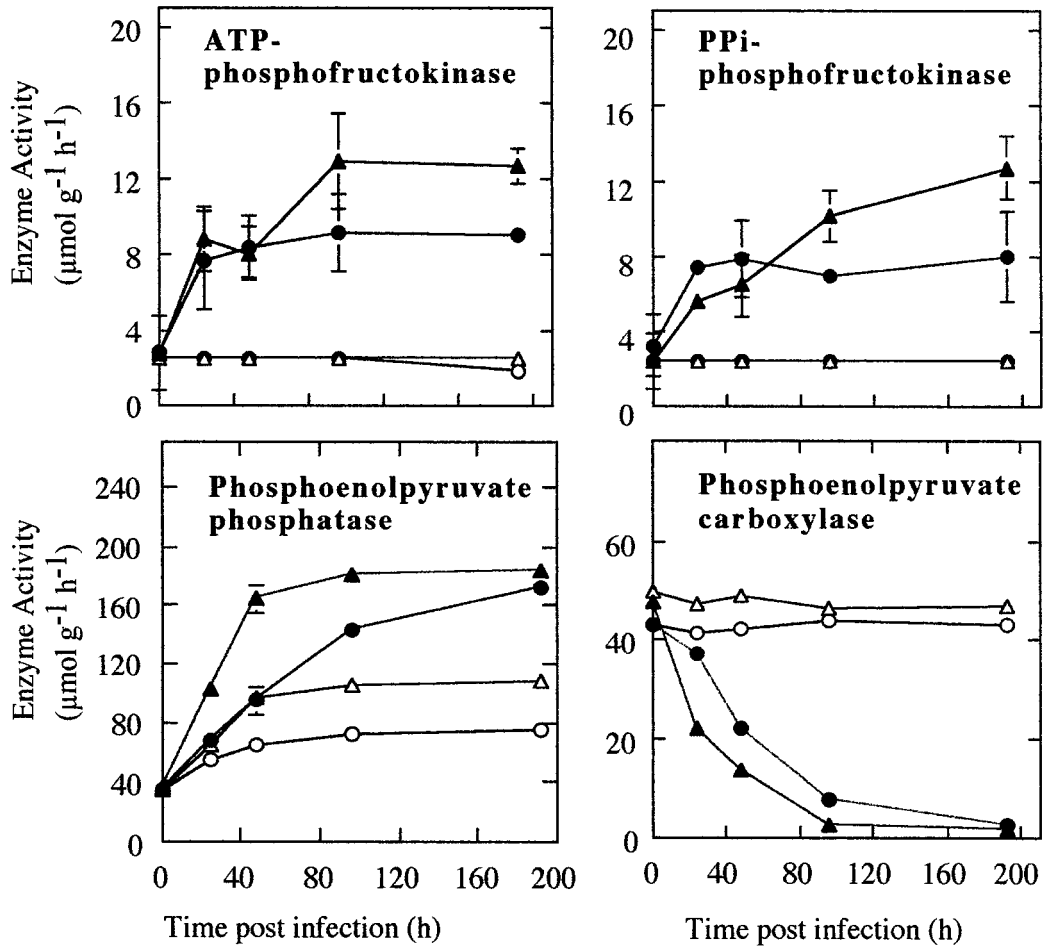

Figure 2. The effect of sheath blight infection on the activities of glycolytic pathway enzymes in resistant (R) and susceptible (S) rice selection lines (infected plants: ▲ R, ● S; uninfected plants: △ R, ○ S). Bars indicate (±) standard deviations.

activities had increased more than two-fold. However, there were no changes in the healthy control plants for both ATP- and pyrophosphate-dependent phosphofructokinase attributing the increase to infection. ATP-dependent phosphofructokinase catalyzes the maintenance pathway and pyrophosphate-dependent phosphofructokinase catalyzes the adaptive pathway. During response to an environmental perturbation plants adapt by regulating the pyrophosphate-dependent phosphofructokinase catalyzed pathway. Plants use fructose-2,6-bisphosphate to regulate the adaptive pathway at the reversible pyrophosphate-dependent phosphofructokinase step (Black et al.,1987). Pyrophosphate is used in place of ATP to enhance the ATP yield of glycolytic pathway (Plaxton, 1996) and is a byproduct of syntheses, such as nucleic acids and proteins. Therefore, pyrophosphate-dependent phosphofructokinase is activated when such macromolecule syntheses occur in the cells (Kubota and Ashihara, 1990). The high pyrophosphate-dependent phosphofructokinase activity observed in the infected sheaths could be an indication of high glycolytic activity.

Phosphoenolpyruvate phosphatase activity increased with infection while phosphoenolpyruvate carboxylase and pyruvate kinase activities declined to barely detectable levels. Phosphoenolpyruvate phosphatase is inhibited by orthophosphate and its specific activity increases following orthophosphate deprivation (Plaxton, 1996), whereby cellular ATP and ADP levels decrease, then pyrophosphate-dependent phosphofructokinase may function as a glycolytic

enzyme and/or orthophosphate generating enzyme. Phosphoenolpyruvate carboxylase functions to replenish TCA cycle intermediates consumed in amino acid biosynthesis. Regulation of phosphoenolpyruvate carboxylase in C_3 plants is not well understood, although evidence linking phosphoenolpyruvate carboxylase to assimilation of NH_4^+ into amino acids was reported by Schuller et al. (1990). Among the important amino acids that are replenished through TCA intermediates are phenylalanine and tyrosine: key enzymes in the synthesis of lignin (Bell, 1981). More detailed studies are required to determine the effect of sheath blight infection on phosphoenolpyruvate carboxylase in rice plants.

The products of plant disease resistance genes are postulated to recognize invading pathogens and rapidly trigger host defense responses. Based on the results from the present study, we conclude that infection of rice plants with the sheath blight fungus causes an activation of pentose phosphate and glycolytic pathways. Similar results have been reported in tobacco plants after the application of the elicitor cryptogein produced by *Phytophthora cryptogea* (Pugin et al.,1997). An interaction between cucumber mosaic virus and cotyledons of the marrow plants increased the rate of respiration and the capacities of the pentose phosphate pathway, glycolysis and the Krebs cycle (Tesci et al., 1994).

These results indicate that adaptation to sheath blight disease by the rice plant involves adjustments in fundamental pathways of carbon metabolism and that these adjustments are interrelated and could be controlled at the level of gene expression. The pentose phosphate and glycolytic pathways provide erythrose-4-phosphate and phosphoenolpyruvate, respectively, precursors for lignin, phenylpropanoids and phytoalexins involved in plant defense mechanisms. We propose that genes coding for the key enzymes within the pentose phosphate and glycolytic pathways could be a useful tool in studies geared towards enhancing resistance of rice plants to sheath blight disease.

Acknowledgements

We would like to thank Dr. Hiroshi Ashihara for very useful comments and Dr. Dennis Grab for valuable assistance during the preparation of this manuscript.

References

Bell AA (1981) Biochemical mechanism of disease resistance. Ann Rev Plant Physiol 32: 21–81

Black CC, Mustardy L, Sung SS, Kormanic PP, Xu D-P and Paz N (1987) Regulation and roles for alternative pathways of hexose metabolism in plants. Physiol Plantarum 69: 387–394

Bonman JM, Khush GS and Nelson RJ (1992) Breeding for resistance to pests. Annu Rev Phytopathol 30: 507–528

Chen ZX, Siva H and Klessig DF (1993) Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science 262: 1883–1886

Danson J, Wasano K and Nose A (1999) Changes in carbohydrates and related enzyme activities in sheath blight-infected resistant and susceptible rice cultivars. SABRAO J 31(1): 23–31

Hrazdina G (1994) Compartmentation in phenolic metabolism. Acta Horti 381: 86–96

Khush GS and Virmani SS (1985) Breeding rice for disease resistance. In: Russell GE (ed.) Progress in Plant Breeding (pp 239–279) Butterworths, London

Koch KE (1996) Carbohydrate-modulated gene expression in plants. Annu Rev Plant Physiol Plant Mol Biol 47: 509–540

Kubota K and Ashihara H (1990) Identification of non-equilibrium glycolytic reactions in suspension-cultured plant cells. Biochim Biophys Acta 1036: 138–142

Levine A, Tenhaken R, Dixon R and Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79: 583–593

Li Z, Pinson SRM, Marchetti MA, Stansel JW and Park WD (1995) Characterization of quantitative trait loci (QTLs) in cultivated rice contributing to field resistance to sheath blight (*Rhizoctonia solani*). Theor Appl Genet 91: 382–388

Pieterse CM, van Wees SC, van Pelt JA, Knoester M, Laan R, Gerrits H, Weisbeek PJ and van Loon LC (1998) A novel signaling pathway controlling induced systemic resistance in Arabidopsis. Plant Cell 9: 1571–1580

Pugin A, Frachisse JM, Tavernier E, Bligny R, Gout E, Douce R and Guern J (1997) Early events induced by the elicitor cryptogein in tobacco cells: involvement of a plasma membrane NADPH oxidase and activation of glycolysis and the pentose phosphate pathway. Plant Cell 9: 2077–2091

Plaxton WC (1990) Glycolysis. In: Dey PM and Harborne JB (eds) Methods in Plant Biochemistry, Vol 3 (pp 145–173) Academic Press, London

Plaxton WC (1996) The organization and regulation of plant glycolysis. Annu Rev Plant Physiol Plant Mol Biol 47: 185–214

Schuller KA, Plaxton WC and Turpin DH (1990) Regulation of phosphoenolpyruvate carboxylase from the green alga *Selenastrum minutum*: properties associated with replenishment of tricarboxylic acid cycle intermediates during ammonium assimilation. Plant Physiol 93: 1303–1311

Sindelarova M, Sindelar L and Burketova L (1997) Dynamic changes in the activities of glucose-6-phosphate dehydrogenase, riboluse bisphosphate carboxylase and ribonuclease in

tobacco leaves, leaf discs and mesophyll protoplasts in relation to TMV multiplication. Physiol Mol Plant Pathol 15: 99–109

Tesci LI, Maule AJ, Smith AM and Richard LC (1994) Metabolic alterations in cotyledons of *Cucubita pepo* infected by cucumber mosaic virus. J Experi Bot 45(280): 1541–1551

Wasano K, Oro S and Kido Y (1983) The syringe inoculation method for selecting rice plants resistant to sheath blight, *Rhizoctonia solani* Kuhn. Japan J Trop Agr 27(3): 131–139

Xie QJ, Linscombe SD, Rush MC and Jodari-Karimi F (1992) Registration of LSBR-33 and LSBR-5 sheath blight resistant germplasm lines of rice. Crop Sci 32: 507