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Stimulation of Phenylpropanoid Biosynthesis in Apple (*Malus domestica* Borkh.) by Abiotic Elicitors

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Summary

Phenylpropanoids are involved in natural defence reactions of apple (*Malus domestica*) against various diseases. An induced phenylpropanoid biosynthesis prior to pathogen attack may, therefore, support resistance mechanisms. In two model systems, the induction of phenylpropanoid biosynthesis was investigated under different light and carbohydrate conditions and after abiotic stressor treatment by measuring the activity of the key enzyme phenylalanine ammonia lyase and by estimating the accumulation of chlorogenic acid and flavan 3-ols. It could be demonstrated that Milsana Tee, Mycosan, lime sulphure, Triton-X-100 and 2,6-dichloroisonicotinic acid (INA) may act as elicitors of phenylpropanoid biosynthesis and are, therefore, candidates for resistance induction in apple.

Zusammenfassung

Stimulierung der Flavanolbiosynthese des Apfels (*Malus domestica*) durch abiotische Elicitoren. Phenylpropanoide sind beteiligt an natürlichen Abwehrreaktionen des Apfels (*Malus domestica*) gegen verschiedene Pathogene. Eine Induktion der Phenylpropanoidbiosynthese vor Infektionen könnte deshalb die Resistenzreaktionen unterstützen. In Modellsystemen wurden potentielle Induktoren auf Apfelblätter appliziert und ihr Effekt auf die Aktivität des Schlüsselenzyms Phenylalaninammonium-Lyase gemessen sowie auf die Akkumulation der Chlorogensäure und Flavan 3-ole. Dabei wurden auch Licht und Kohlenhydratversorgung variiert. Es konnte gezeigt werden, dass Milsana Tee, Mycosan, Netzschwefel, Triton-X-100 und 2,6-Dichloroisonicotinsäure (INA) als Elicitoren des Phenylpropanoidstoffwechsles agieren können und damit als Resistenzinduktoren in Frage kommen.

Key words. catechin – flavonoids – phenylalanine ammonia lyase – plant protection – procyanidin

Introduction

Phenlypropanoids are often involved in plants' defence against biotic and abiotic stress (GEIBEL et al. 1994). Their synthesis is often stimulated after pathogen attack (NICHOLSON and HAMMERSCHMIDT 1992; KUC 1995) as a part of an active defence. It also could be found that an altered and enhanced flavonoid metabolism by exogenously applied bioregulators improved the resistance of several Rosaceaous species (ROEMMELT et al. 2003; SCHLANGEN et al. 2003; GOSCH et al. 2003). Particularly in apple, phenolic compounds and especially flavanols play a role in resistance to the fungus *Venturia inaequalis* causing scab (TREUTTER and FEUCHT 1990a, b). An inhibition of the phenylpropanoid pathway in leaves of the scab-resistant cultivar 'Sir Prize' with the phenylalanine ammonia lyase (PAL) inhibitor AOPP (L-2-aminooxy-3-phenylpropionic acid) reduced the content of phenolic compounds and the plant showed clear scab symptoms (MAYR et al. 1997). Further evidence for the role of flavonoids in scab defence is given by the observation that susceptible cultivars accumulate flavanols after scab infection (MAYR et al. 1995a). These compounds were also inducible by abiotic stress (MAYR et al. 1995a). Here we report the effects of different abiotic stressors, which are discussed as resistance inducers, on synthesis and accumulation of phenolic compounds. PAL activity and the accumulation of chlorogenic acid as the predominant hydroxycinnamic acid derivative in apple leaves and flavan 3-ols as endproducts of the flavonoid pathway were determined. The intention of the experiments was to elucidate whether so-called resistance inducers may act via the phenylpropanoid pathway.

Material and Methods

For all experiments leaves were taken from M9 rootstocks grown in the greenhouse, frozen in liquid nitrogen and stored at –20 °C.

Extraction and determination of phenylalanine ammonia lyase activity

Extraction and determination of PAL-activity was carried out according to MESSNER et al. (1991) and FÜNFGELDER et al. (1994). Protein content was determined according to BRADFORD (1976) using BSA as standard.

Extraction and determination of phenolic compounds

Frozen leaves were lyophilised and ground in a mortar. The fine powder was extracted with methanol containing 6-methoxyflavone as an internal standard for 30 min in a cooled water bath using sonication. After centrifugation the supernatant was evaporated, the residue redissolved in small quantities of methanol and injected for HPLC-analysis. The phenolic compounds were separated on a column (250 x 4 mm I.D.) pre-packed with Hypersil ODS, 3 µm particle size, following a stepwise gradient using mixtures of formic acid (5 % in water) and methanol from 95:5, v/v to 10:90, v/v with a flow rate of 0.5 ml per min (TREUTTER et al. 1994). Chlorogenic acid was detected at 280 nm whereas the flavan 3-ols were estimated at 640 nm after post column derivatisation with DMACA (4-dimethylaminocinnamaldehyde) (TREUTTER 1989). Peak identification was published elsewhere (MAYR et al. 1995b).

Leaf disc system

15 mm diameter discs were taken from the youngest fully expanded leaves (fourth leaf from shoot apex). The leaf discs were maintained in petri dishes at 23–25 °C under continuous light (70 µmol m^{-2} s^{-1} white fluorescent light) on water with or without 0.1 M sucrose. Fifteen hours after starting the experiments, one half of the leaf discs was treated with the anionic detergent Triton X-100. PAL-activity was determined just before treatment and 15 h, 18 h, 21 h, 24 h, 27 h, 39 h, 63 h and 87 h after Triton addition. Content in phenolic compounds was measured after 3, 6 and 11 days.

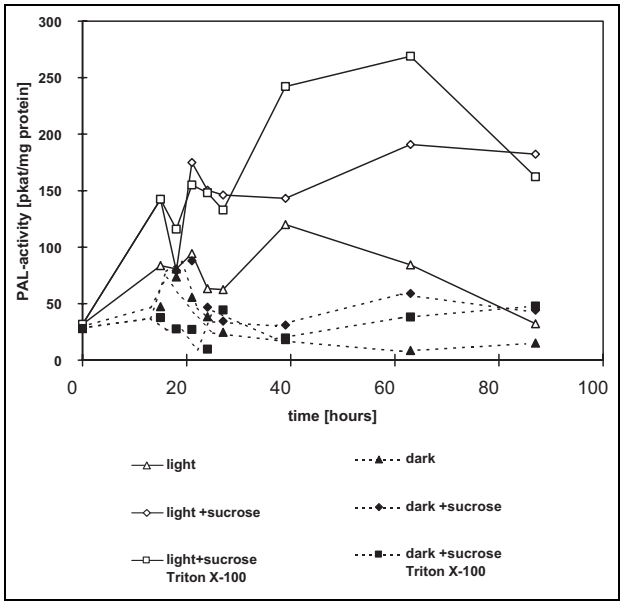


Fig. 1. PAL-activity of apple leaf discs under light (full lines) or under darkness (dotted lines) with or without 0.1 M sucrose and after treatment with 0.1 % Triton X-100 or distilled water. Each data point represents the mean of 15 pooled leaf discs.

Shoot tip system

Shoot tips (tip and the three youngest leaves) of potted M9 apple rootstocks were treated with Milsana Tee (BASF, 1 %), Mycosan (Andermatt Biocontrol, 1 %), lime sulphure (0.2 %) and with INA (2,6-dichloroisonicotinic acid, Ciba Geigy, 0.6 mM) to test their eliciting potential. Each trial was replicated three times; control leaves were treated with de-ionized water. PAL-activity was determined after 15 h and 24 h, phenolic compounds 3 days after treatment.

Results

Using the apple leaf disc system, the activity of phenylalanine ammonia lyase was determined in light and darkness with or without additional sucrose supply. At the beginning of the experiments, a PAL-activity of 30 pkat mg^{-1} protein was measured. After 20 to 24 h, a first peak in PAL activity could be determined in darkness as well as in light. Generally, the level of activity was smaller in darkness (50 pkat mg^{-1} protein) than under light conditions (100 pkat mg^{-1} protein). In light, PAL-activity reached a second maximum after 39 hours with 120 pkat mg^{-1} protein. In darkness, PAL-activity decreased to the starting level without a second maximum (Fig. 1). Additional sucrose supply enhanced PAL-activity both in darkness and light, but the extent of increase was much greater under continuous light (200 pkat mg^{-1} protein) (Fig. 1).

When compared with the experiments under darkness, light conditions (with and without sucrose) increased PAL-activity, which was followed by an increase of chlorogenic acid (maximum after 3 days) and of monomeric and oligomeric flavanols (maxima after 11 days, Table 1). The sucrose supply enhanced formation of these compounds with maximal contents after

Table 1. Concentration of phenolic compounds (mg g^{-1} dry weight) in leaf discs cultivated in light or darkness with or without sucrose (0.1 M) supply and Triton X-100 treatment. Each data point represents the mean of 45 pooled discs.

phenolic compound (mg g^{-1} dry weight)	time (days)	light			darkness	
		– sucrose	+ sucrose	+ sucrose + Triton X-100	– sucrose	+ sucrose
chlorogenic acid	3	3.4	4.4	5.0	0.2	0.1
	6	2.2	5.4	8.2	0.2	0.2
	11	2.5	2.3	9.1	0.2	0.3
monomeric flavanols	3	1.4	2.5	2.3	0.1	0.01
	6	0.8	7.6	5.0	0.1	0.07
	11	1.6	1.3	7.8	0.1	0.06
oligomeric flavanols	3	2.1	2.6	2.2	1.2	0.7
	6	1.6	4.5	3.0	1.6	0.7
	11	2.1	0.9	4.7	1.3	0.6

6 days. The content of phenolic compounds of the discs cultivated in the dark decreased dramatically which could not be prevented by additional sucrose supply (Table 1).

The treatment with the non-ionic tenside Triton X-100, as an abiotic stressor, in a concentration of 0.1% at time 15 h enhanced the PAL-activity to a maximum of approximately 270 pkat mg^{-1} protein between 24 and 48 h after treatment in those leaf discs cultivated in light on sucrose containing medium (Fig. 1). The contents of chlorogenic acid and flavanols increased continuously within 11 days and exceeded the content of those leaf discs exclusively treated with sucrose (Table 1).

In order to avoid the wound response, which occurred in the leaf disk system, the effect of different abiotic stressors on the synthesis and accumulation of phenolic compounds was tested with the shoot tip system. The PAL-activity of shoot tips 15 h and 24 h after treatment is shown in Fig. 2. Milsana Tee showed a slight stimulation of PAL activity at 15 as well as 24 h after treatment, whereas lime sulphure and Mycosan exhibited a stronger response after 24 h 2,6-dichloroisonicotinic acid (INA) increased PAL-activity by about 60 % which could be measured at both time intervals.

Three days after treatment, the content of chlorogenic acid, of monomeric flavan 3-ols (catechin, epicatechin) and of oligomeric flavan 3-ols (procyanidins) was measured in the shoot tips (Fig. 3). A spray with Milsana Tee led to increased contents of monomeric (catechin and epicatechin) and oligomeric flavanols up to 30% above the water control, whereas chlorogenic acid remained at the control level. The spray with Mycosan or lime sulphure increased chlorogenic acid by 250% and 150%, respectively. Lime sulphure and INA could slightly stimulate the accumulation of monomeric flavanols (catechin, epicatechin). Further prolonged studies with INA exhibited increased amounts of chlorogenic acid, monomeric and oligomeric flavanols within 9 days after INA-application in the treated shoot tips and, moreover, within 14 days in the newly grown but not directly treated shoot tips (Table 2).

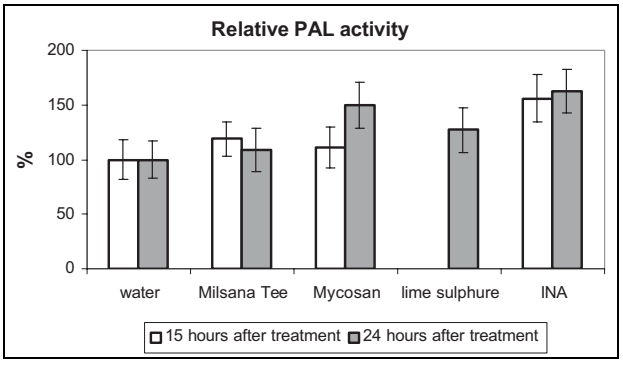


Fig. 2. PAL-activity of apple shoot tips 15 h and 24 h after treatment with abiotic elicitors compared to water treatment (100 %). Lime sulphur was not measured at 15 h. The vertical line represents the standard deviation.

Discussion

The leaf disc system has been chosen for investigations of the phenylpropanoid metabolism of strawberry (CREASY 1968) or sunflower (CREASY et al. 1974). We used this system for apple leaves to investigate the stimulatory effect of the abiotic effector Triton-X-100 on PAL-activity and the accumulation of phenylpropanoids and flavonoids. A spectrophotometric method for measuring the PAL-activity was adapted to apple leaves. The enzyme was previously characterised by optima of pH (8.2) and temperature (55 °C) and showed its highest activity in the youngest leaves (FÜNFGELDER et al. 1994). We found a relation between the increase of chlorogenic acid and flavan 3-ols with a preceding increase of PAL-activity after chemical stress caused by the non-ionic tenside Triton X-100 when sucrose was available and under light conditions.

Unfortunately, in the leaf disc model system the effects of the different inducing agents (light, sucrose and chemical stress) were overlapped by the wound response. The first maximum in PAL-activity may therefore be explained as a wound reaction of the leaf discs due to the preparation of the discs at the beginning of the experiments.

Accumulation of monomeric and oligomeric flavanols in apple tissues as a response to wounding or chemical stress has been described (FEUCHT et al. 1985; MAYR et al. 1995a).

Further studies were performed with shoot tips without wounding of the tissue. The aim of these studies was to elucidate the effect of potential resistance inducers on the phenylpropanoid biosynthesis and accumulation. This was measured by an increase in PAL-activity and in most cases the enhanced enzyme activity was followed by an accumulation of chlorogenic acid and/or flavanols. A weak but rapid induction of PAL-activity by Milsana Tee was shown 15 h after treatment. The analysis of phenolic compounds after 3 days showed a slight accumulation of monomeric and oligomeric flavanols. The plant protectant Mycosan acted slower. PAL-activity was induced 24 h after treatment and only chlorogenic acid accumulated, whereas flavanols did not increase during the experiment. Enhanced resistance by treatment with abiotic elicitors is a well-known phenomenon. For plant protectants, such as Milsana Tee and Mycosan, which are defined as preparations increasing the plant's defence potential without any direct interaction with pathogens, the mechanism of resistance induction is yet unclear. A spray with the *Reynoutria sacchalinensis* extract Milsana prior to an infection with different fungi or bacteria induced the resistance of *Cucumis sativus* and tobacco against these pathogens. In this case the resistance was not only correlated with increased activities of chitinases and glucanases, but also with the enzyme activities of peroxidases, phenoloxidases and PAL (SCHNEIDER and ULLRICH 1994). The mechanism of resistance induction by Milsana is not yet known but a glycolipid with tenside properties was assumed to be the acting agent (JESPERS and DE WAARD 1993). The induction of phenylpropanoid biosynthesis by the anionic tenside Triton X100 is shown in these experiments and also by MAYR et al. (1995a). Mycosan, which also contains some tenside components, is successful-

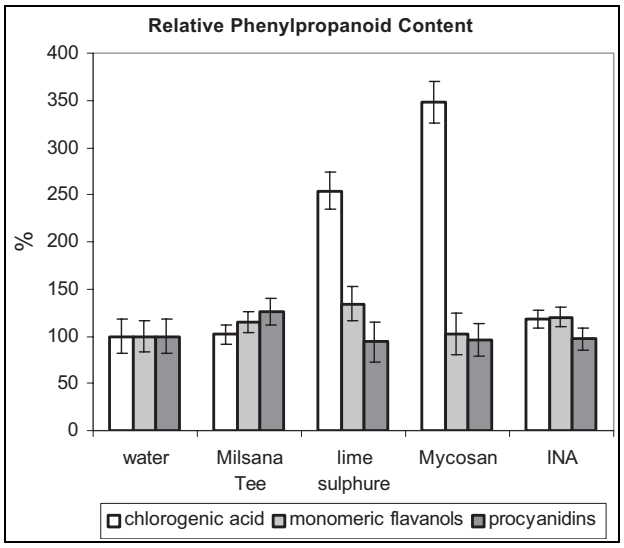


Fig. 3. Relative concentrations of chlorogenic acid and flavanols of apple shoot tips 3 days after treatment with abiotic elicitors compared to the water control (100 %); mean of 3 analyses with standard deviation (vertical line).

ly used against apple scab with a similar degree of effectiveness as lime sulphure or copper sprays (BOSSHARD et al. 1994). Furthermore, also sulphure itself could be the working agent. In our experiments, lime sulphure applied in the non-fungitoxic concentration of 0.2% as well as the sulphure containing plant protectant Mycosan were able to induce PAL-activity in the shoot tip model system.

2,6-Dichloroisonicotinic acid (INA) is a well-known inducer of systemic acquired resistance acting via salicylic acid (KESSMANN et al. 1994a, b). In the apple shoot tip system PAL-activity is highly induced by INA. Surprisingly, only a weak response on phenylpropanoids could be detected after 3 days, but after 9 days and even in the young newly formed leaves a marked accumulation was measured. Therefore, it may be postulated that also in apple INA might act both locally and systemically.

The results generally show that the activity of phenylalanine ammonia lyase in apple leaves could be used as a biochemical marker for the accumulation of chlorogenic acid as well as of monomeric and oligomeric flavanols. However, there is no clear quantitative correlation between PAL-activity and the concentrations of chlorogenic acid and flavanols within the time intervals used in the experiment. This may be due to a differing substrate supply for PAL and/or the competition of other phenylpropanoid pathways such as lignification. In total, Milsana Tee, Mycosan, lime sulphure and INA have been found to act as elicitors of PAL-activity and phenylpropanoid biosynthesis and are, therefore, candidates to induce resistance in apple.

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Table 2. Concentrations of phenolic compounds (mg g^{-1} dry weight) in shoot tips after treatment with 2,6-dichloroisonicotinic acid (0.6 mM).

phenolic compound (mg g^{-1} dry weight)	time (days)	water control	2,6-dichloroisonicotinic acid (% of water control)
chlorogenic acid	9	0.17 ± 0.03**	0.27 ± 0.07 (157 %)
	14*	0.23 ± 0.02	0.35 ± 0.04 (149 %)
monomeric flavanols	9	0.33 ± 0.06	0.49 ± 0.06 (147 %)
	14*	0.37 ± 0.05	0.71 ± 0.13 (193 %)
oligomeric flavanols	9	7.12 ± 1.30	8.87 ± 1.21 (125 %)
	14*	8.15 ± 0.87	17.65 ± 2.28 (217 %)

*: new-grown leaves, non-treated; **: standard deviation. Each data point represents the mean of the analyses of 3 shoot tips.

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