BIOLOGIA PLANTARUM 54 (3): 403-414, 2010

The responsiveness of the *IAA2* promoter to IAA and IBA is differentially affected in *Arabidopsis* roots and shoots by flavonoids

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Abstract

The structural features of flavonoids which are involved in the modulation of auxin distribution in *Arabidopsis thaliana* were evaluated. An auxin-inducible promoter *IAA2* fused to a reporter gene (*GUS*) was used to monitor the tissue responsiveness to auxins. The following aspects were investigated: *1*) the influence of flavonoids (quercetin, naringenin, kaempferol, myricetin and isorhamnetin) on the distribution of indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) in roots and leaves, *2*) differences in flavonoid uptake into roots and shoots depending on flavonoid concentration in the medium, and *3*) influence of structurally different flavonoids on the gravitropic response and growth of roots. The same flavonoids differently affected IAA and IBA distribution in leaves and roots. There were several structural requirements for the flavonoids which resulted in the changes of auxin response/distribution. Great differences between the ability of shoots and roots to take up quercetin were showed. Also, flavonoids influenced gravitropism and root growth of *Arabidopsis* seedlings in a structure-dependent manner.

Additional key words: isorhamnetin, kaempferol, myricetin, naringenin, phenolics, plant hormones, quercetin.

Introduction

Auxins are multifunctional plant hormones known to regulate many processes during plant growth and development (Davies 2004). They induce cell elongation and root branching, determine polarity in developing embryos, initiate development of lateral organs and formation of vascular tissue as well as plant architecture during growth (Costa and Dolan 2000, Reinhardt *et al*. 2000, Swarup *et al*. 2000). The content of indole-3-acetic acid (IAA) is regulated by a complex balance among biosynthesis, conjugation, catabolism and transport (Bartel *et al*. 2001, Ljung *et al*. 2002, Woodward and Bartel 2005, Delker *et al.* 2008). IAA is synthesized in the growing apical shoot region and is transported basipetally by a chemiosmotic polar transport mechanism that is regulated at the cellular level (Muday and DeLong 2001, Muday and Murphy 2002, Geisler and Murphy 2006). The polarity of auxin transport is believed to be controlled by the localization of auxin transport proteins, with both putative efflux carriers and influx carriers having asymmetric distributions (Gälweiler *et al*. 1998, Marchant *et al*. 1999, Murphy *et al*. 2002, Swarup *et al*. 2001, Young *et al*. 2006). The characterization of several *Arabidopsis thaliana* mutants in auxin influx and efflux carriers showed that these proteins are involved in the correct distribution of auxins within the plant (Geldner *et al*. 2001, Dharmasiri *et al*. 2006, Geisler and Murphy 2006). Recent evidence suggests that, in addition to the phytotropin *N*-(1-naphthyl) phthalamic acid (NPA; Zazimalova and Napier 2003), flavonoids may be involved in the regulation of auxin transport (Buer and Muday 2004, Peer *et al*. 2004).

Flavonoid effects on auxin transport were firstly suggested by Jacobs and Rubery (1988) in experiments where they displaced NPA from its binding site of the

Received 2 October 2008, *accepted* 9 June 2009.
Abbreviations: DMSO - dimethylsulphoxide; DPBA - diphenylboric acid 2-amino-ethylester; GAPDH - glycerinaldehyde-3-phosphate; GUS - β-glucuronidase; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; I - isorhamnetin; K - kaempferol; M - myricetin; MES - (2-morpholinoethanesulfonic acid) buffer; MU - 4-methyl umbelliferone; NPA - *N*-(1-naphthyl)phthalamic acid; N - naringenin; Q - quercetin; *tt4* - transparent testa 4; T - taxifolin; X-Glu - 5-bromo-4-chloro-3-indolyl glucuronide.
Acknowledgements: This work was supported by the German Ministry for Science and Education (BMBF; WTZ grant No. HRV 01/025) and by the German Academic Exchange Service (DAAD, PPP with Croatia). We would like to thank Kerstin Jentschel and Silvia Heinze for technical assistance.
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auxin transport protein by different flavonoids. Since then, several lines of evidence have accumulated that auxin transport could be modulated by flavonoids, although the exact mode of action is not yet clear. In a *transparent testa 4* (*tt4*) mutant, which is flavonoid deficient, the auxin transport rate in the mutant shoots was faster than in wild type (Brown *et al*. 2001, Buer and Muday 2004). In whole seedlings the pattern of auxin accumulation was altered, presumably due to higher amount of auxin that escaped from the roots in mutant seedlings compared to wild type (Murphy *et al*. 2000). In all experiments described above, the addition of naringenin was able to restore proper auxin accumulation. In addition, the pattern of IAA efflux carrier localization was altered in flavonoid deficient mutants (Peer *et al*. 2004). The correct localization of IAA efflux carriers, and subsequently the correct IAA distribution, was successfully restored by treatment with exogenous flavonoids.

Schwalm *et al*. (2003) examined the pattern of auxin and flavonoid distribution in tumors induced by *Agrobacterium tumefaciens* and found that chalcone synthase-dependent flavonoid aglycone formation is probably the endogenous regulator of the basipetal auxin flux and thus regulates auxin levels in tumors. Also, flavonoid deficient roots were unable to initiate root nodules, after inoculation with rhizobia (Wasson *et al*. 2006). Nodule formation was restored by the addition of naringenin. The flavonoid deficient roots had an increased auxin transport rate (Wasson *et al*. 2006). In petunia, flavonoids blocked presumably auxin efflux from germinating male gametophyte, thus increasing its intracellular content which in turn facilitates pollen tube polar growth (Kovaleva *et al*. 2007). *Arabidopsis* plants which accumulate several flavonol glycosides showed reduced growth which correlated with the level of inhibition of auxin transport (Besseau *et al*. 2007).

Indole-3-butyric acid (IBA) is an endogenous auxin in many plant species, among them *Arabidopsis thaliana* (Epstein and Ludwig-Müller 1993, Ludwig-Müller *et al*. 1993). Several physiological effects of IBA may be mediated by the hormone itself (Ludwig-Müller *et al*. 1995, 1997), but there is also evidence that IBA acts *via* the conversion to IAA by β-oxidation (Zolman *et al*. 2000, 2001, Zolman and Bartel 2004). Recent evidence suggests that IAA and IBA are transported differently (Rashotte *et al*. 2003).

Auxin-responsive promoters or elements coupled to a suitable reporter gene have been used to characterize the auxin response (Gee *et al*. 1991, Hagen *et al*. 1991, Larkin *et al*. 1996) and they are frequently used to get a readout of auxin distribution in tissues as well (Dubrovsky *et al*. 2008, Weijers *et al*. 2005). In this work *IAA2::GUS Arabidopsis* plants (*Arabidopsis* containing the auxin-responsive promoter *IAA2* coupled to the β-glucuronidase reporter gene) exposed to IAA or IBA were treated with structurally related flavonols (isorhamnetin, kaempferol, myricetin, and quercetin) and flavanones (taxifolin, naringenin) as well as with two synthetic derivatives of naringenin. This way we wanted to evaluate the structural features of flavonoids needed to modulate the auxin response and distribution. Since NPA acts as a competitive inhibitor of flavonoid binding, we also carried out an experiment on plants treated with this phytotropin. The following aspects were investigated: *1*) distribution of the two different auxins IAA and IBA in roots and leaves, *2*) differences in flavonoid uptake in roots and shoots depending of flavonoid concentration in medium and *3*) influence of structurally different flavonoids on auxin distribution as well as gravitropic response and growth of roots.

Materials and methods

Plants: The *Arabidopsis IAA2*-promoter-GUS fusion (*IAA2::GUS*) was kindly provided by Dr. Jennifer Normanly (Amherst, MA, USA). All other *Arabidopsis* ecotypes and mutants were from the Nottingham Arabidopsis Stock Center (NASC). Seedlings were grown on Murashige and Skoog (1962; MS) agar medium in a growth chamber at 16-h photoperiod with irradiance of *ca*. 100 μmol m^{-2} s^{-1} (*Philips* fluorescent tubes *TL55 daylight* and *TL32 Warmton de Luxe*) and temperature of 23 °C. Three-week old seedlings were used in all experiments.

Flavonoids: All tested flavonoids (quercetin, naringenin, kaempferol, myricetin and isorhamnetin) were dissolved in dimethylsulphoxide (DMSO) as 0.01 M stock solutions and stored at 4 °C. Stock solutions were diluted in MS medium to achieve final concentrations of 10^{-5} M and 10^{-4} M. Isorhamnetin was purchased from *Fluka* (Buch**,** Switzerland) and all other flavonoids from *Sigma* (Munich, Germany). 6-Dimethyl-allylnaringenin and 8-prenylnaringenin were synthesized according to Gester *et al*. (2001) and provided by Prof. Dr. Peter Metz, Technische Universität, Dresden, Germany.

Seedling growth and gravitropism under the influence of flavonoids: *Arabidopsis thaliana* seedlings were grown on MS agar (controls) or MS agar supplemented with 10^{-4} M or 10^{-5} M of various flavonoids. Control plants had equivalent DMSO concentration as treated plants. After 3 weeks the length of root as well as fresh mass of 30 plants were measured. For gravitropic response the plates were placed in a vertical position during this time. To measure the gravitropic curvature, the plates were turned in a 90° angle, and after an additional 24 h of growth the angle of gravitropic curvature was measured in 30 seedlings per treatment.

Analysis of quercetin in shoots and roots: Stock

solutions of quercetin were diluted in liquid MS medium to achieve concentrations of 10^{-3}, 10^{-4} and 10^{-5} M. To remove undissolved flavonoid particles the medium was centrifuged for 5 min at 10 000 *g* at room temperature. The concentration of quercetin actually present in the supernatant was determined by HPLC before it was taken for plant incubation. For each treatment 20 plants were incubated for 60 min at room temperature. Control plants were incubated in MS medium supplemented with 0.1 % DMSO at the same conditions. After incubation plants were rinsed two times with 96 % ethanol and afterwards washed two times with 6.25 % aqueous solution of DMSO and dried on filter-paper. After this procedure shoots were separated from roots and the tissue was homogenized with 96 % ethanol (1:10, m/v) using *TissueLyser* (1 min, 30 Hz). The homogenate was left for approximately 20 h (over night) at 4 °C and then centrifuged (20 min, 10 000 *g*, 4 °C) and the supernatant was subjected to HPLC analysis. The plants which were grown in the greenhouse were submitted to the same extraction procedure to determine the endogenous concentration of quercetin in *Arabidopsis* plants.

HPLC system (*Agilent 1100*, *Agilent Technologies*, Santa Clara, USA) equipped with a quaternary pump, multiwave UV/Vis detector, autosampler, fraction collector and 5 μm *Zorbax RX-C18* (250 × 4.6 mm, *Agilent Technologies*) column was used. Injection volume was 0.1 cm^3 and the constant flow rate was 1.0 cm^3 min^{-1}. Quercetin was identified by UV/Vis spectroscopy and by HPLC chromatography with authentic standards. The multiwave UV/Vis detector was set at 268, 280, 310, 350, and 374 nm. A three-solvent gradient elution was performed. The solvent compositions used were A - water : ACN : formic acid (94:5:1; v/v), B - water : ACN : methanol : formic acid (50:24.5:24.5:1) and C - ACN : formic acid (99:1). Prior to each run, the system was equilibrated to 90/10/0 (A/B/C). The solvent composition changed according to the following gradient: 90/10/0 at 0 min, 70/30/0 at 10 min, 0/100/0 at 20 min, 0/0/100 at 36 min and 0/0/100 at 41 min. The concentration of quercetin was determined based on the chromatographic data of the standard. The calibration curve (peak area *vs*. concentration) was obtained for a wide linear concentration range.

Distribution of quercetin and kaempferol in *Arabidopsis* wild type and *tt4* seedlings: *Arabidopsis* wild type and *tt4* seedlings (3 weeks old) were incubated for 1 h in 0.1 M MES buffer, pH 6.0, supplemented with either 100 μM quercetin or kaempferol. After the plants were rinsed thoroughly several times with distilled H_2O, flavonoids were visualized *in vivo* by the fluorescence of flavonoid-conjugated diphenylboric acid 2-amino-ethylester (DPBA) after excitation with blue radiation. DPBA fluoresces orange under wavelength of 366 nm when bound to quercetin and green when bound to kaempferol (Buer and Muday 2004). The plants were stained for 5 min using a saturated aqueous solution of DPBA (0.5 %, m/v) to which 0.005 % (v/v) *Triton X-100* was added. Seedlings were then washed for 5 min with 0.1 M sodium phosphate buffer (pH 7.0) containing 0.005 % (v/v) *Triton X-100*, and directly photographed under a binocular microscope.

Determination of β-glucuronidase activity: The pattern of auxin distribution was determined by histochemical staining of plants with 5-bromo-4-chloro-3-indolyl glucuronid (X-Gluc) (Jefferson 1987). After 1 h incubation in liquid MS medium or IAA (10^{-4} M) supplemented MS medium with or without flavonoids (10^{-4} or 10^{-5} M) the plants were incubated at 37 °C for 1 h in 0.1 M $NaPO_3$ buffer, pH 7.4, containing 10 mM Na_2EDTA, 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$, 0.5 % *Triton X-100* and 1 mM of substrate (X-Gluc dissolved in DMSO). Plants were further incubated in 100 % acetone (30 min), and transferred to a $NaPO_3$ buffer without the substrate over night to block the reaction. After each incubation step, plants were rinsed with water. In parallel experiments plants were incubated in MS containing 10^{-4} M IBA, to check if *IAA2* promoter is IBA inducible. Also the plants were incubated in MS containing 10^{-4} M NPA, (*OlChemIm*, Olomouc, Czech Republic), to compare the effect of artificial auxin transport blocker to the effect of different naturally occurring flavonoids. Every sample was tested in three replicates.

Real time RT-PCR of the *IAA2* gene: Total RNA from *Arabidopsis* seedlings was isolated using RNeasy plant mini kit (*Qiagen*, Hilden, Germany). Plants were incubated in liquid MS medium (MS) supplemented with flavonoids (10^{-5} M) and IAA or IBA (10^{-4} M). In control experiments total RNA was isolated from seedlings incubated in liquid MS medium or in MS medium containing 10^{-4} M IAA or IBA, respectively. Since our real time RT-PCR results proved that *IAA2* expression does not increase significantly after 1 h incubation period (data not shown), plants in all of our experiments were incubated for 1 h. Each group of plants incubated under the different conditions consisted of six intact *Arabidopsis* plants.

Reverse transcription polymerase chain reaction (RT-PCR) with random hexamer primers was performed using the *GeneAmp* RNA PCR kit (*Applied Biosystems,* Foster City, USA) according to the manufacturer's instructions. Primers for two housekeeping genes actin and glycerin-aldehyde-3-phosphate (GAPDH) and primers for the IAA2 gene used in real time PCR were designed using Primer Express software (IAA2: F 5'-CCT CCT ACC AAA ACT CAA ATC GTT-3', R 5'-CGT AGC TCA CAC TGT TGT TGT TCT-3'; ACTIN: F 5'-TGA GAG ATT CAG ATG CCC AGA-3', R 5'-TGG ATT CCA GCA GCT TCC AT-3'; His2a: F 5'-GAC AAG AAG AAG CCT ATC ACT CGT T-3', R 5'-CAG ACG ATG CAC CCT ACC AA-3'; GAPDH F 5'-GCG GTG CCA AGA AAG AAG TTA TAA TTT-3', R 5- TGC TCG TTT ACT CCA ACA ACA AA-3'). Real time PCR was performed in an *ABI PRISM 7700* sequence detector in a 0.025 cm^3 final volume containing

0.012 cm^3 *Usybr Green PCR Master Mix* (*Applied Biosystems*), 150 nM actin primers (150 nM GAPDH, 200 nM His2a), 100 nM IAA2 primers and 0.005 cm^3 of 5 times diluted cDNA. Amplification was performed using the following cycling conditions: 2 min 50 °C, 10 min 95 °C, and 40 two-step cycles of 15 s at 95 °C and 60 s at 60 °C. Triplicate real time RT-PCR analyses were executed for each sample, and the obtained threshold cycle values (Ct) were averaged. According to the comparative Ct method gene expression was normalized to the expression of the house keeping gene, yielding the ΔCt value. The average ΔCt value obtained from non-treated samples (MSO) was then subtracted from the average ΔCt value of each sample subjected to the experimental conditions described, yielding the ΔΔCt value. The gene expression level, normalized to the housekeeping gene and relative to the control sample, was calculated by $2^{-\Delta\Delta Ct}$.

Results

The *IAA2* promoter is equally induced in *Arabidopsis* seedlings by IAA and IBA: The *IAA2* promoter was chosen because it generally gave stronger signals than the synthetic *DR5* promoter under our experimental conditions. *IAA2::GUS Arabidopsis* plants were treated for various incubation times with different concentrations of IAA or IBA. Histochemical GUS staining of plant roots was weakly visible in lateral root tips in control plants (Fig. 1*A*) and increased firstly in lateral roots in auxin treated plants. After 1 h incubation with either 10^{-4} M IAA or IBA a strong blue colour was observed throughout the root, especially in the vascular tissue (Fig. 1*B*,*C*). Since the staining intensity did not increase with longer incubation time (data not shown), all plants used in our experiments were incubated for 1 h with 10^{-4} M of the respective auxin in the following

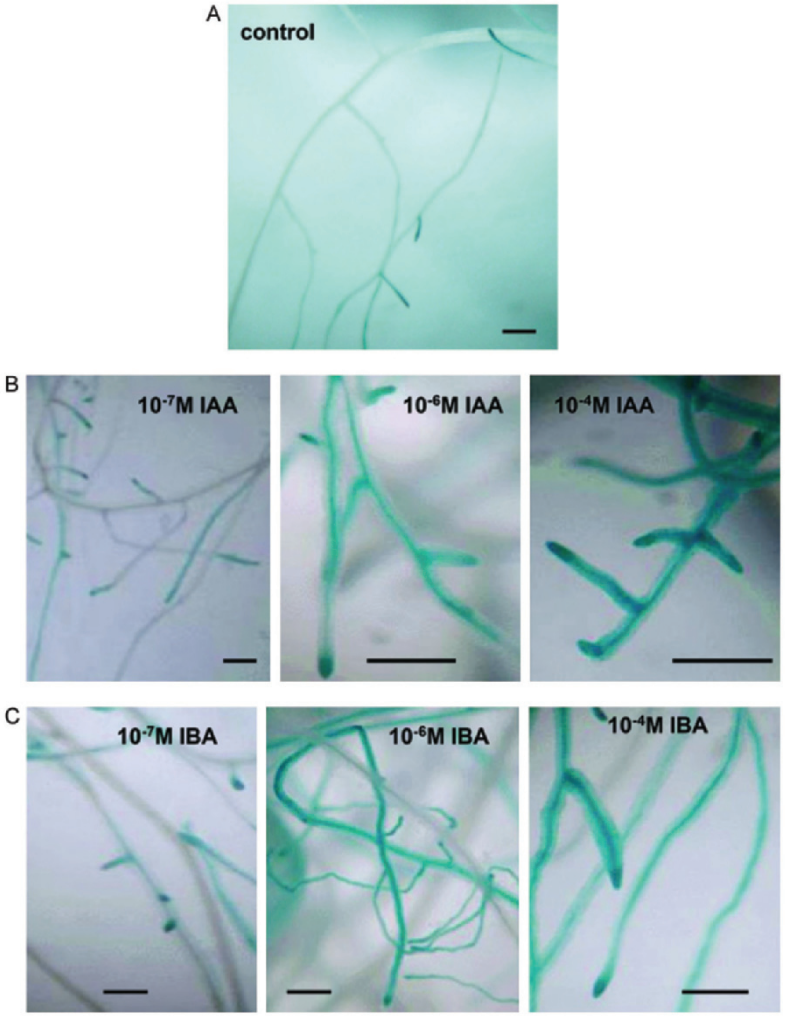

Fig. 1. The distribution of auxin shown by GUS staining in *IAA2::GUS* roots of *Arabidopsis thaliana* in control plants (*A*), plants incubated for 1 h with 10^{-7} M, 10^{-6} M, and 10^{-4} M IAA (*B*) and IBA (*C*). The *scale bar* is always 1 cm.

Table 1. Flavonoids are taken up into shoots and roots of *Arabidopsis* seedlings. Three-week-old *Arabidopsis* seedlings were incubated for 1 h in MS medium in which quercetin stock solutions (0.01, 0.1 and 1 M) were diluted (1:1000, v/v). The really achieved concentration of quercetin in such quercetin supplemented MS medium as well as achieved concentrations of quercetin in shoots and roots after incubation were determined by HPLC.

	Medium			Shoots			Roots		
Declared concentration of quercetin [M]	0.01	0.10	1.00	0.01	0.10	1.00	0.01	0.10	1.00
Real concentration of quercetin [μM]	2.93	4.14	5.94	2.72	3.71	5.43	60.32	112.43	108.23

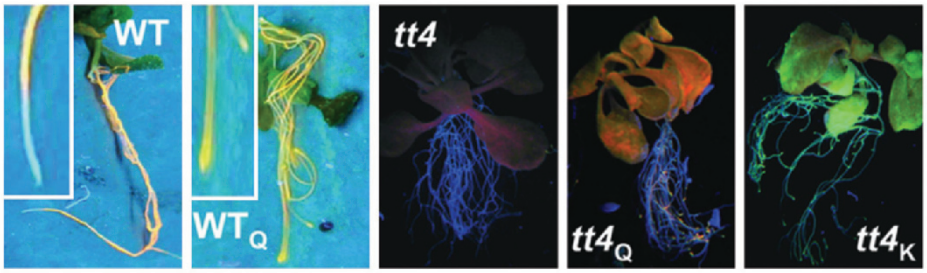

Fig. 2. *In vivo* staining of quercetin with DPBA after incubation of wild type (WT) and *tt4* seedlings for 1 h with 10^{-4} M quercetin (Q) or kaempferol (K). Controls were incubated for the same period with buffer (MES buffer, pH 6.0). The inserts show the root tip of wild type without and with quercetin at a higher magnification for better comparison.

experiments with flavonoids, and the short incubation time should also rule out a herbicidal effect of flavonoids on the tissue.

Quercetin is taken up by *Arabidopsis* seedlings: To analyze whether the flavonoids can enter the seedling tissue and how this process is influenced by the flavonoid concentration in the medium, we incubated three-week-old *Arabidopsis* seedlings with 10^{-5}, 10^{-4} and 10^{-3} M quercetin for 1 h and determined the concentration of quercetin in shoots and roots. The really achieved concentrations of quercetin in the medium in which the flavonoid stock solutions were dissolved (1:1000, v/v) was also determined. HPLC analysis revealed that concentrations of quercetin really achieved in the medium were significantly lower compared to expected concentrations (Table 1). Concentrations of quercetin determined in quercetin treated shoots were in perfect correlation with real concentrations of quercetin in medium. On the other side, quercetin concentrations in quercetin treated roots were more than 20 fold higher compared to real concentrations of quercetin in medium or to those in quercetin treated shoots. According to the results of HPLC analysis quercetin was below the detection limit in control plants incubated in MS medium without quercetin as well as in untreated greenhouse-grown plants. It should be pointed out that HPLC analysis revealed traces of quercetin in hydrolyzed extracts of both of these control plants (data not shown) indicating that quercetin is present in the glycosylated form. Additionally, we performed experiments using *in vivo* staining with DPBA after incubation of wild type and *tt4* seedlings with 100 μM quercetin or kaempferol for 1 h (Fig. 2). *tt4* plants were used because in the flavonoid free background the staining was better visible, but we also wanted to demonstrate that the uptake was not restricted to the mutant. Enrichment of quercetin and kaempferol was observed in root tips of wild type (only shown for quercetin because kaempferol was overlaid by the fluorescence of quercetin) and *tt4*, however, distribution of both flavonols throughout the root system was observed. Also the leaves accumulated flavonols as shown for *tt4* seedlings.

Influence of flavonoids on auxin distribution: We used auxins at 10^{-4} M concentration because the staining patterns were best visible and changes in auxin distribution caused by flavonoids could be better monitored. Leaves from *IAA2::GUS* control plants (MS incubated) showed a slight blue colour due to the auxin naturally present in the seedlings. However, the seedlings had to be removed carefully from the MS medium because the *IAA2* promoter was easily inducible by damaging the plants. Incubation of *Arabidopsis* seedlings with 10^{-4} M IAA resulted in an increased blue staining of the leaves (Fig. 3*A*). In most cases the leaves were completely stained, however, in some cases only the lower part of the leaves and the vascular bundle showed GUS activity. Treatment with flavonoids at equimolar concentration (10^{-4} M) reduced the GUS staining to control level; only the lower part of the rosette leaves was stained and the staining was limited to the vasculature (Fig. 3*B*). Although all investigated flavonoids modulated IAA distribution, quercetin seemed to be the most effective substance. Also, the effect was concentration dependent, *i.e.* flavonoids at 10^{-5} M concentration did not have a visible effect (data not shown). The phytotropin NPA also reduced GUS staining in the leaves (Fig. 3*B*) in

a similar manner as did quercetin and the other flavonoids.

After treatment with IBA and flavonoids we observed only slight effects on the distribution of IBA within the rosette leaves, but the GUS staining was more pronounced in the main vasculature and the petiole (Fig. 3*C*). Again, incubation of the seedlings with 10^{-4} M IBA resulted in an increase in blue colour, which was more or less evenly distributed throughout the leaves. The treatment with quercetin is shown as an example (Fig. 3*C*), but also other tested flavonoids had the same effect (data not shown). To our knowledge this is the first report about effects of flavonoids on IBA distribution in plants. In the experiments presented here higher concentrations of auxins and flavonoids were used to make our observations visible for documentation, but in Fig. 3*C* we also show an example for induction of *IAA2::GUS* with IBA at 10^{-5} M and 10^{-4} M quercetin. Even though the GUS staining was less well developed when lower concentrations of IBA were used, the effect of quercetin on the distribution of GUS activity could be seen. As with higher concentrations, quercetin reduced GUS staining in the leaf and it was restricted to the petiole and basis of the main vascular bundle.

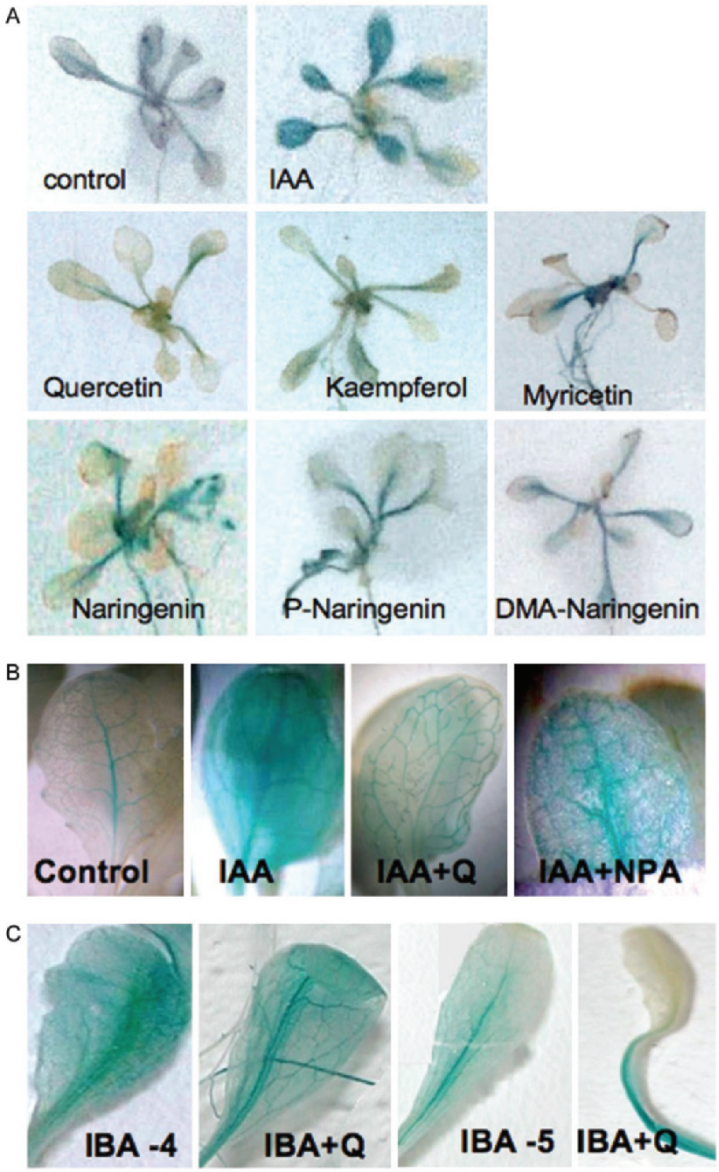

Fig. 3. *A* - Visualization of the IAA distribution in rosettes of *IAA2::GUS Arabidopsis thaliana* plants under control conditions and after treatment with either 10^{-4} M IAA alone or in combination with various flavonoids at 10^{-4} M. *B* - Close up picture of leaves of *IAA2::GUS* plants under control conditions and after treatment with 10^{-4} M IAA, IAA together with equimolar concentrations of quercetin (Q), and IAA together with equimolar concentrations of the phytotropin naphthylphthalamic acid (NPA). *C* - Close up picture of leaves of *IAA2::GUS* plants after treatment with 10^{-4} M IBA and 10^{-4} M IBA together with 10^{-4} M quercetin (Q) as well as 10^{-5} M IBA and 10^{-5} M IBA together with 10^{-4} M quercetin (Q).

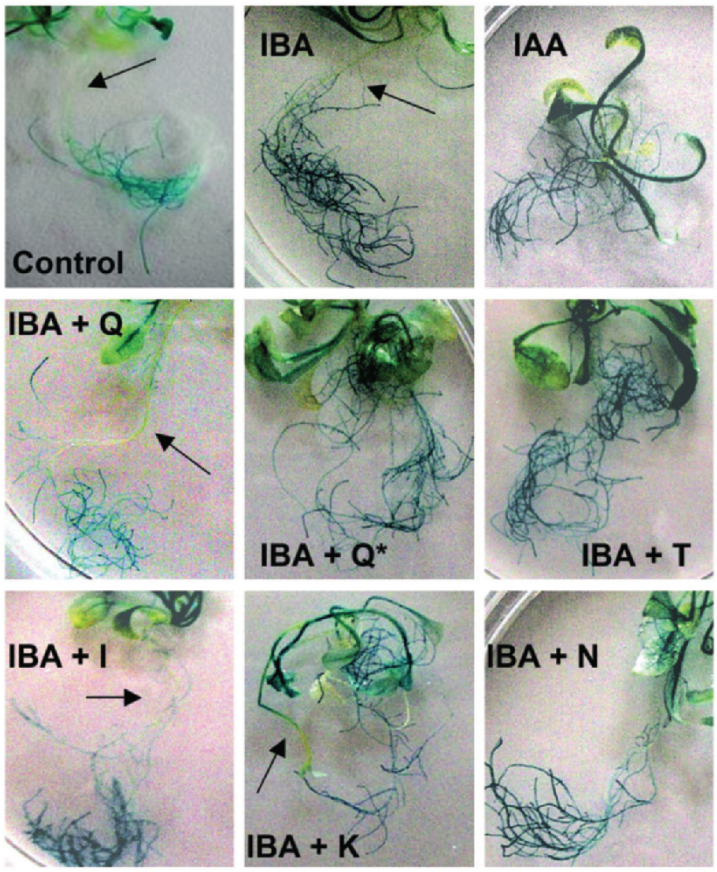

Fig. 4. Visualization of the auxin distribution in roots of *IAA2::GUS* plants under control conditions and after treatment with either 10^{-4} M IAA and IBA alone or with IBA in combination with various flavonoids at concentrations 10^{-4} M (Q - quercetin, T - taxifolin, I - isorhamnetin, K - kaempferol, N - naringenin) or 10^{-5} M quercetin (Q*). The same results were obtained when IAA was used (data not shown). The *arrows* mark the unstained regions in the control and in flavonoid treated roots.

Table 2. Real time RT-PCR analysis of *IAA2* expression in *IAA2::GUS Arabidopsis* plants incubated in MS medium supplemented with 10^{-5} M IAA/IBA with addition of 10^{-5} M flavonoids (Q - quercetin, K - kaempferol, M - myricetin, T - taxifolin, I - isorhamnetin, N - naringenin, 8PN - 8-prenylnaringenin) or in MS medium supplemented with flavonoids only. As an internal control the actin housekeeping gene was used, but the same results were obtained with GAPDH (see Materials and methods). Relative fold ratios are presented as mean ± SE of three experiments. Data obtained for negative (plants incubated in MS medium) and positive controls (plants incubated in MS medium supplemented with 10^{-4} M IAA/IBA) are following: negative control 1; positive controls 11.51 ± 3.53 (IAA), 7.72 ± 2.07 (IBA).

	Q	K	M	T	I	N	8PN
MS + IAA + flavonoids	10.66 ± 0.54	11.48 ± 0.64	10.60 ± 0.77	12.55 ± 1.43	9.66 ± 0.94	11.49 ± 2.15	8.74 ± 0.81
MS + IBA + flavonoids	3.11 ± 0.54	1.59 ± 0.19	2.02 ± 0.42	1.93 ± 0.29	2.50 ± 0.47	1.27 ± 0.06	2.68 ± 0.66
MS + flavonoids	7.51 ± 2.43	9.28 ± 0.99	6.11 ± 0.84	6.46 ± 1.48	7.96 ± 2.08	7.98 ± 3.79	7.65 ± 1.87

Interestingly, the roots of control plants were stained in the basal part to some extent already without auxin treatment, whereas the upper part did not show any staining, as indicated by an *arrow* (Fig. 4). Incubation with both auxins resulted in blue staining of whole roots, especially of their lower parts. The staining in the part of roots was completely reduced after simultaneous treatment with both auxins and flavonoids (Fig. 4, see *arrows*) and again this was concentration dependent as shown for quercetin. In contrast to our results obtained for leaves, this effect in roots was dependent on the flavonoid structure. While the flavonol quercetin was most active, kaempferol and isorhamnetin gave similar results, but much weaker in their reduction of GUS

staining, and the two flavanones naringenin and taxifolin were not effective (Fig. 4). The same results were obtained for IAA (data not shown).

Rosmarinic acid and caffeic acid, two strong antioxidants, did not inhibit auxin-induced GUS activity in any of the tested tissues (data not shown) showing that the inhibitory effect is not simply due to the antioxidative activity.

Expression of *IAA2* after auxin and flavonoid treatment: To test the hypothesis that flavonoids affect auxin transport but not the expression of the *IAA2* gene, we examined *IAA2* expression by RT-PCR. Plants were incubated in MS medium containing 10^{-4} M IAA or IBA with or without addition of various flavonoids at 10^{-5} M concentration. The reaction was performed using three housekeeping genes (histone, actin and GAPDH), but since the expression of the histone gene was induced by flavonoids we excluded it from the analysis. While the addition of IAA and IBA induced *IAA2* gene expression, the addition of flavonoids did not further enhance this effect and did not induce *IAA2* gene expression when applied alone (Table 2). These data were confirmed with 10^{-4} M of flavonoid treatment by RT-PCR (data not shown). The results suggest that tested flavonoids affect auxin distribution but have no effect on *IAA2* gene expression.

Effect of long term flavonoid treatment on seedling growth and gravitropism: In addition to the above experiments, where the seedlings were treated only for short periods with auxin and flavonoids, we have also investigated the long term effect of flavonoids on seedling growth and gravitropism. All flavonoids inhibited root but not shoot growth at both concentrations (10^{-5} and 10^{-4} M) tested. Most effective in this sense were isorhamnetin and myricetin at a concentration of 10^{-4} M (Table 3). The fresh mass of plants growing on media supplemented with flavonoids (10^{-4} M), was significantly reduced, except with naringenin (Table 3). Lower concentration (10^{-5} M) of most flavonoids tested did not cause any significant changes in fresh mass of plants. Quercetin and naringenin at lower concentration slightly promoted growth.

Because auxin transport is important for the gravitropic response and because effects of flavonoids on the gravitropic bending were previously reported, we tested the flavonoids used in this study for their ability to inhibit the gravitropic curvature. At concentrations of 10^{-5} M only quercetin, and to some extent kaempferol, inhibited the gravitropic response whereas 10^{-4} M quercetin, kaempferol and myricetin reduced the bending angle (Table 3). Even at this rather high concentration naringenin and isorhamnetin did not inhibit the gravitropic response.

Table 3. Growth of *Arabidopsis thaliana* seedlings on media containing flavonoids. *Arabidopsis* seedlings were grown on MS medium (controls) or MS medium supplemented with 10^{-5} M or 10^{-4} M flavonoids (Q - quercetin, N - naringenin, K - kaempferol, M - myricetin, I - isorhamnetin). Control plants had equivalent DMSO concentration as flavonoid treated plants. Per treatment 30 seedlings were measured and the mean values are given. The length of root and fresh mass were measured after 3 weeks. To measure the gravitropic curvature the plates were turned in a 90° angle and after an additional 24 h of growth the bending angle of 30 seedlings was measured.

Flavonoids	Q	N	K	M	I	Q	N	K	M	I
Concentration	10^{-5} M					10^{-4} M				
Root length [% of controls]	88.3	60.5	27.4	47.2	30.6	65.8	55.1	38.4	18.3	20.0
Fresh mass [% of controls]	122.5	121.4	77.4	115.7	92.1	45.5	95.6	52.7	54.3	56.5
Angle of curvature [degrees]	32.5	85.8	43.1	65.2	78.4	5.3	80.6	2.7	2.6	87.3

Discussion

Flavonoids play a number of roles in plants, such as pigmentation (Grotewold *et al*. 1994), male fertility (Winkel-Shirley 2001b), seed dormancy (Debeaujon *et al*. 2000), protection from UV-radiation (Winkel-Shirley 2001b), and in plant-microbe and plant-plant interaction defining host specificity and recognition (Winkel-Shirley 2001b). Recently, it has been suggested that flavonoids act as regulators of polar auxin transport (Murphy *et al*. 2000, Brown *et al*. 2001, Peer *et al*. 2004, Buer and Muday 2004, Besseau *et al*. 2007). They may be involved in the induction of root nodule formation or growth by increasing auxin content through efflux inhibition (Mathesius 2001). Similar hypothesis have been brought forward for *Agrobacterium tumefaciens* infection of plant tissues and tumor formation (Schwalm *et al*. 2003).

The use of auxin-inducible promoter-reporter systems is widely used in the literature to monitor auxin contents and distribution (*e.g*. Ulmasov *et al*. 1997, Sabatini *et al*. 1999, Weijers *et al*. 2005, Schwalm *et al*. 2003, Dubrovsky *et al*. 2008). However, in reality the GUS activity monitors the response of the auxin-inducible promoter to endogenous and applied auxin and only indirectly auxin accumulation. This has to be kept in mind when interpreting the findings presented here. The expression of the auxin-inducible *IAA2::GUS* marker (Luschnig *et al*. 1998, Swarup *et al*. 2001, Marchant *et al*. 2002) has been used in this study to monitor auxin

distribution/responsiveness within the plant tissue under the influence of different flavonoids. It was possible to use *IAA2* because flavonoids themselves did not alter *IAA2* gene expression significantly (Table 2). We hypothesized that the distribution of GUS activity should be altered if auxin transport inhibitors were applied simultaneously with auxin. Auxin could be trapped within the cells, thus increasing GUS levels. Buer and Muday (2004) showed that the differences in *DR5::GUS* expression in Col and *tt4*(2YY6) were not due to differences in sensitivity but rather to differences in auxin transport. Since it has been described that IAA and IBA are transported differently in *Arabidopsis* (Rashotte *et al*. 2003) we have compared IAA and IBA for their altered distribution after flavonoid application. IBA, like 2,4-D, does not seem to use the IAA efflux carrier(s), as shown by experiments using the *eir1*/*agr1*/*pin2* mutant (Utsuno *et al*. 1998, Poupart and Waddell 2000). In the *Arabidopsis rib1* mutant the transport of IBA was reduced to approximately 60 % of that in wild type plants, but IAA transport was unaltered (Poupart *et al*. 2005).

In *Arabidopsis* the biosynthetic pathway of flavonoids leads from naringenin to the formation of quercetin and kaempferol (Winkel-Shirley 2001a). The flavonoids used in this study were the flavanone naringenin, its chemical derivatives 8-prenyl and 6-dimethylallyl-narigenin as well as taxifolin, a naturally occurring flavanone in other plant species. In addition, the flavonols quercetin, kaempferol, myricetin and isorhamnetin were employed. We were interested to test which structural features of the flavonoids seem to be required for induction of the investigated physiological responses in *Arabidopsis*. Our results show that *Arabidopsis* seedlings can take up quercetin in shoots and roots but with different capacities (Table 1, Fig. 2). Moreover, roots were shown to be able to take up quercetin against a concentration gradient suggesting active transport of this substance into the root. The mechanism of this transport remains to be elucidated. Buer *et al*. (2007) also showed that flavonoids are taken up and distributed/transported in *Arabidopsis*. Flavonoids accumulated in root tips of plants with light-grown shoots and light-shielded roots, consistent with shoot-to-root flavonoid movement. Flavonoids also accumulated in tissues distal to the application site, indicating uptake and movement systems. Naringenin was taken up at the root tip, midroot, or cotyledons and traveled long distances *via* cell-to-cell pathway to distal tissues, whereas kaempferol and quercetin were only taken up at the root tip (Buer *et al*. 2007). However, since differential permeation of plant tissues to the flavonoid compounds used occurred, we cannot completely rule out that some of the effects described here are due to differential uptake.

In most cases leaves of *Arabidopsis* seedlings were completely stained after IAA/IBA application. Treatment with flavonoids at equimolar concentrations (10^{-4} M) reduced the GUS staining to control levels in the IAA treated leaves. Mostly the vasculature and the lower part of the rosette leaf was stained (Fig. 3*B*). With respect to altered auxin response in leaves quercetin seemed to be the most effective substance in modulating IAA transport/efflux. NPA also reduced GUS staining in the leaves (Fig. 3*B*) in a similar manner as did quercetin. The inhibition may be attributed to the structural similarity of NPA to flavonoids, but this has yet to be established. On the contrary, when IBA was used the effect was not so pronounced (Fig. 3*C*) indicating different mechanisms of distribution for IAA and IBA in leaves. The situation was different for roots. While the upper part of control roots adjacent to the hypocotyls did not show staining without auxin treatment, the basal part of control roots stained to some extent already without auxin treatment (Fig. 4). After IAA/IBA application the whole root system stained blue. Only the auxin signal in that part of the root which did not stain in control roots was affected by flavonoids and this was independent from the auxin used (Fig. 4). While the flavonols quercetin, kaempferol and isorhamnetin gave similar results in reduction of GUS staining, the two flavanones naringenin and taxifolin were not effective. These results indicate that *1*) there is a tissue specific effect, *2*) in leaves there is an auxin type dependent effect, and *3*) in roots there is a flavonoid structure dependent effect on auxin distribution; the double bond between C2-C3 position seems to be essential whereas the hydroxyl group at C3' could enhance this effect.

Flavonoids are known modulators of auxin transport but the mechanism by which they interfere with auxin efflux complex is not yet clear. Recently, Bailly *et al*. (2008) have shown that these compounds disrupt protein-protein interaction between TWD1 (immunophilin-like) and PGP1 (P-glycoprotein). It is known that TWD1 acts as a positive regulator of PGP mediated auxin efflux. Quercetin was the most effective substance among the flavonoid tested and this fact is in agreement with our findings.

Our previous results (Rusak *et al*. 2005) showed that several biological effects of flavonoids were dependent on their structure. In addition to the C2-C3 double bond, methylation and hydroxylation at the B-ring can change the bioactive properties of the molecule. In general the flavonols were more active than the flavanones in affecting auxin distribution which is also true for their antioxidative properties, leading to the assumption that the C2-C3 double bond is important for some biological activities of flavonoids, although not essential in all cases. Jacobs and Rubery (1988) examined the structure of flavonoids to dissect the specificity in other auxin transport and/or binding assays and pointed out that the C2-C3 bond should be unsaturated because flavonoid molecules with a saturated C2-C3 bond are tetrahedral rather than planar. They also concluded that hydroxyl groups on both A- and B-rings of flavonoids, but not on the central pyran ring, are required for their maximum NPA-like activity. Faulkner and Rubery (1992) suggested that type of substitution at the position C7 of the flavonoid ring-A is functionally important for inhibition of auxin efflux. The fact that all flavonoids investigated

in our study posses hydroxyl groups at C7 position, but only quercetin, kaempferol and isorhamnetin affect GUS staining in IBA treated roots suggests that this structural feature is not essential for this activity.

Auxin transport inhibitors such as NPA lead to decrease in root growth and gravitropism (Parry *et al*. 2001). In addition, mutants in auxin transport proteins (AUX1, PIN family) show various degrees of inhibition of the gravitropic response (Bennett *et al*. 1996, Marchant *et al*. 1999, Friml *et al*. 2002). This corresponds to altered PIN distribution in flavonoid mutant (Peer *et al*. 2004). Also, in addition, flavonoids accumulated in gravistimulated *Arabidopsis* roots (Buer and Muday, 2004). Therefore, the effects of a range of structurally related flavonoids on gravitropism were compared. Only the flavonols quercetin and to some extent kaempferol inhibited the gravitropic response, whereas myricetin was active only at the highest concentration tested (Table 3). Neither naringenin nor isorhamnetin had an effect on the gravitropic response, although they influenced plant growth as determined by root and shoot fresh mass (Table 3). This suggests that the C2-C3 double bond is not essential for the ability of flavonoids to affect gravitropic response. Buer and Muday (2004) showed that the gravitropic response could be restored by 10 μM naringenin, which is in disagreement to our findings here. However, this was only shown for the mutant *tt4*(2YY6) which cannot be compared to the wild type. Brown *et al*. (2001) compared prolonged growth (12 d) of *Arabidopsis* roots on medium supplemented with 100 μM naringenin. They concluded that high doses of flavonoids are sufficient to almost completely inhibit growth and gravity response. However, other authors (Levizou *et al.* 2004) have shown that flavonoids could have inhibitory, but also an inducible effect on root growth and this indicates again that these effects depend on the chemical structure. In addition, we have shown that, due to low water solubility of quercetin, dissolving of high concentrated flavonoid stock solutions in the medium results in significantly lower concentrations of quercetin in the solutions than we expected (Table 1). This should be kept in mind in all investigations of biological activities of quercetin in all systems in which aqueous medium is used for flavonoid treatment.

In conclusion, the experiments carried out in this study illustrated that *1*) auxin distribution is differently affected by the same flavonoid in different tissues; *2*) the distribution of IAA and IBA is differentially influenced by the same flavonoid; *3*) there are several structural requirements for the flavonoids, which result in the inhibition of auxin distribution; and *4*) flavonoids affect the root gravitropic response in a structure-dependent manner. Further experiments are necessary to elucidate the mode of action of flavonoids on IAA and IBA distribution in *Arabidopsis*.

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