Flavonoids induced in cells undergoing nodule organogenesis in white clover are regulators of auxin breakdown by peroxidase

Ulrike Mathesius
Genomic Interactions Group, Research School of Biological Sciences, Australian National University, GPO Box 475, Canberra, ACT 2601, Australia

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Abstract
It was tested whether flavonoids that specifically accumulate in cells undergoing early nodule organogenesis could affect auxin turnover by a peroxidase to explain local changes in auxin distribution that occur during nodule formation in white clover (Trifolium repens cv. Haifa). A fluorometric assay was developed to determine the kinetics of indole-acetic acid (IAA) breakdown rates by a horseradish peroxidase in vitro. Three flavonoid compounds, which had previously been localized and identified, were purified from root tissue and their tissue concentrations estimated. A derivative of 7,4'-dihydroxyflavone (DHF), as well as free DHF, strongly inhibited auxin breakdown by peroxidase at concentrations estimated in the root tissue. Formononetin, an isoflavonoid accumulating in nodule primordia, accelerated auxin breakdown by peroxidase at concentrations estimated to be present in the roots. These results suggest that local changes in flavonoid accumulation could regulate local auxin levels during nodule organogenesis. The results are consistent with previous observations on the localization of auxin during nodule organogenesis. A model for the interaction of flavonoids with peroxidases is proposed to explain changes auxin during nodule development. A similar mechanism could be involved in lateral root and root gall formation.

Key words: Auxin, flavonoids, lateral root formation, nodulation, organogenesis.

Introduction
Most species of legume plants have the ability to form root nodules, which are specialized plant organs permitting the biological fixation of atmospheric nitrogen. Nodule formation is induced by symbiotic soil bacteria of the genera Rhizobium, Bradyrhizobium, Mesorhizobium, Sinorhizobium, and Azorhizobium, generically called rhizobia. Nodule organogenesis starts with the re-initiation of cell divisions in the root cortex of legume plants. A small group of dividing cells forms a nodule primordium that later differentiates into a nodule invaded by rhizobia (Hirsch, 1992). One central question in the study of nodule organogenesis is what regulates the new initiation of cortical cell divisions and the subsequent differentiation of a nodule.

A class of lipochitin oligosaccharide (LCO) molecules, also called nodulation or Nod factors, is synthesized by rhizobia (Lerouge et al., 1990). LCOs alone are sufficient for the induction of cortical cell divisions and early nodule primordia (Savouré et al., 1994; Schlamann et al., 1998; Spank, 1996). It is not known how LCOs stimulate cell division, but it is likely that LCOs act indirectly by perturbing the plant hormone balance (Hirsch, 1992; Hirsch and Fang, 1994). One particular focus has been on the involvement of auxin during nodulation. Auxin is synthesized in the shoot and transported, in a polar direction, to roots, where auxin levels can be regulated by breakdown, conjugation or transport (Bandurski et al., 1995; Lomax et al., 1995). The involvement of auxin in nodule organogenesis is likely in the stimulation of cell divisions and regulation of root differentiation (Dudits et al., 1993; Wightman et al., 1980), although it is likely...
that cytokinin is involved in addition to auxin (Hirsch and Fang, 1994).

The first indirect evidence for an involvement of auxin in nodulation came from experiments demonstrating that external manipulation of auxin transport with synthetic auxin transport inhibitors can lead to the formation of nodule-like structures (Allen et al., 1953; Hirsch et al., 1989). Recently, transgenic plants carrying an auxin-responsive promoter (GH3) fused to the GUS reporter gene have been used to localize auxin inside white clover roots during nodule organogenesis (Mathesius et al., 1998b). It was shown that rhizobia and their LCOs have a similar effect on the expression of GH3:gusA as a synthetic auxin transport inhibitor, suggesting that LCOs inhibit auxin transport during nodulation. This was supported by studies showing that LCOs reduce the auxin transport capacity in vetch roots (Boot et al., 1999). The expression patterns of GH3:gusA in white clover further indicated that auxin accumulates to high levels in cortical cells before their division and remains high in dividing cells until just after the first rounds of cortical cell divisions. During nodule primordium formation, GH3:gusA expression diminishes in the primordium (Mathesius et al., 1998b).

In the search for a secondary signal that could mediate the action of LCOs on the redistribution of auxin during nodulation, plant flavonoids have been suggested as possible candidates (Hirsch, 1992). Certain flavonoids can inhibit auxin transport (Jacobs and Rubery, 1988) and auxin turnover (Stenlid, 1963) in vitro. However, in these studies it was shown that flavonoid action depends strictly on their structure. With a multitude of flavonoids present in most plants (Stafford, 1990; Wollenweber and Dietz, 1981), it remained to be shown whether rhizobia could induce specific flavonoids during nodulation with action as regulators of auxin transport or turnover. Mathesius et al., therefore, tried to identify flavonoids in single cells during nodule organogenesis using microspectrofluorometry (Mathesius et al., 1998a). It was found that a derivative of 7,4’-dihydroxyflavone (DHF, Fig. 1) accumulates specifically in the vacuoles of a group of cortical cells which later undergo division to form a nodule primordium. These were the same group of cells also showing high expression of GH3:gusA, indicating high auxin levels. During nodule primordium formation, at a stage where GH3:gusA expression diminished, the isoflavonoid formononetin (Fig. 1) accumulated in the dividing cells (Mathesius et al., 1998a).

The strong temporal and spatial correlation between accumulation of specific flavonoids and the localization of auxin using the GH3:gusA reporter construct suggested that flavonoids could act as specific regulators of local auxin levels (Mathesius et al., 1998a, Mathesius et al., 1998b). Various flavonoids and other phenolic compounds had previously been shown to regulate the activity of IAA oxidases, including peroxidases, which regulate auxin breakdown in plants (reviewed by McClure, 1975; Rao, 1990; Schneider and Wightman, 1974). However, these studies have not connected the site of flavonoid location to a localized change in auxin levels.

To test the above hypothesis, the flavonoids which are locally induced by rhizobia were extracted from root tissue, purified using HPLC and tested for their ability to regulate IAA oxidase activity. A commercially available, purified horseradish peroxidase was used which is a strong IAA oxidase. Although horseradish peroxidase has a variety of substrates in the cell, its properties as an IAA oxidase have been extensively studied, making it a useful enzyme for IAA oxidase studies (Hinman and Lang, 1965; Schneider and Wightman, 1974). Based on the specific fluorescence properties of the auxin IAA and its breakdown products (Burnett and Audus, 1964; Hinman and Lang, 1965), a fast fluorometric assay was adapted for the quantification of IAA during the reaction with peroxidase in vitro.

### Materials and methods

#### Plant growth conditions

White clover plants (Trifolium repens cv. Haifa) were grown to maturity in potting mix in a glasshouse at temperatures between 26 °C (day) and 19 °C (night). Young leaves were excised from those plants, and roots were generated from the leaves (following a method by Rolfe and McVler, 1996). All so-called ‘rooted leaf’ plantlets were grown on sterile Petri dishes on Jensen’s medium (Jensen, 1942) in a growth chamber in an 8 h night, 16 h day cycle at 22 °C (day) and 18 °C (night). The light

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**Formononetin**

Fig. 1. Chemical structures of flavonoids induced by R. leguminosarum bv. trifoli in white clover roots.
intensity in the growth chamber was 120 \, \text{\mu E m}^{-2} \, \text{s}^{-1}. The roots were covered with dark paper to avoid light exposure. Roots were harvested at a length of 3–4 cm, which they reached after approximately 2 weeks of culture.

**Quantification of flavonoids using HPLC**

Roots of axenically-grown, rooted leaves were harvested, weighed and immediately frozen in liquid nitrogen and finely ground, after addition of glass powder (Schott Glass Jena, Germany), using a mortar and pestle. The powder was mixed with HPLC-grade methanol (BDH Chemicals, Pool, UK), using 2 ml of methanol for every 100 mg of fresh weight tissue. After vigorous shaking and centrifugation for 15 min at 7000 rpm at 4 °C, the supernatant was removed, evaporated under a nitrogen gas stream and the remaining substance redissolved in HPLC-grade methanol at 100 \, \text{\mu g} \, \text{ml}^{-1} \, \text{tissue}. The components of the root extracts were separated on a Hewlett Packard 1090 liquid chromatograph equipped with a C-18 column (Alltech Altima, 250 × 4.6 mm) using the following solvents: A = ultra pure water (Millipore, Australia) containing 0.09% trifluoroacetic acid; B = acetonitrile (HPLC purified) containing 0.09% trifluoroacetic acid. The column was eluted isocratically with solvent A for 5 min and a linear gradient of 0–100% B between 5 min and 25 min at ambient temperature with a flow rate of 1 ml min\(^{-1}\). Eluting compounds were monitored between 250 nm and 450 nm using a diode array detector. The different peaks of the HPLC chromatogram had previously been identified as DHF, a DHF derivative and formononetin and localized inside the roots (Mathiesius et al., 1998a). Commercially available DHF (Indofine Chemicals, Sommerville, NJ, USA) and formononetin (ICN Biomedicals, Plainview, NY, USA) were injected at different concentrations and the resulting peak areas calibrated against flavonoid concentrations. This calibration was used to determine the concentration of the extracted compounds in the injected volume of extract. Tissue concentrations of the extracted flavonoids were then determined by calculating the amount of flavonoid compound \( \text{g}^{-1} \) of tissue fresh weight, assuming fresh weight to be totally due to water content. This calculation slightly underestimated flavonoid concentrations, because the fresh weight also contained the small fraction of dry tissue weight, which was not measured here. All extractions were done three times and the results averaged.

**IAA oxidase assay**

The effect of flavonoids purified from white clover roots on the activity of horseradish peroxidase (Sigma Chemicals, St Louis, MO, USA), an IAA oxidase, was measured in a fluorometric assay using an SLM Aminco 8100 Spectrofluorometer (Rochester, New York). IAA exhibits typical fluorescence in the ultra violet range (Burnett and Audus, 1964). A solution of IAA in 0.1 M Na-phosphate buffer (pH 6; the pH was chosen for maximum activity of the peroxidase (Hinman and Lang, 1965)) was placed in a quartz cuvette and the fluorescence excitation and emission spectra measured at that pH. The excitation maximum at 285 nm was used for all assays as the excitation wavelength and the fluorescence emission intensity was measured at 357 nm, the emission maximum, using a 2 nm bandpass filter. The fluorescence emission was linearly correlated with the IAA concentration between 10\(^{-4}\) and 10\(^{-5}\) M IAA (Fig. 2A). 10\(^{-5}\) M IAA was used as the starting concentration, because at this concentration the breakdown rate was fast enough for a 10 min assay. At 10\(^{-6}\) M or lower concentrations of auxin, the breakdown rate was slower by several orders of magnitude which would have been impractical for a fast assay. Fluorescence emission intensity was then measured every minute for 2 s, over a 10 min period at 25 °C. The solution was retained in the cuvette in the dark and the sample was only illuminated during the 2 s measurement time. To the IAA solution, 5 × 10\(^{-7}\) M horseradish peroxidase and a flavonoid compound (both dissolved in the described phosphate

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**Fig. 2.** Fluorescence characteristics of IAA. (A) Standard curves for the fluorescence intensity of IAA detected at 357 nm. IAA fluorescence was linearly correlated to the IAA concentration. Fluorescence intensity of IAA was reduced after addition of 5 × 10\(^{-7}\) M horseradish peroxidase (P) or after addition of P and flavonoids to the IAA solution. A separate standard curve was measured for each added compound and used to calculate the IAA concentration from fluorescence units. The examples shown here are the curves for the highest tested concentration of each purified flavonoid. (B) Fluorescence spectrum of IAA recorded over a 10 min reaction period with peroxidase. The fluorescence emission spectrum does not change in the presence of horseradish peroxidase, whereas the total intensity decreases over time.
buffer) were added at time zero to test their effect on auxin breakdown.

**Statistical analysis**

To analyse the differences in the kinetic curves of IAA breakdown by peroxidase compared to the curves produced after the addition of flavonoid compounds, linear mixed effects modelling was used as implemented in S-Plus (Math Soft, 1999). A polynomial of degree three was used, different for each treatment, to model the changes in fluorescence over time. A random constant term, a linear term and a quadratic term was used, different for each of the three replicas.

**Results**

**Quantification of flavonoid contents in roots**

The concentrations of DHF and formononetin in whole roots were estimated at 1 μM and 10 μM, respectively. Tissue concentrations of the DHF derivative (in the following named DHF-X) was calibrated against DHF because both compounds show almost identical absorbance spectra (Mathesius et al., 1998a). The estimated concentrations of DHF-X from root extracts ranged between 10 μM and 100 μM, depending on the samples, averaged over the whole root.

**Fluorometric assay for the quantification of auxin during the reaction with peroxidase**

The fluorometric assay for the quantification of IAA showed a linear correlation between IAA concentration and fluorescence intensity in a concentration range between $10^{-5}$ and $10^{-6}$ M IAA (Fig. 2A). The addition of horseradish peroxidase or flavonoids to the solution of IAA diminished the fluorescence intensity due to their own absorbance, so that calibration curves for IAA in the presence of peroxidase and each flavonoid were established (Fig. 2A). The fluorescence emission spectrum of auxin did not change in the presence of peroxidase over the course of the peroxidase reaction (Fig. 2B), making it possible reliably to correlate fluorescence intensity to free levels of IAA. The addition of flavonoids at the concentration used had no effect on the emission spectrum of IAA (data not shown).

**Activity of flavonoids as regulators of IAA oxidase**

The ability of DHF, DHF-X and formononetin to affect the peroxidase-catalysed breakdown of IAA was measured in vitro. All experiments were repeated independently three times and showed almost identical results. A solution of IAA alone lost approximately 10% of its fluorescence intensity over the 10 min period, presumably due to unspecific oxidation or photodestruction (Fig. 3A, B, C). Addition of peroxidase to the IAA solution caused a rapid decrease of fluorescence intensity.
to approximately 50% of the initial IAA concentration over 10 min before reaching a saturation point (Fig. 3A, B, C).

Addition of 10 μM, 1 μM and 0.5 μM DHF to the solution of IAA and peroxidase significantly ($P < 0.0001$) inhibited the peroxidase activity over the 10 min period (Fig. 3A). The inhibition of IAA oxidase activity was strongest at 1 μM DHF, slightly less at 10 μM and weaker at 0.5 μM. Surprisingly, DHF at 0.1 μM significantly ($P < 0.0001$) accelerated the rate of IAA breakdown compared to the activity of peroxidase alone.

DHF-X almost completely ($P < 0.0001$) inhibited IAA breakdown during the 10 min incubation time at estimated concentrations of 20, 10 or 5 μM. The strongest inhibition was caused by a concentration of 10 μM. At 1 μM, the rate of breakdown was significantly ($P < 0.0001$) slower than after addition of peroxidase alone, but faster than after addition of 5 μM and above. At 0.1 μM, IAA breakdown was slightly faster than after addition of peroxidase alone, but the kinetic curve was not significantly different from the one produced by peroxidase alone ($P > 0.05$) (Fig. 3B).

The addition of formononetin at 100 nM to a solution containing IAA and peroxidase did not cause any significant ($P > 0.05$) change in the rate of IAA breakdown compared to addition of peroxidase alone (Fig. 3C). At 1 μM, formononetin caused a very small, but statistically significant ($P < 0.0001$), inhibition of IAA breakdown, although the shape of the kinetic curves in the presence and absence of this concentration of formononetin were not significantly different. Formononetin at 10 μM very significantly ($P < 0.0001$) increased the rate of IAA breakdown to the extent that the final IAA concentration was reached approximately twice as fast as with peroxidase alone (Fig. 3C).

**Discussion**

*Flavonoids of white clover roots affect IAA breakdown by a peroxidase*

The results show that flavonoids isolated from white clover root tissue had strong effects on the breakdown of the naturally occurring auxin IAA by a horseradish peroxidase. The horseradish peroxidase used in these experiments catalysed a rapid decrease in IAA breakdown over a 10 min incubation time. Both DHF and DHF-X, shown to accumulate in nodule progenitor cells (Mathiesius et al., 1998a) inhibited the IAA breakdown at concentrations between 1 and 10 μM for DHF and between 5 to 20 μM (estimated) for DHF-X. The rate of IAA breakdown in the presence of high DHF and DHF-X concentrations were in some cases even lower than the breakdown in the absence of peroxidase. This effect could be due to inhibition of unspecific oxidation of IAA by flavonoids, which have anti-oxidative properties (Wagner et al., 1988). At low concentrations (100 nM), DHF and DHF-X accelerated IAA breakdown by peroxidase. The tissue concentrations of DHF and DHF-X were estimated to be 1 μM and 10–100 μM, respectively, averaged over the whole roots. Local concentrations are likely to be higher because the distribution of the DHF derivative was found mainly in cells located just behind the root tip and in small groups of inner cortex cells (Mathiesius et al., 1998a). Therefore, DHF and DHF-X are likely to cause an inhibition of IAA breakdown in the groups of cells within which they are accumulating.

Similar results were obtained in *in vitro* assays with ferulic acid (Gortner et al., 1958) and with *p*-hydroxybenzoic acid (Pilet, 1966), which inhibited IAA oxidase at high concentrations, but accelerated IAA breakdown at low concentrations. Other flavonoids and phenolic compounds found to inhibit IAA oxidase include quercetin (Furuya et al., 1962; Stenlid, 1963), myricetin, morin, rutin, and taxifolin (Stenlid, 1963).

In contrast to the inhibition of IAA breakdown by DHF and DHF-X, formononetin accelerated IAA breakdown at concentrations of 10 μM, but not at lower concentrations. The concentration of formononetin in the root extracts was estimated at 10 μM, but again, the actual concentration in nodule primordium cells, that had previously been shown to accumulate formononetin (Mathiesius et al., 1998a), could be higher than 10 μM. Therefore, IAA oxidase activity is likely to be stimulated at the actual concentration of formononetin in the nodule primordium cells.

Other stimulators of horseradish peroxidase include *p*-hydroxybenzoic acid (Pilet, 1966), kaempferol derivatives (Furuya et al., 1962), methyl-umbelliferone (Andreae and Andreae, 1953), apigenin, daidzein, naringenin, biochanin A, and genistein at 0.3–30 μM (Stenlid, 1963). However, other studies show inhibitory action of genistein (at concentrations of 10–40 μM) on IAA oxidase (Ferrer et al., 1992), and the differences between different studies could be due to the IAA oxidases used for the assay which were isolated from different species and from crude extracts.

**Possible mechanisms for flavonoid action as IAA oxidase regulators**

There are several possible mechanisms by which flavonoids could regulate IAA oxidase, and both the structure and the concentration of each flavonoid could determine whether it inhibits or activates IAA oxidase. Flavonoids that inhibit IAA oxidase could act as alternative substrates for a peroxidase and protect IAA from oxidation (Kefeli and Dashek, 1984). For example, the coumarin scopoletin was shown to be an alternative
substrate for IAA oxidase (Andreae, 1952). Flavonoids could also inhibit IAA oxidation by scavenging hydrogen peroxide, which is produced in an initial oxidase action preceding peroxidase activity and is necessary for IAA oxidation (Galston et al., 1950; Grambow and Langenbeck-Schwich, 1983). Flavonoids also have strong anti-oxidative properties (Wagner et al., 1988; Hodnick et al., 1988) and could inhibit the formation of an IAA radical during IAA oxidation (Gelinas and Postlethwait, 1969). The acceleration of IAA oxidation could be due to an allosteric effect of flavonoids on peroxidase (Furuya et al., 1962). Alternatively, flavonoids in their antioxidative function could keep \( \text{Mn}^{2+} \), a cofactor of IAA oxidase, and \( \text{Fe}^{3+} \), which is part of the functional heme group of peroxidase, in a reduced and therefore active state (Schneider and Wightman, 1974).

**Local induction of specific flavonoids could be a mechanism to regulate auxin distribution during organogenesis**

So far, it is unknown how the plant regulates the distribution of auxin during developmental processes. For both lateral root (Greenwood et al., 1973) and nodule formation (Mathesius et al., 1998b), it has been shown that auxin accumulates in the first dividing cells of lateral root and nodule primordia, respectively. Later, during lateral root and nodule differentiation, auxin

![Diagram of auxin transport and localization](image)

**Fig. 4.** Model for the role of *Rhizobium*-induced flavonoids in the regulation of the auxin balance during nodule formation in white clover. The model combines the previous data on identity and localization of flavonoids and auxin (Mathesius et al., 1998a, b) with data on the possible link between flavonoid action and auxin turnover presented in this paper. *Rhizobium leguminosarum* bv. *trifolii* induces the synthesis of a DHF derivative (black) in the nodule progenitor cells, evident within 14 h post inoculation (p.i.). At the same time, rhizobia inhibit auxin transport in the root by a so far unknown mechanism. Presumably as a consequence, auxin levels rise (grey) in all cortex cells (around 24 h p.i.), but are reduced by concurrent activity of IAA oxidase(s). Cells containing high levels of a DHF derivative retain high auxin levels because the DHF derivative inhibits the action of IAA oxidase(s). After several rounds of cell divisions (around 72–96 h p.i.), the DHF derivative disappears from the nodule primordium and formononetin (stripes) accumulates. Formononetin stimulates or, at least does not inhibit, IAA oxidase activity. Therefore, auxin is destroyed in the nodule primordium by IAA oxidase. Some auxin remains in the periphery of the nodule.
levels diminish, presumably to allow differentiation and cell elongation to proceed (Laskowski et al., 1995). IAA oxidases are likely to be involved in regulation of auxin levels in the tissue (Schneider and Wightman, 1974), however, their activity would have to be regulated locally for local changes in auxin accumulation and breakdown to occur. The data presented here suggest that flavonoids locally induced during nodule organogenesis could affect auxin breakdown by IAA oxidase in small groups of cells. Flavonoid compounds would be very adaptable IAA oxidase regulators, because slight variations in their structure (Fig. 1) could lead to substantial changes in their activity. In Fig. 4, a model is suggested to explain local changes in auxin levels during nodule organogenesis through the regulation of IAA oxidases by flavonoids whose accumulation overlaps temporally and spatially with that of auxin.

Recently, it was shown that the same flavonoids accumulate during the early stages of lateral root development as during nodule development (Mathiesius et al., 2000), and similar flavonoid accumulation has been reported during lateral root formation in subterranean clover (Djordjevic et al., 1997). Similarly, flavonoids are accumulated in cells undergoing development into a root gall induced by parasitic nematodes, and again, the accumulation of flavonoid compounds overlaps spatially and temporally with changes in auxin distribution (Hutangura et al., 1999). Therefore, it is possible that the proposed regulation of auxin breakdown by local accumulation of specific flavonoids is a common mechanism in different organogenesis programmes, whether induced by plant developmental signals, symbiotic rhizobia or parasitic nematodes.

The suggested model assumes that peroxidase can come freely into contact with flavonoids in the cells and that the IAA oxidation in the cell is catalysed by an IAA oxidase that is similarly regulated by flavonoids as the horseradish peroxidase used in this assay. Different isofoms of peroxidases occur in most plants and these can be cell wall-bound or located in membranes, vacuoles or the cytoplasm (Mäder, 1992; Schneider and Wightman, 1974). DHF and formononetin could be located in membranes because they are non-polar. Thereby, they could have an effect on membrane-bound or cytoplasmic peroxidases which could metabolize cytoplasmically located IAA. Both free DHF and formononetin were found in root extracts (Mathiesius et al., 1998a) and could have non-local effects because they would not be restricted to the vacuole. The glycosidic DHF derivative was located in the vacuoles (Mathiesius et al., 1998a) and might therefore affect vacuolar peroxidases or peroxidases located in the tonoplast. However, IAA located in the vacuoles is probably conjugated and might therefore be protected from peroxidase attack (Cohen and Bandurski, 1982). Future experiments could attempt to determine the effect of the flavonoid compounds on peroxidases isolated from different cellular compartments of the root.

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