Phenylalanine Ammonia Lyase Under Combined Effects of Enhanced UV-B Radiation and Allelopathy Stress

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This study examined the effects of UV-B radiation and allelochemical stress induced by ferulic acid (FA) on the activity of phenylalanine ammonia lyase (PAL; EC 4.3.1.5) at metabolic and molecular levels in two cucumber genotypes differing in tolerance to cold and disease, in order to determine any interaction between stress effects and genotype response. Stresses were applied simultaneously, sequentially, and singly. In both genotypes, several days of UV radiation retarded growth up to 36%. The effect of FA was not significant. The response to a particular stress, including the effect on PAL activation, was enhanced by simultaneous application of the two stresses. PAL transcription was not correlated with the increase of PAL activity. Exposure to UV-B, FA, and combined UV-B and FA was detrimental to both genotypes but to different extents. The response was not correlated with the genotype of cold and disease sensitivity. PAL activity and its transcription seem to be involved in UV and allelochemical stress, but not related to the plants' tolerance of these stresses.

Key words: Phenylalanine ammonia lyase (PAL), UV-B radiation, ferulic acid, stress combinations, cucumber.

INTRODUCTION

Plant responses to individual stress conditions have been the subject of intensive research. In the natural environment, however, a number of stresses can occur simultaneously; tolerance mechanisms and the response to a stress combination are more complex. Enhanced UV-B radiation (280–315 nm) is potentially damaging for plants. Its biological effect depends on the ratio of UV-B to PAR, the spectral distribution within the UV-B wavelength band, and the genetic profile of the plant. To prevent harmful effects, plants accumulate UV-screening phenolic compounds and antioxidants, and other defense mechanisms are induced (Gitz et al., 1998; Hollosy, 2002; Yao et al., 2006). Various stresses competing with supplemental UV-B irradiation have been shown to modify the effects of UV-B (Conner and Zangori, 1998; Sandermann, 2004). The tolerance response to one stress may be different when different factors co-occur (Mittler, 2006).

Allelochemicals are important inhibitory factors released from the donor plant to the environment; they influence the growth and development of other plants (Lara-Nunez et al., 2006). They are commonly synthesized via the phenylpropanoid pathway (Blokker et al., 2006). Ferulic acid (FA) in the form of feruloyl CoA is an intermediate product of this pathway. Allelochemical stress caused by FA reduces water utilization, inhibits foliar expansion and root elongation, reduces rates of photosynthesis and induces lipid peroxidation. Moreover, FA can activate the phenylpropanoid pathway by inducing the activity of enzymes such as phenylalaninie ammonia lyase (Weir et al., 2004; Politycka and Mielcarz, 2007; dos Santos et al., 2008).

Phenylalanine ammonia lyase (PAL; EC 4.3.1.5) is a crucial enzyme in plant metabolism. It is the gateway from the primary shikimate pathway to the secondary phenylpropanoid pathway. PAL has been described as a tetrameric protein with a molecular weight of ~330 kDa (Maldonado et al., 2006). It catalyzes the nonoxidative formation of *trans*-cinnamic acid via L-deamination of phenylalanine (Hahlbrock and Scheel, 1989; Ritter and Schultz, 2004). *Trans*-cinnamate is an essential substrate for synthesis of phenylpropanoid compounds, which fulfill many

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Abbreviations: PAL – phenylalanine ammonia lyase; FA – ferulic acid; PAR – photosynthetically available radiation; PPFD – photosynthetic photon flux density

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important functions in plant organisms. Lignins, flavonoids, anthocyanins and simple phenolic acids protect plants against biotic and abiotic stresses; this makes PAL an important enzyme which participates in the plant stress response (Maldonado et al., 2006). It is known that PAL activity is induced by, for example, biotic stress, mechanical wounding, UV-B radiation and high/low temperature (Dixon and Paiva, 1995), but PAL responses under stress combinations are insufficiently known.

In this study we examined the effects of enhanced UV-B radiation and allelopathic stress caused by FA on the PAL activity of cucumber, at metabolic and molecular level. The stresses were applied simultaneously, sequentially, and singly. The aim was to determine the response of two cucumber genotypes differing in cold tolerance and disease resistance. Cucumber has been widely used to study acquired resistance and cross-tolerance.

MATERIALS AND METHODS

PLANT MATERIAL

We used two genotypes of cucumber (*Cucumis sativus* L.) selected for cold tolerance and disease resistance (provided by the Nochowo Spójnia Seed Company): no. 1 (susceptible) and no. 14 (tolerant). Seeds imbibed for 24 h were sown in vermiculite in plastic pots (10–12 cm diam.). Seedlings were grown in a growth chamber under a 12 h photoperiod (120 μmol m^{-2} s^{-1} PPFD; Philips 58 W/84 fluorescent sunlamps) at 25/22°C (day/night) and 60–65% RH. PAR was measured with an FF-01 quantum sensor (Sonopan, Poland). Seedlings were watered daily and subirrigated once a week with complete Knop solution. Plants ~20 days old were subjected to UV-B radiation and/or allelochemical stress singly or else combined simultaneously or sequentially. Under sequential treatment, after 48 h of the first stress the next one was applied.

UV-B was supplied for 6 h daily with TL 20W/01 RS Philips lamps at 18 kJ m^{-2} d^{-1} (750 mW m^{-2}) irradiance at canopy leaf level and 3.25 μmol m^{-2} s^{-1} photon flux density at 315 nm. Irradiance level was measured with a VLX 3W (Vilber Lourmat, France) radiometer.

Seedlings were subjected to allelochemical stress by supplying 2 mM solution of ferulic acid (50 cm^3 per pot) directly to the vermiculite. The reagent (Sigma-Aldrich) was dissolved in ethanol (380 mg FA per 10 cm^3) and diluted tenfold with water.

BIOMETRIC PARAMETERS

The leaf area coefficient was measured according to Cho et al. (2007) after 10 days of stress treatment. Leaf length was measured from the lamina tip to the intersection of the lamina and petiole along the lamina midrib, width was measured between the widest lamina lobes, and the two values were multiplied. Then the plants were harvested for fresh and dry weight measurement. Dry weight was measured after heating at 105°C for 48 h. At least five plants were used for each stress treatment.

EXTRACTION AND QUANTIFICATION OF PHENYLALANINE AMMONIA LYASE

Leaf samples were collected successively, always at the same interval of the light period. Extraction was performed according to Cahill and McComb (1992) with modifications. Fresh leaf samples were ground at 4°C with mortar and pestle using 0.1 M Tris-HCL buffer (pH 8.6, 2.5 cm^3 per 0.5 g tissue) containing 10 mM mercaptoethanol and 50 mg Polyclar AT. The homogenates were centrifuged at 12,000 g for 20 min. The incubation mixture contained 0.5 cm^3 80 mM borate buffer (pH 8.9), 0.5 cm^3 30 mM L-phenylalanine and 0.5 cm^3 extract. The reaction was run for 24 h at 30°C and was stopped by adding 1.5 cm^3 of 2 N HCL. *Trans*-cinnamic acid as the reaction product was determined spectrophotometrically (Jasco V-530 UV-VIS) at 290 nm. Activity was expressed as nkat mg^{-1} protein determined according to Bradford (1976).

PAL GENE EXPRESSION ANALYSIS

RNA was isolated from 0.5 g leaf tissues using the RNeasy Plant Kit (Qiagen). DNA was removed using RNase-Free DNase (Qiagen). The RNA concentration was measured with Qubit (Invitrogen) and 3 μg total RNA was used for cDNA synthesis using oligo(dT)19 primer and SuperScript III reverse transcriptase (Invitrogen). The PCR reaction was performed on the synthesized cDNA with cucumber-specific PAL primers PALF 5'GGGAGTTCTTACATGAAAAC 3' and PALR 5'CGATTTCAGCACCTTTAAACC 3'. As control, expression of cucumber actin was analyzed using primers ACTF 5' TGCTGGATTCTGGTGATG-GT 3' and ACTR 5' GCTTCTCTTTCATGTCACGG 3'. The sequences for cucumber phenylalanine ammonia lyase 1 (AF475285) and actin (AB010922) were obtained from GeneBank. Real-time PCR reactions were prepared on an Eppendorf Mastercycler® ep realplex Thermal Cycler.

STATISTICAL ANALYSIS

The experiments were run and analyzed at least three times. The results were subjected to ANOVA and Tukey's HSD range test in Statistica 8.0.

TABLE 1. Restriction of seedling growth of two cucumber genotypes after application of UV-B and FA stress singly and in combination

Stress	Restriction of growth (%)		
	fresh weight	dry weight	leaf area
Genotype 1			
UV-B	26.1	26.35	24.6
FA	4.4	3.9	22.2
UV-B+FA	34.3	28.74	26.97
UV-B→FA	27.1	11.2	31.9
FA→UV-B	22.5	18.6	29.2
Genotype 14			
UV-B	38.6	31.6	37.9
FA	19.4	15.3	23.9
UV-B+FA	45.9	42.2	41.9
UV-B→FA	26.9	23.5	11.2
FA→UV-B	21.7	27.7	13.8

RESULTS

UV-B radiation and FA, acting separately, simultaneously and sequentially, reduced the fresh and dry weight and leaf area in both genotypes (Fig. 1); the percentage of growth restriction is shown in Table 1. Single UV-B stress reduced the growth of cold-tolerant genotype 14 more than 30% and 24–26% in susceptible genotype 1. The effect of FA was minor. The reductions of fresh weight, dry weight and leaf area were highest in the combined stress treatment; in genotypes 1 and 14 the respective decreases were 34% and 46% fresh weight, 28% and 42% dry weight, and 27% and 42% leaf area. Under the stresses acting sequentially, growth reductions in genotype 1 were 22–32% for fresh weight and leaf area, and 11–18% for dry weight. In genotype 14 the growth reduction was similar for fresh and dry weight (22–27%) and much lower for leaf area (11–14%).

UV-B exposure significantly increased PAL activity in genotype 1 (Fig. 2). Under allelochemical stress, PAL activity was almost constant.

Combined stresses induced PAL activity in genotype 1 (Fig. 3); the increase was noted from day 2 of the UV-B+FA treatment, and on day 12 it was three times higher than in control plants. There was no effect on genotype 14.

The effects of sequential stresses are shown in Fig. 4. Under the UV-B→FA treatment, PAL activation occurred at the end of the experiment in plants of genotype 1, where activity was tripled. The effect of FA→UV-B was slighter than under the UV-B→FA treatment. In genotype 14, PAL activity more than
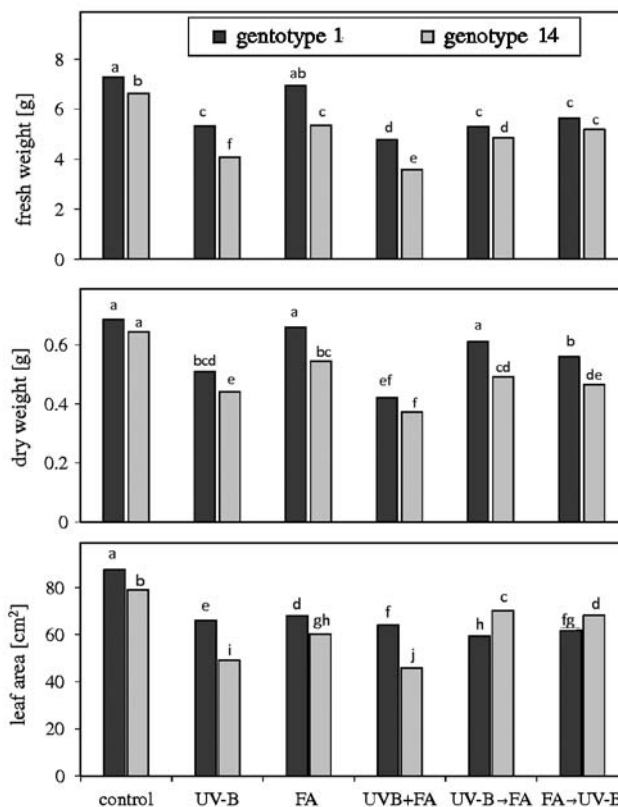

Fig. 1. Biometric parameters of cucumber genotypes 1 and 14 after UV-B stress, FA stress, and combined stress treatment (+ – simultaneously applied, → – sequentially applied).
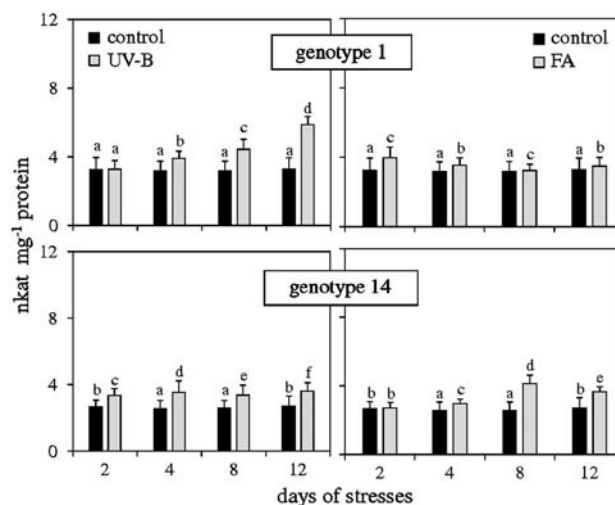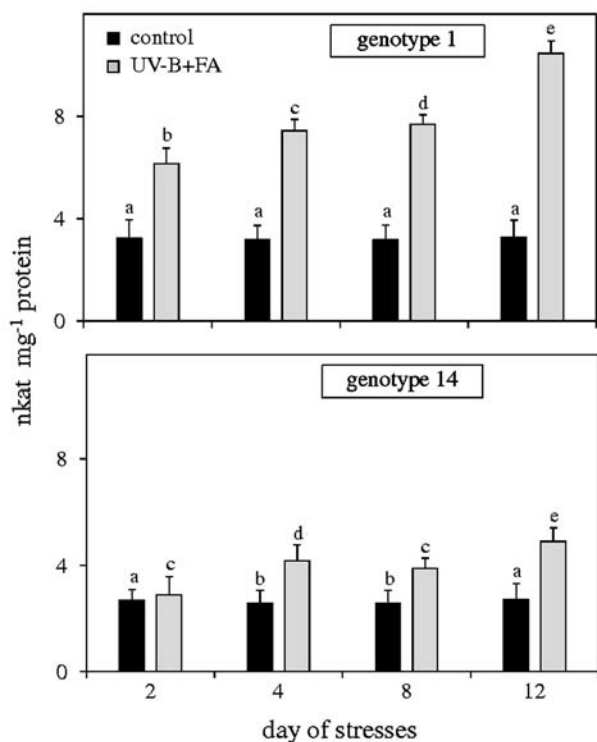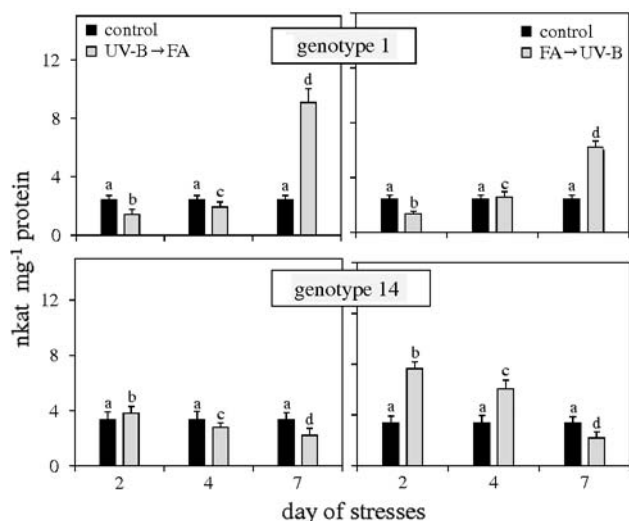

Fig. 2. PAL activity in cucumber seedlings of genotypes 1 and 14 cultured for 12 days under UV-B or FA stress applied singly.

doubled under FA→UV-B but only 2 and 4 days after the second stress, and decreased gradually thereafter. Under UV-B→FA, PAL activity was constant.

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Fig. 3. PAL activity in cucumber seedlings of genotypes 1 and 14 cultured for 12 days under UV-B+FA combined stress.

Fig. 4. PAL activity in cucumber seedlings of genotypes 1 and 14 cultured under UV-B→FA or FA→UV-B sequential stress (2 days first stress, 7 days second stress).

The level of the PAL transcript was strongly related to genotype. In genotype 14, gene expression increased under UV-B and FA (Fig. 5), and under double stresses the effect was similar to the effect of UV-B alone. In genotype 1 the changes were slight,
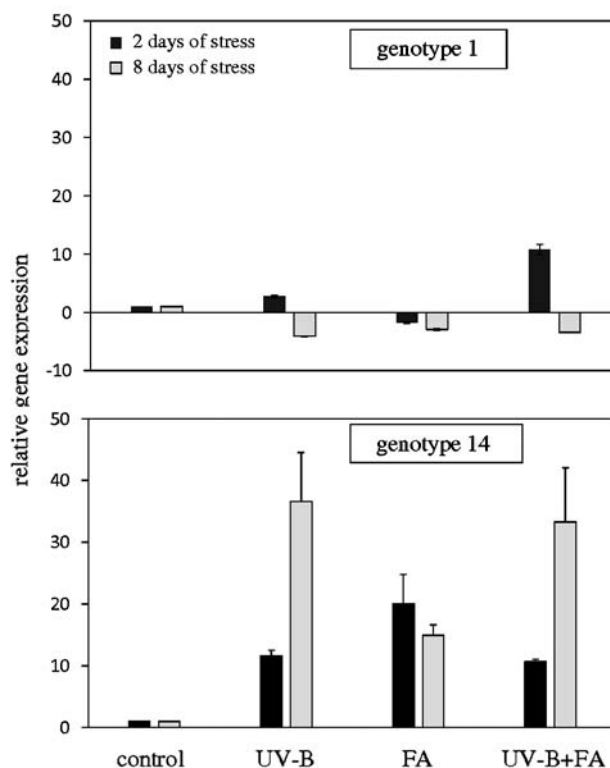
Fig. 5. PAL relative gene expression in cucumber seedlings of genotypes 1 and 14 after UV-B, FA, and UV-B+FA stress treatments.

and after 8 days of all stresses the expression levels in the treatments were similar or below to the control values.

DISCUSSION

In our experiments, enhanced UV radiation and FA, acting singly or in combination, reduced the growth parameters of cucumber genotypes selected for their cold tolerance and disease resistance. However, the responses were not uniform. Genotype 14, with high cold tolerance and disease resistance, proved much more sensitive to both stresses than genotype 1. Genotype 1, sensitive to cold and disease, was more tolerant of UV and allelopathy stress. There are a number of reports of decreased fresh and dry weight in plants exposed to single as well as combined stresses (Kondo and Kawashima, 2000; Hollósy, 2002; Koti et al., 2007). In poplar, enhanced UV-B radiation combined with water deficit significantly lowered both fresh and dry weight (Ren et al., 2007).

An important part of our study was to determine the interaction of UV-B and allelochemical stress, as cucumber is sensitive to phenolic allelo-

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chemicals (Politycka and Mielcarz, 2007; Batish et al., 2008; dos Santos et al., 2008). The effect of FA alone, especially on fresh and dry weight, was much weaker than when it acted simultaneously with UV-B. Its effects in sequential treatment were similar to its effect as a single stress. The biometric changes can be linked directly to inhibition of cell elongation, as has already been reported for FA (dos Santos et al., 2008) and ultraviolet radiation (Kumari et al., 2009).

Phenylalanine ammonia lyase, a key enzyme of the phenylpropanoid pathway, may be involved in the mechanism of protection against radiation stress, as flavonoids are important UV-screening pigments (Lavola et al., 2000; Kumari et al., 2009). We confirmed this effect, but the response of the cucumber genotypes varied in intensity; it was stronger in the more tolerant (no. 1) than in the sensitive (no. 14) genotype. In contrast, FA did not affect the enzyme's activity in either of the genotypes. MacDonald and D'Cunh (2007) found that cinnamic acid derivatives even inhibited the activity. In a study of the effects of allelochemicals on PAL activity in potato and peas, Sato et al. (1982) reported similar results. Politycka and Mielcarz (2007), on the other hand, found that PAL in cucumber roots was activated under the influence of exogenous FA.

Under FA+UV-B the enzyme was induced in genotype 1, whereas in genotype 14 the changes were negligible. Under sequential treatment (UV-B→ FA) its activity was three times higher in genotype 1 than in control plants. Such a response was not observed in genotype 14, that is, the sensitive plants. Under the reverse treatment (FA→UV-B), PAL activation occurred several days after exposure in the more tolerant plants, but in the sensitive plants its activity was highest immediately after the treatment. This effect is difficult to explain and probably is related to differences in metabolism.

The level of PAL transcripts rose in response to both stresses but not in parallel with PAL enzyme activation. UV-B radiation had the greatest effect on PAL gene expression, both singly and in combination with FA, but only in the more sensitive plants (genotype 14). In many crop plants such as wheat (*Triticum aestivum* L.), petunia (*Petunia axillaris*) and parsley (*Petroselinum crispim*), PAL has been shown to be involved in the UV response (Ryan et al., 2002; Zinser et al., 2007; Zhang and Bjorn, 2009). The involvement of PAL in allelopathy is more complex; in general, genes encoding the phenylpropanoid pathway are stimulated under a range of abiotic stresses.

In the two genotypes of cucumber we studied, cold and disease tolerance were not correlated with UV and allelochemical stress sensitivity. UV-B and FA stresses applied singly and in combination reduced cucumber growth. The response to a par-

ticular stress was enhanced when it was applied simultaneously with the other one, and this effect also involved PAL activation. PAL activity and transcription seems to be involved in the response to UV and allelopathy stress but does not seem to be related to the level of cold and disease stress tolerance in cucumber.

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