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Effect of Prohexadione-Ca on Various Fruit Crops: Flavonoid Composition and Substrate Specificity of their Dihydroflavonol 4-Reductases

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Summary

In pome fruit trees, the growth retardant prohexadione-Ca induces the formation of 3-deoxycatechins, which correlates with an increased resistance against fire blight and also against some fungal diseases. The formation of 3-deoxycatechins is due to the channelling of intermediates unusual for pome fruits, which requires the presence of a flavanone 4-reductase (FNR) activity. Here, it is investigated, whether the crop plants strawberry, grapevine, cranberry, cherry, peach, plum, elder, and kiwi fruit react in the same way to prohexadione-Ca application. The presence of FNR activity is shown by enzymatic investigations and the formation of a 3-deoxycatechin after prohexadione-Ca application *in planta*. Furthermore, by cloning of the corresponding cDNAs and studies with the heterologously expressed enzymes it is shown that the DFR enzymes of grapevine, cranberry and strawberry posses also FNR activity. Conclusively, all the crop plants studied have the potential to form 3-deoxyflavonoids. However, the changes in the flavonoid composition observed after prohexadione-Ca treatment were not exactly the same in all species studied here. In kiwi fruit leaves, a strong accumulation of flavanones rather than the formation of large amounts of 3-deoxycatechins is observed.

Zusammenfassung

Wirkung von Prohexadion-Ca auf einige Obstarten: Flavonoidzusammensetzung und Substratspezifität der Dihydroflavonol 4-Reduktasen. Der Wachstumsregulator Prohexadion-Ca kann bei Kernobst die Bildung von 3-Deoxyflavonoiden induzieren, was mit einer erhöhten Resistenz gegen Feuerbrand, aber auch gegen Pilzerkrankungen einhergeht. Das Vorhandensein einer Flavanon 4-Reduktase ist hierbei entscheidend für die Umlenkung von Intermediaten in Richtung der 3-Deoxycatechine. In der vorliegenden Studie wurde untersucht, ob weitere Obstarten wie Erdbeere, Weinrebe, Cranberry, Kirsche, Pfirsich, Pflaume, Holunder und Kiwi gleichermaßen auf eine Prohexadion-Ca Anwendung reagieren. FNR-Aktivität wurde enzymatisch und durch die Bildung eines 3-Deoxycatechins nach Behandlung mit Prohexadion-Ca nachgewiesen. Darüber hinaus wurde durch cDNA-Klonierung und heterologe Expression gezeigt, dass die DFR-Enzyme von Wein, Cranberry und Erdbeere auch FNR-Aktivität besitzen. Generell wurde nachgewiesen, dass alle untersuchten Pflanzen das Potential haben, 3-Deoxyflavonoide zu bilden. Die Veränderungen im Flavonoidstoffwechsel nach Prohexadion-Ca Behandlung waren allerdings in den untersuchten Pflanzen unterschiedlich und führten beispielsweise in Kiwi zu einer starken Akkumulation von Flavanonen.

Key words. *Actinidia* sp. (kiwi fruit) – 3-deoxyflavonoids – flavanone 4-reductase (FNR) – *Fragaria* x *ananassa* (strawberry) – induced resistance – *Prunus domestica* (plum) – *Vitis vinifera* (grapevine) – *Prunus persica* (peach)

Introduction

Pome fruit trees, treated with the plant growth retardant prohexadione-Ca, are significantly less affected by fire blight and scab than control trees. The enhanced resistance is correlated with the occurrence of phenolic compounds in apple and pear leaves, which are not formed under natural conditions (ROEMMELT et al. 2003). Among these, 3-deoxyflavonoids are of special interest, since they are known to act as phytoalexins in some plant species (SNYDER and NICHOLSON 1990; LO et al. 1996). In contrast to the common 3-hydroxyflavonoids, which are widely spread in many plant families, the rare 3-deoxyflavonoids are found only in a few plant species, e.g. Gesneriaceae, *Zea mays*, and *Sorghum* sp.. The committed step in the formation of 3-deoxyflavonoids is the reduction of the oxo-group of flavanones in position 4 by flavanone 4-reductase (FNR), which was shown for the first time by STICH and FORKMANN in the petals of *Sinningia cardinalis* (1988a) and *Columnea*

hybrida (1988b). The resulting flavan 4-ols are the intermediates for 3-deoxyanthocyanins, 3-deoxycatechins and derived phlobaphenes. Recently, it was shown that in *Zea mays* the dihydroflavonol 4-reductase, an enzyme of the common flavonoid pathway, possesses a broad substrate specificity and accepts beside dihydroflavonols also flavanones as substrates (HALBWIRTH et al. 2003). Thus, the dihydroflavonol 4-reductase of maize is involved in the biosynthesis of 3-hydroxyanthocyanins and 3-deoxyanthocyanins, and the anthocyanin type formed depends essentially on the strength of flavanone 3-hydroxylase (FHT) activity. Since dihydroflavonols are the preferred substrates, flavan 4-ols are formed only in maize tissues showing reduced or absent FHT activity. FNR activity was shown to be present in apple and pear leaves as well (HALBWIRTH et al. 2002; FISCHER et al. 2003), although 3-deoxyflavonoids are not formed under natural conditions (ROEMMELT et al., this issue). By cloning of the cDNA and studies with the heterologously expressed enzymes, it could be shown, that the DFR of apple and pear also exhibit FNR activity (FISCHER et al. 2003) and is involved in the formation of the 3-deoxyflavonoids after prohexadione-Ca treatment. As in maize, dihydroflavonols are the preferred substrates for DFR, and flavan 4-ols are formed only when flavanones accumulate after FHT inhibition.

Thus, it was assumed that formation of 3-deoxyflavonoids could be artificially induced in all plants showing, as an essential precondition, FNR activity in the target tissue and that this could be connected with an enhanced resistance against plant pathogens in general. Therefore, we studied the economically important crop species strawberry (*Fragaria* x *ananassa*), grapevine (*Vitis vinifera*), cranberry (*Vaccinium macrocarpon*), cherry (*Prunus avium*), peach (*Prunus persica*), plum (*Prunus domestica*), elder (*Sambucus nigra*), and kiwi fruit (*Actinidia* sp.) for the presence of FNR activity and the substrate specificity of their DFRs. The respective crop plant tissues all represent targets of bacterial and/or fungal pathogens. In kiwi fruit, grapevine, peach and strawberry, the changes in the flavonoid composition after prohexadione-Ca application were investigated in more detail.

Material and Methods

Plant material

Studies on the DFR substrate specificity were performed with almost ripe fruits of *Fragaria* x *ananassa* cv. 'Thetis' and on young leaves of *Vitis vinifera* cv. 'Riesling', *Vaccinium macrocarpon* cv. 'Pilgrim', *Prunus avium* cv. 'Horitschoner Herzkirsche', *Prunus persica*, *Prunus domestica*, *Sambucus nigra* (wild isolate) and *Actinidia arguta*. The plant material was collected during spring and summer 2002, frozen in liquid nitrogen and stored at −80 °C. Prohexadione-Ca treatments were performed on *Vitis vinifera* cv. 'Silvaner' and cv. 'Müller-Thurgau', *Fragaria* x *ananassa* cv. 'Elsanta', *Prunus persica* cv. 'Stark Red Gold', *Actinidia deliciosa* cv. 'Hayward' and *Actinidia arguta*.

Prohexadione-Ca treatment

Vitis vinifera: The grapevine cultivars 'Silvaner' and 'Müller-Thurgau' were treated with 500 ppm prohexadione-Ca at the phenological stages BBCH 68 (end of flower, June 20), BBCH 73 (June 27) or at both stages. Six days after the last treatment, young leaves (with the same age) and young berries were harvested for analyses.

Actinidia deliciosa: Two years old potted plants were treated with 250 ppm prohexadione-Ca (June 5). Comparable young leaves were signed and sampled before (time 0) and 2, 5, and 10 days after prohexadione-Ca application.

Actinidia arguta plants were treated with 250 and 500 ppm prohexadione-Ca. For the analyses, comparable young leaves were harvested 14 days after treatment from 5 plants and extracted as a mixed sample.

Fragaria x *ananassa* plants were sprayed with prohexadione-Ca at a concentration of 250 ppm. The youngest not fully developed leaves at time of the treatment were sampled 11 days later. The leaves were frozen in liquid nitrogen immediately after sampling and lyophilised afterwards. Control leaves and treated leaves of three different plants each were analysed as mixed samples.

Prunus persica: Peach trees were sprayed with 250 ppm prohexadione-Ca (June 5). 20 comparable shoots were chosen and their young leaves collected before (time 0) and 2, 5, and 10 days after prohexadione-Ca application.

Chemicals (Materials)

For spray application of prohexadione-Ca, the formulation BAS 125 10 W (Regalis®, BASF AG, Ludwigshafen, Germany), a wettable granular containing 10 % by weight of prohexadione-Ca was used. (2-^{14}C)-Malonyl-coenzyme A (55 mCi mmol^{-1} was purchased from Amersham International (Freiburg, Germany). (^{14}C)-Naringenin (NAR) was prepared as described (BRITSCH and GRISEBACH 1985), using heterologously expressed chalcone synthase (Schroeder, University of Freiburg, Germany). (^{14}C)-Eriodictyol (ERI) was synthesized from (^{14}C)-NAR (FORKMANN and STOTZ 1981), using F3'H-activity of microsomal preparations from *Tagetes erecta*. (^{14}C)-Dihydrokaempferol (DHK) was prepared from (^{14}C)-NAR with heterologously expressed flavanone 3-hydroxylase (R. Lukacin, University of Marburg, Germany) and used for preparation of (^{14}C)-dihydroquercetin (DHQ) (FORKMANN and STOTZ 1981) with microsomal preparations from *Tagetes erecta*.

Cloning and heterologous expression of the DFR genes

Poly(A)-tailed mRNA was isolated from *Vitis*, using the (MACS mRNA Isolation Kit® (Miltenyi Biotec, 130-075-201). From *Fragaria* and *Vaccinium*, total RNA was isolated with the RNeasy® Plant Mini Kit (Qiagen, 74904). Reverse transcription was done with the SuperScript™ II RNase H- Reverse Transcriptase (Invitrogen, 18064-014) and the oligo(dT)-anchor-primer 5´-GACCACGCGTATCGATGTCGAC(T)16V-3´.
Several RT-PCR primers for the non-coding 5´- and 3´-region were derived from the public GenBank database sequences: *Vitis* DFR (SPARVOLI et al. 1994, X75964), *Fragaria* DFR (MOYANO et al. 1998, AF029685) and *Vaccinium* DFR (POLASHOCK et al. 2002, AF483836). Finally, PCR with the forward primers 5´-CTCGGATTATTTTTGAGAAGTATG-3´

(*Vitis*), 5´-GCACTAACTAAAGCATGGG-3´ (*Fragaria*), 5´-GATATTTGGAACCAGTCATG-3´ (*Vaccinium*) and the reverse primers 5´-CTCTCGCTAGAAAGCCTGACAG-3´ (*Vitis*), 5´-GATAACTTCGACTGGATCGGA-3´ (*Fragaria*), 5´-CCACACAAGCATTTGAAATAGC-3´ (*Vaccinium*) led to PCR products of the expected size. The 3´-ends of the forward primers included the start codon (*Vitis*, *Vaccinium*) and two additional bases (*Fragaria*). In the case of *Fragaria*, the PCR product could not be gained from cDNA from leaves of cv. 'Elsanta', but only from cDNA from fruits of cv. 'Thetis'. Amplifications were achieved with the Taq/Pwo-polymerase system ´Expand High Fidelity PCR System®´ (Roche, 1732641) according to the manufacturer's instructions. Cycling conditions were 1.5 min at 94 °C, 30 x (30 sec at 94 °C, 1 min at 50 °C (*Vitis*) or 51 °C (*Fragaria*, *Vaccinium*), 2 min at 72 °C) and 7 min at 72 °C. After gel analysis the RT-PCR product was ligated into the yeast expression plasmid pYES2.1, using the pYES2.1 TOPO TA Expression Kit® (Invitrogen, K4150-01). The plasmid constructs were first transformed into the *E. coli* strain TOP10 (Invitrogen, C4040-03). Presence and orientation of the insert were tested with PCR, using a plasmid specific and an insert specific primer. The plasmid of a positive clone was used for the transformation of the yeast strain INVSc1 (Invitrogen, C810-00), using the S.c. EasyComp™ Transformation Kit (Invitrogen, K5050-01). After a small scale test for functional DFR expression, sequencing of the respective plasmid was performed by a commercial supplier. Small scale test and preparative functional expression of DFR cDNAs were performed as described (MARTENS et al. 2002).

Enzyme assay

Enzyme preparation as well as enzyme assays were performed as described earlier (FISCHER et al. 2003).

Analyses of phenolic compounds

For extraction of phenolic compounds, lyophilised leaves were ground in a ball mill. The fine powder was extracted with methanol (100 %) containing 6-methoxyflavone as an internal standard for 30 min in a cooled water bath during 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.) prepacked with Hypersil ODS, 3 µm particle size, following a stepwise gradient, using mixtures of solvent A (formic acid, 5 % in water) and solvent B (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). The gradient profile used was: 0–5 min, isocratically, 5 % B in A; 5–15 min, 5–10 % B in A; 15–30 min, isocratically, 10 % B in A; 30–50 min, 10–15 % B in A; 50–70 min, isocratically, 15 % B in A; 70–85 min, 15–20 % B in A; 85–95 min, isocratically, 20 % B in A; 95–110 min, 20–25 % B in A; 110–140 min, 25–30 % B in A; 140–160 min, 30–40 % B in A; 160–175 min, 40–50 % B in A; 175–190 min, 50–90 % B in A. For the selective estimation of flavans, a post-column derivatisation method was employed (TREUTTER et al. 1994). Flavanones, catechins, and procyanidins as well as the novel compound luteoliflavan were identified according to their chromatographic behaviour on HPLC and thin layer chromatography and in comparison with previously isolated standards (MAYR et al. 1995; ROEMMELT et al. 2003). Flavonols were selectively detected at 360 nm and identified by their UV absorbance spectra with a diode array detector. Quantification was performed as follows: Catechin and epicatechin were available as standards, procyanidins were calculated as procyanidin B2, luteoliflavan as catechin, flavonols as rutin, and flavanones as eriodictyol-7-glucoside.

Results

Studies on the DFR substrate specificities

The main results obtained in the studies of DFR substrate specificities from various crop plants are summarised in Table 1. Incubation of DHQ with enzyme

Table 1. Overview of indicators of FNR activity in various crop species. Second column: DFR activity (DHQ as a substrate) and FNR activity (ERI as a substrate) of enzyme preparations from leaves; third column: DFR and FNR activity of heterologously expressed cDNAs; fourth column: luteoliflavan formation after prohexadione-Ca (ProCa) application as indirect evidence for FNR activity.

Crop species	**Enzyme activity measurable**		**Enzyme activity of heterologously expressed DFR**		**Luteoliflavan formation after ProCa treatment**
	DFR	**FNR**	**DFR activity**	**FNR activity**	
Grapevine (*Vitis vinifera)*	+++	++	+++	++	yes
Strawberry *(Fragaria* x *ananassa)*	+++	+	+++	++	yes
Cranberry (*Vaccinium macrocarpon*)	+	+	+++	+++	yes
Peach *(Prunus persica)*	+++	+	n.d.	n.d.	yes
Kiwi fruit (*Actinidia* sp.)	+++	+++	n.d.	n.d.	yes
Plum *(Prunus domestica)*	+++	+	n.d.	n.d.	n.d.
Cherry *(Prunus avium)*	+++	+	n.d.	n.d.	n.d.
Elder *(Sambucus nigra)*	+	+	n.d.	n.d.	n.d.

n.d.: not determined, +++: high activity, ++: average activity, +: low activity

preparations of grapevine, strawberry, kiwi fruit, plum, peach, cherry, cranberry, and elder, respectively, in the presence of NADPH, led to the formation of leucocyanidin (DFR activity). In the case of the elder enzyme, conversion rates were relatively low (approx. 20 %), whereas all other extracts tested showed rather high activities (65 % and above). Incubation of ERI with the respective enzyme preparations in the presence of NADPH led to the formation of luteoforol (FNR activity). In the case of strawberry, FNR activity could be detected only in fruits (*F.* x *ananassa* cv. 'Thetis'). Highest FNR activities were observed in leaves of kiwi fruit (conversion rate approx. 60 %) and grapevine (conversion rate approx. 30 %), whereas FNR activities in peach, plum and elder leaves showed conversion rates below 15 %.

The FNR activity was also studied in a molecular biological approach. It was tested, if, like in apple and pear, the DFR enzymes possess an additional FNR activity. For this, three crop species were chosen: strawberry, grapevine and cranberry. The respective cDNA was derived from RNA prepared from leaves of grapevine and cranberry. Since no suitable cDNA could be obtained from leaves of strawberry, cDNA prepared from fruits was used. The DFR cDNAs were cloned and used for heterologous expression in yeast. Incubation of the dihydroflavonols DHK and DHQ with the heterologously expressed enzymes from strawberry, grapevine and cranberry in the presence of NADPH led to the formation of the respective leucoanthocyanidins, which verifies the cDNAs as DFRs. High conversion rates were observed with both substrates, but DHQ seemed to be preferred. Incubation of the respective enzyme preparations with the flavanones NAR and ERI in the presence of NADPH led to the formation of the corresponding flavan 4-ols. In all three cases, higher conversion rates were observed with NAR as a substrate, but with the exception of strawberry, the differences were rather small.

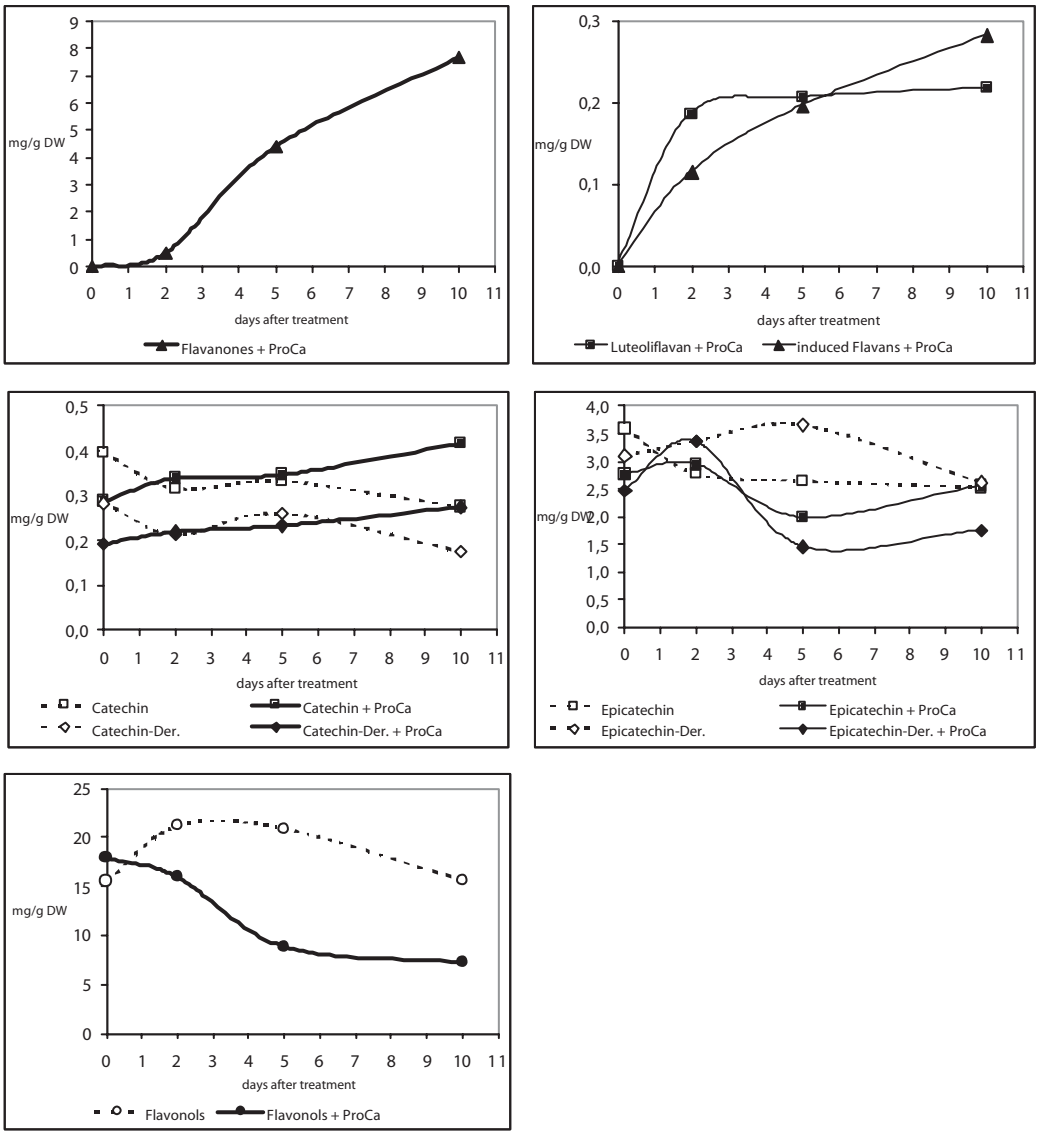


Fig. 1 Time course of flavonoids of kiwi fruit leaves (*Actinidia deliciosa*) treated with prohexadione-Ca (ProCa). For each date, comparable leaves from 2 plants were harvested and analysed as mixed sample. Luteoliflavan and flavanones could not be detected in samples from untreated plants.

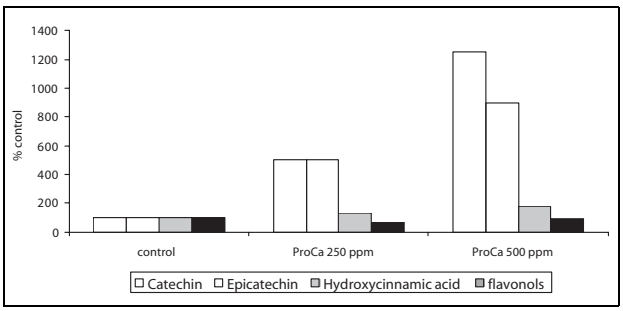


Fig. 2 Effect of Prohexadione-Ca on the composition of phenolic compounds of *Actinidia arguta* leaves.

the same time, a pronounced accumulation of flavanones up to 7 mg g^{-1} DW was found. On the other hand, the flavonols were reduced by about 10 mg g^{-1} DW (Fig. 1). Epicatechin and the epicatechin derived procyanidins also decreased at day 5 after treatments with increasing tendency thereafter, while catechin and its derivatives remained on low but rather constant levels during the first 5 days and increased later on (Fig. 1). As a response to prohexadione-Ca treatment, luteoliflavan and some flavanones could be detected also in *Actinidia arguta* leaves (data not shown), whereas the amount of flavonols decreased (Fig. 2). Catechin and epicatechin strongly accumulated. Hydroxycinnamic

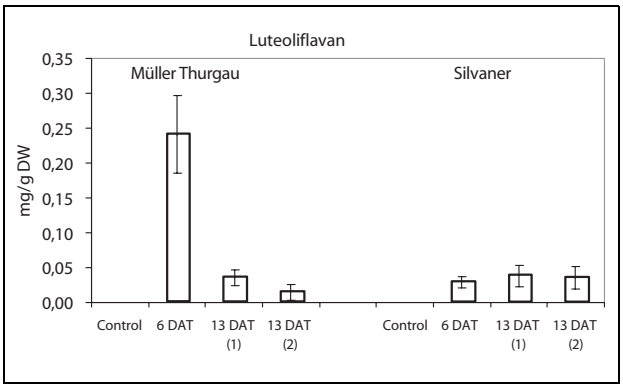


Fig. 3a

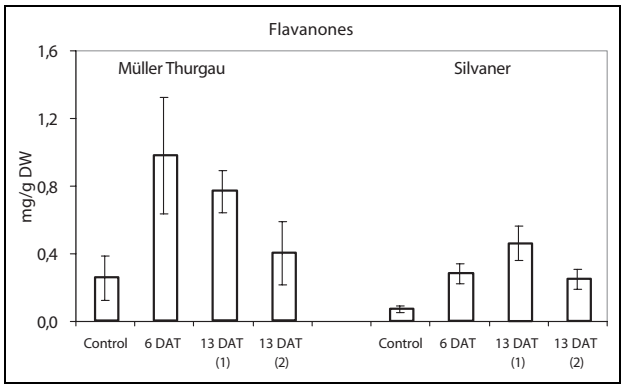


Fig. 3b

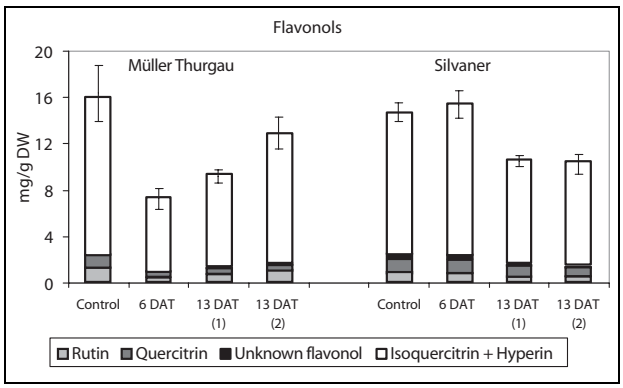


Fig. 3c

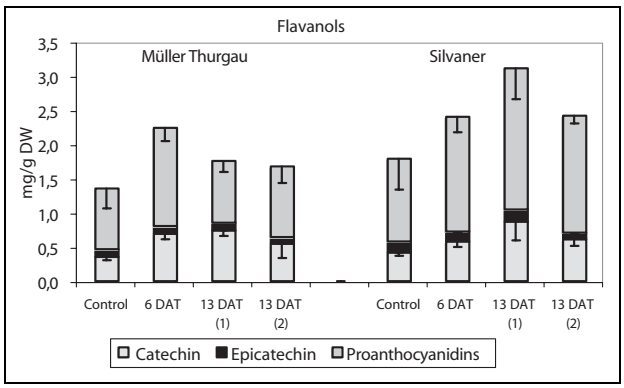


Fig. 3d

Fig. 3 Concentration of luteoliflavan, flavanones, flavanols and flavonols in grapevine leaves of cvs. 'Müller Thurgau' and 'Silvaner' treated once (1) or twice (2) with prohexadione-Ca, 6 and 13 days after treatment (DAT). The vertical line indicates the standard deviation. The vertical lines in Fig. 3c represent the standard deviation of catechin and proanthocyanidins, respectively.

Flavonoid Analyses

Flavonoid analyses were performed on leaves of kiwi fruit, grapevine, peach, cranberry, and strawberry. In these plants, luteoliflavan could be detected only after prohexadione-Ca treatment (Table 1). The influence of prohexadione-Ca on the general flavonoid composition in the respective plant species is shown in Fig. 1–5. Beside luteoliflavan, the leaves of *Actinidia deliciosa* produced some other new, but not yet identified flavans after application of prohexadione-Ca. Together, they reached a concentration of 0.5 mg g^{-1} DW (Fig. 1). At

acids tendentially increased, particularly in those leaves, which were treated with the higher prohexadione-Ca concentration of 500 ppm (Fig. 2). Grapevine leaves treated with prohexadione-Ca also formed luteoliflavan and flavanones at low concentrations (Fig. 3a, 3b). The concentration of flavonols decreased (Fig. 3c), whereas catechin, epicatechin and procyanidins increased (Fig. 3d). In peach leaves, the prohexadione-Ca application also led to an accumulation of luteoliflavan (Table 1, Fig. 4) and of a not yet identified flavanone. Whereas the concentrations of catechin and procyanidins remained rather unaffected, flavonols markedly de-

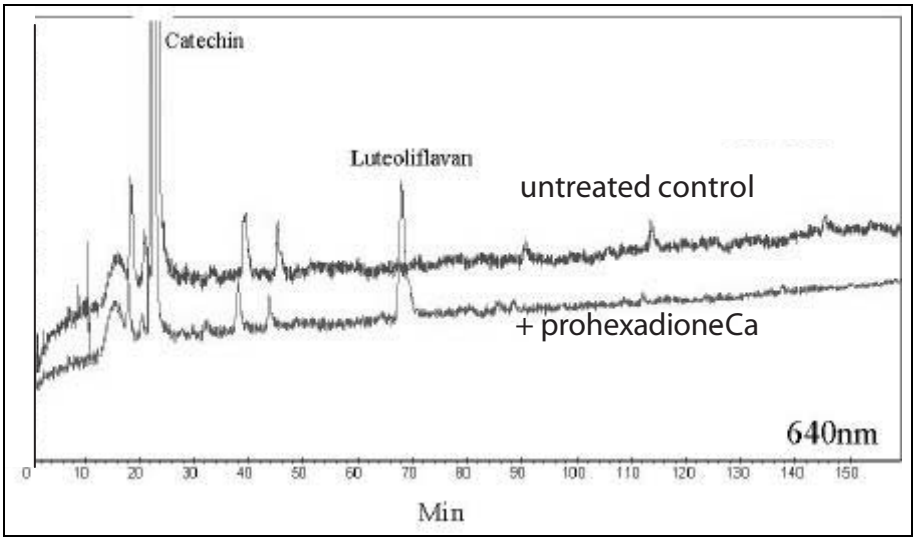


Fig. 4 HPLC-profiles of flavans of peach leaves (*Prunus persica*) with post column derivatisation and detection at 640 nm. Upper chromatogram: untreated control; lower chromatogram: leaves were extracted 5 days after treatment with prohexadione-Ca.

creased (Fig. 5). Strawberry leaves treated with the dioxygenase inhibitor exhibited only a weak response in that luteoliflavan occurred in the chromatogram (not shown).

Discussion

The occurrence of 3-deoxyflavonoids in apple after prohexadione-Ca treatment can be connected with an enhanced resistance against pathogens (ROEMMELT et al. 2002, 2003) as it is known for the 3-deoxyanthocyanins of *Sorghum* (SNYDER and NICHOLSON 1990; LO et al. 1996). Therefore, the interest focussed on the molecular mechanism underlying the effect of prohexadione-Ca (FISCHER et al. 2003; ROEMMELT et al., this issue). Essential preconditions are the inhibition of the FHT with a subsequent accumulation of flavanones and the formation of flavan 4-ols by FNR activity. Here, studies were performed with various fruit crops to investigate whether they have the potential to respond to prohexadione-Ca application in the same way as pome fruit trees. The presence of FNR activity was investigated *in vitro* by testing crude enzyme preparations and *in planta* by treatment with prohexadione-Ca and subsequent screening for luteoliflavan formation. The investigations revealed the presence of FNR activity in all plants tested (Table 1). Furthermore, analyses of the flavonoid composition showed major changes in the leaves of the treated plants. The concentration of flavonols decreased much more than the concentration of catechins, as found for grapevine, *Actinidia deliciosa*, and peach (Fig. 1, 3, 5). This is probably due to the fact that apart from FHT another 2-oxoglutarate dependent dioxygenase, flavonol synthase (FLS), is involved in the formation of flavonols, which is affected by prohexadione-Ca as well. In all three plants, the DFRs clearly prefer dihydroflavonols as substrates and compete with the affected FLSs for their common substrates. Consequently, flavonol concentrations decreased heavily. In peach, only a moderate luteoliflavan accumulation was observed, which corresponds well with the relatively low FNR activity detected. The drastic decrease of flavonols was accompanied by only little formation of the newly formed compounds flavanones

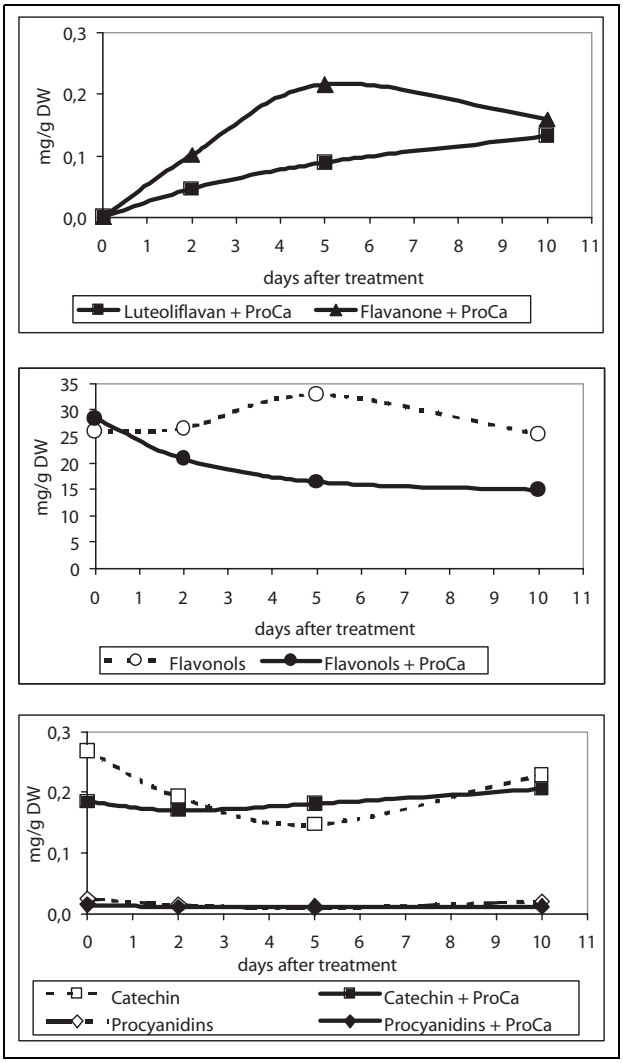


Fig. 5 Time course effect of prohexadione-Ca on the content of flavonoids in peach leaves (*Prunus persica*). For each date, comparable leaves from 2 plants were harvested and analysed as mixed sample. Luteoliflavan and flavanones could not be detected in samples from untreated plants.

and luteoliflavan (about 0.3 mg g^{-1} DW in maximum). This could be interpreted as a hint that FLS is more inhibited by prohexadione-Ca than FHT. However, no biochemical proof is available yet and various other factors such as changes in the concentration of hydroxycinnamic acids as described for apple must also be considered. In grapevine leaves, the concentrations of catechin, epicatechin and procyanidins increased. An enhanced flavonoid biosynthesis as a general stress response might be involved. Several spray treatments have been shown to stimulate PAL activity unspecifically (MICHALEK 1996). The response of the two grapevine cultivars differed quantitatively; cv. 'Müller Thurgau' accumulated more luteoliflavan and flavanones as cv. 'Silvaner' did.

The extent of changes in the composition of phenolic compounds obviously depends on the balance of biosynthesis and metabolism of intermediates, which is mainly due to the characteristics of the enzymes involved. Under natural conditions, flavanones are not detectable in the leaves of the examined plants. By the inhibition of 2-oxoglutarate dependent dioxygenases, metabolic bottlenecks occur with FHT being the first. Thus, the expectable consequence is the accumulation of flavanones. The plants studied here, however, react in different ways probably depending on the substrate affinity and/or specificity of the enzymes involved. Whereas in peach leaves, the amounts of flavanones and luteoliflavan were balanced (Fig. 5), grapevine leaves (Fig. 3b) and, particularly, kiwi fruit leaves (Fig. 1) accumulated high amounts of flavanones, which are not yet identified but probably are glycosylated. Since kiwi fruit leaves show both high F3'H activity and FNS II activity as well as high conversion rates of eriodictyol to luteoforol, the high concentrations of flavanones are rather surprising. However, it could be speculated that there is a strong competition between glucosyltransferases or other modifying enzymes and DFR/FNR, thus, shifting the balance towards flavanones.

The heterologously expressed DFR enzymes of grapevine, strawberry, and cranberry showed substrate specificities which were significantly different from those observed with the enzymes of apple and pear (FISCHER et al. 2003). In contrast to the DFRs of apple and pear, which clearly preferred eriodictyol as substrate and accepted naringenin, if any, only to a minor extent (conversion rates below 10 %), the enzymes of grapevine, strawberry and cranberry clearly showed high conversion rates with both naringenin and eriodictyol, although naringenin seemed to be slightly preferred as a substrate. In contrast to the DFR of pear, which showed a distinct preference for DHQ, the enzymes of grapevine, strawberry and cranberry accepted DHK and DHQ to almost the same extent as it was described for the DFR from apple.

In order to gain more insight into the in vivo mechanism with respect to the significance of DFR/FNR activity and substrate specificity, complete identification of all flavanones occurring in the various plants studied would be necessary. In general, it can be concluded, that the potential of 3-deoxyflavan formation is more widespread in plants than expected and offers new chances for resistance induction. Further on, it must be mentioned that the overall change in flavonoid composition due to prohexadione-Ca treatment may have several physiological consequences. The reduction of flavonols, for instance, must be noticed carefully since they are known as effective antioxidants (ELSTNER et al. 1993) and UV-protectants (SCHNABL et al. 1986). On the other hand, the accumulation of different metabolites as well as the possible activation of the general flavonoid pathway, which was also found in some experiments, may explain the broad and unspecific prevention of plant diseases by prohexadione-Ca (RADEMACHER and KOBER, this issue).

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References

BRITSCH, L. and H. GRISEBACH 1985: Improved preparation and assay of chalcone synthase. Phytochemistry **24**, 1975–1976.

ELSTNER, E. F., W. OßWALD, R. VOLPERT and H. SCHEMPP 1993: Phenolic antioxidants. In: GEIBEL, M., D. TREUTTER and W. FEUCHT (Eds.): International Symposium on Natural Phenols in Plant Resistance. Freising-Weihenstephan. Acta Hort. **381**, 304–335.

FISCHER, T. C., H. HALBWIRTH, B. MEISEL, K. STICH and G. FORKMANN 2003: Molecular cloning, substrate specificity of the functionally expressed dihydroflavonol 4-reductases from *Malus domestica* and *Pyrus communis* cultivars and the consequences for flavonoid metabolism. Archives of Biochemistry and Biophysics **412**, 223–230.

FORKMANN, G. and G. STOTZ 1981: Genetic control of flavanone 3-hydroxylase activity and flavonoid 3'-hydroxylase activity in *Antirrhinum majus* (snapdragon), J. of Biosciences **36c**, 411–416.

HALBWIRTH, H., W. KAMPAN, K. STICH, T. C. FISCHER, B. MEISEL, G. FORKMANN and W. RADEMACHER 2002: Biochemical and molecular biological investigations with respect to induction of fire blight resistance in apple and pear by transiently altering the flavonoid metabolism with specific enzyme inhibitors. Acta Hort. **590**, 485–492.

HALBWIRTH, H., S. MARTENS, U. WIENAND, G. FORKMANN and K. STICH 2003: Biochemical formation of anthocyanins in silk tissue of *Zea mays*. Plant Science **164**, 489–495.

LO, S.-C., I. WEIERGANG, C. BONHAM, J. HIPSKIND, K. WOOD and R. L. NICHOLSON 1996: Phytoalexin accumulation in sorghum: identification of a methyl ether of luteolinidin. Physiological and Molecular Plant Pathology **49**, 21–31.

MARTENS, S., T. TEERI and G. FORKMANN 2002: Heterologous expression of dihydroflavonol 4-reductases

from various plants. FEBS Letters **531**, 453–458.
MAYR, U., D. TREUTTER, C. SANTOS-BUELGA, H. BAUER and W. FEUCHT 1995: Developmental changes in the phenol concentrations of 'Golden Delicious' apple fruits and leaves. Phytochemistry **38**, 1151–1155.
MICHALEK, S. 1996: Stimulierung der Phenolbiosynthese des Apfels (*Malus domestica*) im Hinblick auf Induzierte Resistenz. PhD thesis Technische Universität München, Shaker Verlag, Aachen.
MOYANO, E., I. PORTERO-ROBLES, N. MEDINA-ESCOBAR, V. VALPUESTA, J. MUNOZ-BLANCO and J. L. CABALLERO 1998: A fruit-specific putative dihydroflavonol 4-reductase gene is differentially expressed in strawberry during the ripening process. Plant Physiol. **117**, 711–716.
POLASHOCK, J. J., R. J. GRIESBACH, R. F. SULLIVAN and N. VORSA N. 2002: Cloning of a cDNA encoding the cranberry dihydroflavonol-4-reductase (DFR) and expression in transgenic tobacco. Plant Sci. **163**, 241–251.
ROEMMELT, S., S. PETEREK, D. TREUTTER, W. RADEMACHER, J. B. SPEAKMAN, C. ANDREOTTI, G. COSTA, G. SPONZA, L. TORTORETO, C. BAZZI, H. HALBWIRTH, N. ZIMMERMANN, K. STICH and FORKMANN G. 2002: Alteration of phenylpropanoid biosynthesis of fruit trees as a tool for enhancement of fire blight resistance. Acta Horticulturae **590**, 477–484.
ROEMMELT, S., N. ZIMMERMANN, W. RADEMACHER and D. TREUTTER 2003: Formation of novel flavonoids in apple (*Malus* x domestica) treated with the 2-oxoglutarate-dependent dioxygenase inhibitor prohexadione-Ca. Phytochemistry, in press.
SCHNABL, H., G. WEISSENBÖCK and H. SCHARF 1986: In vivo microspectrophotometric characterization of flavonol glycosides in *Vicia faba* guard and epidermal cells. J. Exp. Bot. **37**, 61–72.
SNYDER, B. A. and R. L. NICHOLSON 1990: Synthesis of phytoalexins in sorghum as a site-specific response to fungal ingress. Science **248**, 1637–1639.
SPARVOLI, F., C. MARTIN, A. SCIENZA, G. GAVAZZI and C. TONELLI 1994: Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape (*Vitis vinifera* L.). Plant Mol. Biol. **24**, 743–755.
STICH, K. and G. FORKMANN 1988a: Biosynthesis of 3-deoxyanthocyanins with flower extracts from *Sinningia cardinalis*. Phytochemistry **27**, 785–789.
STICH, K. and G. FORKMANN 1988b: Studies on columnidin biosynthesis with flower extracts from *Columnea hybrida*. Zeitschrift für Naturforschung, C: Journal of Biosciences **43**, 311–314.
TREUTTER, D., C. SANTOS-BUELGA, M. GUTMANN and H. KOLODZIEJ 1994: Identification of flavan-3-ols and procyanidins by high performance liquid chromatography and chemical reaction detection. J. Chrom. **667**, 290–297.

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