Anthranilate Synthase and Chorismate Mutase Activities in Stem Rust-Inoculated and Elicitor-Treated Resistant, Moderately Resistant, and Susceptible Near-Isogenic Wheat Lines

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Anthranilate synthase and chorismate mutase activities which control the flow of substrate from chorismate into the tryptophan pathway and into the phenylalanine/tyrosine pathway, respectively, were examined in three near isogenic wheat lines of *Triticum aestivum* L. (cv. Prelude Sr5, highly resistant to stem rust infection; cv. Prelude Sr24, moderately resistant; cv. Prelude srx, susceptible). The activities of both enzymes were found to increase in response to inoculation with the stem rust fungus *Puccinia graminis* f. sp. *tritici* or treatment with Pgt elicitor. Thus, both the tryptophan branch and the phenylalanine branch appear to contribute to the resistance response in wheat leaves. Only the cytosolic but not the plastidic fraction of the enzyme activities appears to be affected by fungal infection or elicitor treatment. Some differences with respect to degree and time dependency of enzyme activation were noticed between the three wheat lines following inoculation but not after treatment with the Pgt elicitor.

Introduction

Highly resistant wheat plants respond to inoculation with *Puccinia graminis* f. sp. *tritici* (Pgt) with a complex pattern of defense reactions, *e.g.* alterations in the activities of phenylalanine ammonia-lyase (PAL), 4-coumarate-CoA-ligase, cinnamoyl alcohol dehydrogenase and peroxidase (Moerschbacher *et al.,* 1988, 1990), and lipoxygenase (Ocampo *et al.,* 1986). These alterations contribute to the hypersensitive response (HR) which results in lignification and host cell death. In moderately resistant wheat plants the processes conferring resistance are far less known. In this case, certain members of the benzoxazinone family, which probably originate from the anthranilate/tryptophan pathways and which strongly increase in concentration following fungal infection, appear to act as phytoalexins (Bücker and Grambow, 1990).

Abbreviations: AS, anthranilate synthase; CM, chorismate mutase; DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; HR, hypersensitive response; LOX, lipoxygenase; PAL, phenylalanine ammonia-lyase; Pgt, *Puccinia graminis* f. sp. *tritici.*

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The apparent differences in secondary metabolism in the various wheat lines led us to study the relative activities of anthranilate synthase (AS) and chorismate mutase (CM) in stem rust-inoculated and elicitor-treated leaves of different near-isogenic wheat cultivars representing a highly resistant (*Triticum aestivum* cv. Prelude Sr5), a moderately resistant (Prelude Sr24), and a susceptible wheat line (Prelude srx). CM is the key enzyme controlling the entry of chorismate into the phenylalanine branch which finally leads to the formation of lignin precursors involved in HR in the highly resistant line. AS is the key enzyme controlling the entry of chorismate into the tryptophan branch which, eventually, may lead to the formation of benzoxazinones in the moderately resistant wheat line.

Materials and Methods

Plants and fungus

Three near-isogenic lines of *Triticum aestivum* L. (cultivar Prelude Sr5; cv. Prelude Sr24; and cv. Prelude srx) which originally had been kindly provided by R. Rohringer, Agricultural Research Station, Winnipeg, Canada, were analyzed. With

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respect to their responses following inoculation with *Puccinia graminis* f. sp. *tritici*, E. & H., race 32, these lines are classified as follows: Prelude Sr5, infection type 0 (highly resistant); Prelude Sr24, infection type 1–2 (moderately resistant); and Prelude srx, infection type 3 (susceptible).

The pathogen *Puccinia graminis* was recultured as described (Kogel *et al.*, 1985). The wheat plants were cultivated with some minor modifications according to Moerschbacher *et al.* (1988): seeds were soaked in the dark for 36 h at 25 °C on wet filter paper in glass Petri dishes, transferred into soil (type 2, Torfwerk Brill, Neuenhaus, Germany), and grown in a growth chamber under controlled conditions (light period 16 h, humidity 60%, temperature 20/18 °C day/night, light intensity 18,000 lx).

Axenically grown mycelium of *Puccinia graminis* was provided by M. Fasters, RWTH Aachen.

Inoculation

The wheat plants were inoculated using a slightly modified method of Moerschbacher *et al.* (1988): 7-day-old plants (including time for soaking the seeds) were inoculated by spraying them with water and subsequently with a suspension of uredospores in 1,1,2-trichlorotrifluoroethane (1 g/50 ml per 325 seedlings) from a distance of 30–40 cm. After harvesting the zero-hour-sample, the plants were transferred to a plastic tent which was carefully sealed to maintain high humidity conditions. The tents were placed in the growth chamber and kept in the dark for 16 h. 2 h after onset of the light period, the inoculation tent was removed. The density of infection was about 100 pustules/cm^2 in the compatible interaction. Controls were treated in the same way with the exception of spraying them only with water.

Application of elicitor

Pgt elicitor was isolated from the germ tubes of *Puccinia graminis* according to Moerschbacher *et al.* (1986a). Approximately 100 µl of the preparation, adjusted to 40 µg/ml glucose equivalents, were injected into the intercellular space of the wheat leaves using a method of Kogel *et al.* (1985). At this concentration, the Pgt elicitor causes a half-maximum increase of PAL activity (Moerschbacher *et al.*, 1986b).

Extraction of enzymes

Anthranilate synthase: 6 primary leaves were pulverized under liquid nitrogen. In the case of elicitor treatment only the infiltrated areas, 2.5 cm to each side of the injection site, were used for extraction. After addition of 50 mg Polyclar AT to the pulverized material, the enzyme was extracted at 4 °C for 10 min on a magnetic stirrer in 5 ml of 0.1 M Tris-HCl, pH 7.5, with 0.1 M EDTA, 2 mM DTT, 20 mM glutamine, 4 mM $MgCl_2$, and 10% glycerol. After filtration and centrifugation (10 min, 12,000×*g*), the supernatant was used in the enzyme test. The protein concentration of the extract was estimated according to Bradford (1976).

Chorismate mutase: For the extraction of chorismate mutase, the extraction procedure was slightly modified in that 0.5 M citrate/phosphate buffer, pH 8.0, with 1 mM Trp, 10 mM DTT and 1 mM EDTA was used. After centrifugation, the extract was purified on a PD 10 column (Pharmacia, Sephadex G25) which was equilibrated with 0.1 M citrate/phosphate, pH 7.5.

Estimation of enzyme activity

Anthranilate synthase: 0.5 ml of substrate solution (0.7 µM chorismate in 0.1 M citrate/phosphate buffer, pH 7.5) were added to 0.5 ml of the extract containing the enzyme in addition to glutamine, $MgCl_2$ *etc.* (see above). After incubation (1 h at 30 °C) the reaction was stopped by inactivating the enzymes in boiling water. Controls were inactivated prior to incubation. Precipitated proteins were removed by centrifugation (10 min at 12,000×*g* in an Eppendorf centrifuge). The supernatant was submitted to chromatographic analysis.

Quantification of anthranilate was performed by a newly developed procedure based on reversed phase HPLC (Kromasil 5C18, 250×4.6 mm) using a linear acetonitrile/water gradient (15% to 40% acetonitrile in water in 15 min). The water contained 5 mM tetrabutylammonium phosphate and the pH was adjusted to 7.5 with NaOH. The flow rate was 1 ml/min. Anthranilate was detected with a fluorescence detector (ISCO FL-2a; excitation filter 240–410 nm, emission filter 430–470 nm). In this system, anthranilate was eluted with a retention time of 10.9 min.

At a later stage, the HPLC analysis of anthranilate was modified in favour of an isocratic but similarly efficient procedure which was published in the course of our experiments (Poulsen *et al.*, 1991). In this case, anthranilate was eluted with water/methanol (65:35) containing 50 mM H_3PO_3, adjusted to pH 2.5 with 6 N NaOH before addition of methanol. Under these conditions, the retention time of anthranilate was 12.2 min at a flow rate of 1 ml/min.

Chorismate mutase

0.1 ml of substrate solution (2 µM chorismate in 0.1 M citrate/phosphate buffer, pH 7.5) were added to 0.1 ml of enzyme extract and incubated for 30 min at 30 °C. Then the reaction was terminated by addition of 0.1 ml of 3 N HCl and left standing for another 30 min at 30 °C to convert the product of chorismate mutase, prephenate, into phenyl pyruvate. Then 0.2 ml of 3 N NaOH were added and the absorbance at 320 nm was measured to determine the concentration of phenyl pyruvate. In the controls, HCl was added prior to incubation.

Cell fractionation

Fractionation into a plastidic and a non-plastidic fraction ("cytosolic fraction") was performed according to Schmittmann (1991). Primary wheat leaves (5 g) were cut into 0.5 cm segments and homogenized (3 s) in 100 ml of cold suspension buffer (0.4 M sucrose; 10 mM Tricine/NaOH, pH 8.0; 10 mM NaCl; 40 mM sodium ascorbate) using a Waring blendor. Following filtration through 2 gaze layers, the filtrate was centrifuged (5 min, 1000×*g*, 4 °C). The supernatant corre-

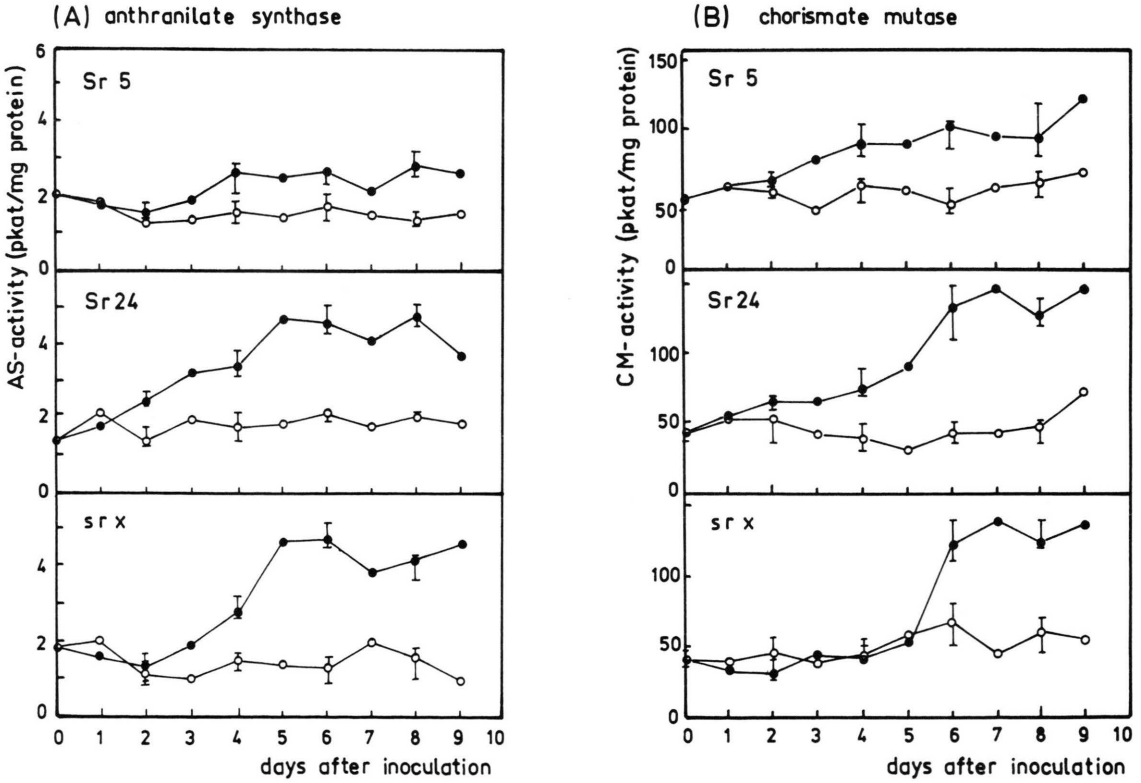

Fig. 1. (A) Anthranilate synthase and (B) chorismate mutase activities in stem rust inoculated (●—●) and control (○—○) leaves of the three near-isogenic wheat lines Prelude Sr5 (highly resistant), Prelude Sr24 (moderately resistant), and Prelude srx (susceptible). Vertical bars represent the range of data obtained in three independent experiments.

sponding to the non-plastidic fraction was separated from the pellet. The latter was carefully suspended in 20 ml of suspension buffer (same composition as above but without sodium ascorbate) and again centrifuged (same conditions as above). The pellet was suspended in 6 ml of distilled water to osmotically destroy the plastids and to release the plastidic fraction of enzymes. Chlorophyll content was measured according to Arnon (1949) in both the non-plastidic and plastidic fraction in order to calculate the relative portion of plastidic enzymes in the non-plastidic fraction. Both fractions were further purified by centrifugation (10 min, 12,000×*g*) and by gel filtration of the supernatants using PD 10 columns (Sephadex G 25; Pharmacia) which had been equilibrated before with 0.1 M Tris/HCl buffer, pH 7.5 (containing 2 mM DTT; 0.1 M EDTA; 20 mM glutamine; 4 mM $MgCl_2$; and 10% glycerol) for purification of anthranilate synthase, or with 0.1 M citrate/phosphate buffer, pH 7.5 (containing 0.1 M sodium hydrogen phosphate; 1 mM EDTA; and 10 mM DTT) for purification of chorismate mutase. Application to the columns and elution of the protein fractions were performed according to the manufacturer's instructions. The purified enzyme extracts were used for the estimation of protein contents and enzyme activities.

Results

Both AS and CM activities could be measured in extracts of stem rust inoculated and uninoculated wheat leaves (Fig. 1 and Fig. 2). Generally, the specific activity of CM was 25 to 30 times higher than AS activity.

Both AS and CM activities began to increase about 2 days after inoculation with the stem rust fungus in the Prelude Sr 24 wheat isoline and, somewhat less pronounced, in the Prelude Sr 5 isoline (Fig. 1). In the fully susceptible isoline Prelude srx the increase in AS and especially in CM activi-

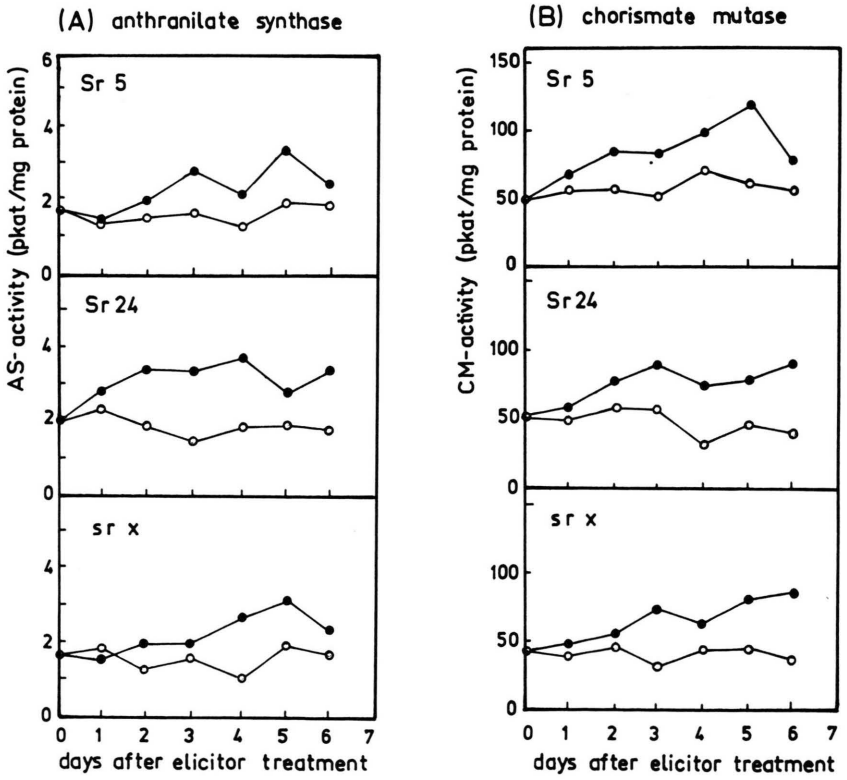

Fig. 2. (A) Anthranilate synthase and (B) chorismate mutase activities in elicitor-treated (●—●) and H_2O-treated control (○—○) leaves of the Prelude Sr 5, Prelude Sr 24, and Prelude srx wheat lines. Data represent mean values of two independent experiments.

ties appeared to be suppressed during the early stages of infection, but then the increase was comparable in size with the Sr24 isoline.

AS and CM activities increased also in response to treatment of the wheat leaves with the Pgt elicitor. In this case, however, there were no significant differences observed between the various wheat lines (Fig. 2).

Enzyme activities in the cytosolic and plastidic fractions

When AS and CM activities in response to inoculation or elicitor treatment were measured separately in the chloroplast fraction and in the remaining "cytosolic" fraction, alterations in the activities were only observed in the latter. The results presented in Fig. 3 and Fig. 4 which were obtained 3 days following treatment, more or less reflect the differences found in the whole leaf extracts. The specific enzyme activities of both the cytosolic and plastidic fractions were in the same range as in the whole leaf extracts. Data from stages later than 3 days after treatment are not available due to unreliable chloroplast isolation from treated leaves.

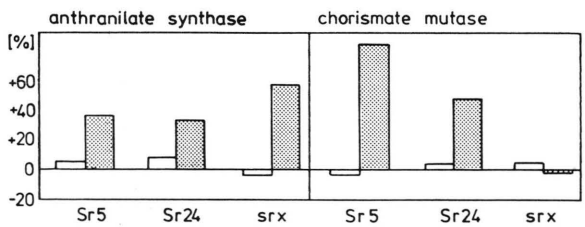

Fig. 3. Relative alterations in (A) anthranilate synthase- and (B) chorismate mutase-specific activities in the plastidic (□) and cytosolic (▩) fractions of stem rust inoculated leaves of the Prelude Sr5, Prelude Sr24, and Prelude srx wheat lines 3 days after inoculation (controls = 100%).

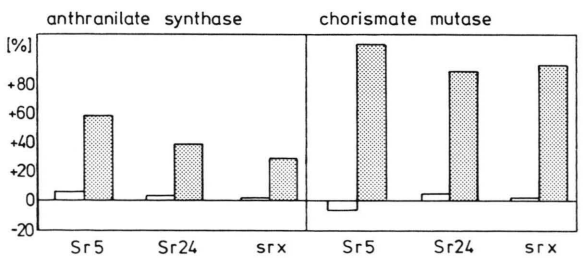

Fig. 4. Relative alterations in (A) anthranilate synthase- and (B) chorismate mutase-specific activities in the plastidic (□) and cytosolic (▩) fractions of elicitor-treated leaves of the Prelude Sr5, Prelude Sr24, and Prelude srx wheat lines 3 days after treatment (controls = 100%; mean values of two independent experiments).

Enzyme activities in axenically grown fungal mycelium

In a preliminary attempt to estimate CM and AS activities in very small amounts of axenically grown stem rust mycelium it was found that, indeed, fairly high activities of both enzymes are present in the extract of fungal mycelium (AS: 10 pkat/mg protein; CM: 90 pkat/mg protein).

Protein content

The protein content of the wheat leaves was found to remain constant throughout the experiments. Only in inoculated Prelude srx and Prelude Sr24 leaves the protein content increased 1.3- to 1.5-fold due to mycelial growth at later stages of the infection.

Discussion

The specific activities of both AS and CM were found to increase in all three wheat cultivars investigated in response to infection with the stem rust fungus or in response to treatment with the Pgt elicitor. In case of the elicitor, there were no obvious differences observed between the three wheat lines. This is in accordance with earlier findings that the activity of the Pgt elicitor is independent of the type of the interaction and, thus, does not confer specificity (Moerschbacher *et al.*, 1989).

With respect to the highly resistant Prelude Sr5 isoline which does not allow any mycelial growth of the fungus, the effect of inoculation with fungal spores on the enzyme activities was comparable in size to the effect of elicitor treatment. Most probably, this reflects the action of the elicitor in the normal host/pathogen interaction.

However, AS and CM activities reached higher values at later stages of the infection in the moderately resistant Prelude Sr24 and susceptible Prelude srx lines compared to the resistant line or to the elicitor-treated leaves. Apparently, this late increase correlates with the growth of the fungal

mycelium and may in part represent the contribution of the pathogen to AS and CM activities. In fact, high activities of both enzymes were measured in extracts of axenically grown stem rust mycelium.

It should be emphasized that in the fully susceptible cultivat Prelude srx, in contrast to Prelude Sr 5 and Sr 24, the proportion of CM activity which would be expected to emerge as a result of elicitor action during the earlier stages of the infection (day 1–5), appears to be suppressed. Such a pattern of enzyme induction which is fully consistent with results obtained in measurements of PAL activities in Prelude Sr 5 and srx cultivars (Moerschbacher *et al.,* 1988) and also in Prelude Sr 24 (Moerschbacher, personal communication), may point to the existence of a mechanism counteracting elicitor activity in the susceptible cultivar.

A great number of investigations has established the role of PAL and the phenylpropanoid metabolism in defense reactions in many incompatible plant/pathogen interactions. In contrast, only a few studies have envisaged the possible involvement of the shikimate pathway including the chorismate branch in defense responses. Thus, treatment with fungal elicitor-induced mRNA encoding 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase, the first enzyme of the shikimate pathway, in suspension-cultured parsley cells (Henstrand *et al.,* 1992). In arabidopsis, DAHP synthase genes were differentially induced by wounding and pathogenic attack (Keith *et al.,* 1991). Also, CM levels were increased in potato tubers in response to wounding (Kuroki and Conn, 1989). In Arabidopsis, one of two identified AS genes was activated by wounding and bacterial infiltration while the other was expressed at a constitutive level (Niyogi and Fink, 1992). Our results suggest an extensive involvement of the biosynthetic pathways leading to aromatic compounds also in the resistance response in the wheat/stem rust system. Probably, not only PAL and subsequent activities leading to lignin formation (see Introduction) but also AS and CM and, thus, both the tryptophan and phenylalanine branch contribute to the defense reactions. On the other hand, our results also indicate, that the strongly different responses to the pathogen of the highly resistant and lignifying wheat line Prelude Sr 5 compared to the moderately resistant and benzoxazinone-producing wheat line Prelude Sr 24 do not show any apparent correlation with alterations in the CM/AS ratio. Thus, these differences must depend on other and to date unknown biochemical events.

AS and CM act at the branch point between tryptophan biosynthesis on one hand, and phenylalanine and tyrosine biosynthesis on the other. Their obvious involvement in the control of substrate flux among the three aromatic amino acids has led to considerable efforts to understand their regulation in microorganisms and, more recently, also in higher plants. Both enzymes have been reported to be present in multiple forms in a number of plant species and it has been suggested that their plastidic forms are feedback-regulated by aromatic amino acids while the cytosolic forms are not (see review by Poulsen and Verpoorte, 1991). However, the existence of a cytosolic in addition to a plastidic shikimate pathway including CM and AS has been questioned in view of the fact that, without exception, cDNAs of CM, AS and other key enzymes of the shikimate pathway from higher plants which have been characterized to date encode proteins with N-terminal plastid-specific transit peptides (Görlach *et al.,* 1993a, 1993b; Eberhard *et al.,* 1993; Niyogi and Fink, 1992). Apparently, the relationship between the plastidic and cytosolic groups of the various enzymes is far from clear and awaits further elucidation.

It was not the aim of this study to identify or characterize AS and CM isoforms. Nevertheless, we could detect both AS and CM activities in the plastidic fraction and in the remaining "cytosolic" fraction. Our results suggest that, at least under the experimental conditions, only the cytosolic fractions of CM and AS are affected by the inoculation or elicitor treatments. These findings may be at variance with other results which, in contrast, point to an involvement of the plastidic isoform of DAHP synthase, the entry enzyme of the shikimate pathway, in response to environmental challenge (McCue and Conn, 1989; Muday and Herrmann, 1992). Considering the unsolved problems with respect to the subcellular localization and function of the complete shikimate pathway or of individual enzymes (see above), however, much more information will be necessary to understand the meaning of the various results obtained.

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