International Journal of Plant Developmental Biology ©2011 Global Science Books

Effect of Flavonoid-mediated Free IAA Regulation on Growth and Development of *in Vitro*-Grown Tobacco Seedlings

Monika Mahajan • Vinay Kumar • Sudesh Kumar Yadav*

Plant Metabolic Engineering Laboratory, Biotechnology Division, CSIR-Institute of Himalayan Bioresource Technology, Council of Scientific and Industrial Research, Palampur-176061 (HP), India

Corresponding author: * **skyt@rediffmail.com; sudeshkumar@ihbt.res.in**

ABSTRACT

The role of exogenously applied two flavonoids, quercetin and epicatechin, was assessed on plant growth and development. For this, tobacco (*Nicotiana tabacum*) seedlings were supplemented with 50 and 100 μM epicatechin and quercetin individually and their shoot and root parts were analyzed for total flavonoids content, transcriptional regulation of flavonoid biosynthesis pathway, morphological and anatomical responses. Exogenous application of these two flavonoids increased the level of total flavonoids in the shoot and decreased it in the root. Similarly the transcript expression of genes encoding key regulatory enzymes of flavonoid biosynthesis pathway was increased in the shoot and decreased or not affected in the root. Interestingly, chalcone synthase (*CHS*) expression decreased in the shoot and increased in the root. Seedlings exposed to flavonoids were observed for the following changes: inhibition of lateral and adventitious roots, reduced plant growth and leaf expansion. Further, flavonoids increased the number of parallel vascular strands in the central and petiolar region of the leaf and decreased the cell size of various regions in the leaf. These observations were similar to the responses produced by synthetic auxin transport inhibitors. Therefore, free indole-3-acetic acid (IAA) content was estimated in tobacco seedlings. Exposure to flavonoids enhanced the endogenous free IAA content in tobacco shoots. In tobacco roots, exposure to only 50 μM epicatechin and quercetin enhanced the free IAA content, while 100 μM exposure decreased the free IAA content. Results suggest that recorded changes could be the consequence of decreased basipetal free IAA transport due to higher flavonoid content in tobacco seedlings.

Keywords: epicatechin, flavonoids, IAA, quercetin, tobacco, transcript expression

INTRODUCTION

Flavonoids are widespread throughout the plant kingdom. They serve a variety of ecological and physiological functions in plants (Stafford 1990; Mathesius *et al*. 1998a; Debeaujon *et al*. 2000). Some flavonoids are considered as regulators for endogenous auxin transport that affect plant development (Jacobs and Rubery 1988; Brown *et al*. 2001; Taylor and Grotewold 2005). Indole acetic acid (IAA), one of the endogenous auxin has been considered as a critical determinant in controlling plant growth (Casimiro *et al*. 2003; Friml 2003; Grebe 2004). Further, the young developing leaves have been documented as an important source of auxin for rest of the plant (Ljung *et al*. 2001). Auxin moves from cell to cell in a highly polar fashion, with a basipetal polarity in the stems and a more complex polarity in the roots (Lomax *et al*. 1995). Auxin enters into root from shoot and transported through the central tissue of the root towards the tip. There it combines with apically synthesized auxin (Ljung *et al*. 2005), and transported basipetally through lateral root caps and epidermis (Swarup and Bennett 2003). This strong bias in the direction of transport within a tissue has resulted from asymmetry in the cellular localization of an influx and efflux apparatus that contained auxin importer AUX1 and PIN-type efflux facilitators, respectively (Muller *et al*. 1998; Swarup *et al*. 2001; Friml 2003).

Flavonoids have also been screened for their ability to block binding of a synthetic auxin transport inhibitor, naphthylphthalamic acid (NPA) and to inhibit auxin transport from hypocotyls. Qercetin, kaempferol, and bestatin have been found to be the most active flavonoids that compete with NPA for binding to the same protein (Jacobs and Rubery 1988; Rubery and Jacobs 1990).

Quercetin produces developmental alterations similar to those produced by NPA in wheat embryos (Fischer *et al*. 1997). The change in endogenous flavonoid concentration leads to the change in auxin transport. This suggests that flavonoids are endogenous regulators of auxin transport. The *tt* Arabidopsis mutant has been used effectively to analyze the effects of individual flavonoids on auxin transport and growth characteristics (Koornneef 1990; Shirley *et al*. 1995). The *tt4* alleles contained mutations in the gene encoding chalcone synthase (CHS), the first enzyme of the flavonoid biosynthesis. Mutant also showed alteration in auxin transport in seedlings and inflorescence tissue as well as increased number of lateral roots (Murphy *et al*. 2000; Brown *et al*. 2001). However, treatment of *tt4* mutant seedlings with naringenin, a flavonoid precursor could restore flavonoid biosynthesis. In Arabidopsis, lateral root formation is reduced upon inhibition of auxin transport (Reed *et al*. 1998) and promoted upon increase in auxin transport (Casimiro *et al*. 2001). The mechanisms underlying root growth and architecture are well studied in *Arabidopsis thaliana* (Benkova and Hejatko 2009; Fukaki and Tasaka 2009). However, several specifics of legume roots suggest that regulatory mechanisms involved in determining root growth and architecture may differ (Gonzalez-Rizzo *et al*. 2009). The compact root architecture1 (*CRA1*) gene has been reported to regulate lignification, flavonoid production, and polar auxin transport in *Medicago trunculata* (Laffont *et al*. 2010).

The vascular tissue of a plant is composed of continuous network of vascular bundles. These are primarily composed of phloem and xylem tissues and translocate dissolved photo assimilates and minerals, respectively (Nelson and Dengler 1997). All types of vascular tissues differentiated from provascular cells. These cells are arranged in con-

Received: 30 May, 2011. Accepted: 25 July, 2011.

Original Research Paper

tinuous strands of narrow cells through coordinated, oriented divisions, parallel to the axis of the emerging strand. Auxin is well known for inducing vascular differentiation (Lyndon 1990). However, the molecular mechanism controlling this differentiation during plant organ development is not known. Importantly, transgenic plants overproducing auxin have increased the amount of vascular tissue (Klee *et al*. 1987).

A large number of environmental factors like light, wounding, pathogens, symbiotic bacteria, and development regulate the synthesis of enzymes that control flavonoid biosynthesis (Feinbaum *et al*. 1991; Kubasek *et al*. 1992; Yang *et al*. 1992; Shirley 1996; Sakuta 2000). Changes in the endogenous flavonoid concentration altered auxin transport. Flavonoids have been co-localized to the tissues that transport auxin (Murphy *et al*. 2000; Peer *et al*. 2001) and to the plasma membrane (Peer *et al*. 2001), where auxin transport inhibitor binding site is localized (Dixon *et al*. 1996). Therefore, flavonoids have the characteristics that make them suitable endogenous regulators of auxin transport.

This study was conducted to see the influence of two flavonoids; quercetin and epicatechin on plant growth and development. Also, to check whether flavonoids induced changes are mediated through the regulation of auxin/free IAA transport or content. For this, tobacco seedlings were grown on MS media containing epicatechin and quercetin. Flavonoids content, expression levels of flavonoid pathway genes and free IAA content was estimated in root and shoot tissues of exposed tobacco seedlings. Higher doses of these flavonoids led to the inhibition of the lateral and adventitious root formation and also produced developmental alterations. Probable mechanism of flavonoid induced changes in plants has been documented.

MATERIALS AND METHODS

Chemicals

Quercetin, epicatechin, DMSO, PVPP and 2,4-dinitrophenylhydrazine (2,4-DNPH) were purchased from Sigma-Aldrich USA. Tween 20 and mercuric chloride were procured from Himedia. Absolute ethanol was purchased from Merck. All other chemicals used were of high analytical grade.

Tobacco seedling and flavonoid treatment

Tobacco (*Nicotiana tabacum* L. cv Xanthi) seeds obtained from ICGEB, New Delhi were firstly treated with detergent 10% Tween-20 for 5 min and thereafter with 70% ethanol for 30 sec. Seeds were then surface sterilized with 0.001% mercuric chloride for 3 min and washed thrice with sterile distilled water. Seeds were grown in petri plates containing full strength Murashige and Skoog medium (MS; Murashige and Skoog 1962) with 3% sucrose and 0.6% agar at 25 ± 2°C for 7 days. By this time cotyledons had emerged and roots had reached a length of 1-1.5 cm. These 7 day old tobacco seedlings were transferred to new plates containing MS media or MS plus quercetin and MS plus epicatechin. Quercetin and epicatechin were dissolved in dimethylsulfoxide (DMSO) and were added at 50°C to molten sterile MS media. These two flavonoids were used at two different concentrations 50 and 100 µM. Seedlings were allowed to grow for next 21 days. Seedlings were carefully removed from the plates and their root and shoot parts were separated and stored in liquid nitrogen for further use.

Total flavonoid estimation

Hundred milligrams of tissue was extracted with 2.5 ml of 95% ethanol under 200 rpm shaking for 24 h. After filtration, the filtrate was used for flavonoid estimation. Total flavonoids comprise of flavones, flavonols and flavanones. The flavones and flavonols were estimated following aluminum chloride colorimetric method (Woisky and Salatino 1998). The reaction mixture contained sample extract, 95% ethanol, 10% aluminum chloride, and 1M potassium acetate. After incubation at room temperature for 30 min, the absorbance was read at 415 nm with UV-Vis spectrophotometer.

The flavanones were estimated following the 2,4-DNPH colorimetric method described earlier (Nagy and Grancai 1996). One millilitre extract was reacted with 2 ml of 1% 2,4-DNPH and 2 ml of methanol at 50°C for 50 min. After cooling at room temperature, the reaction mixture was mixed with 5 ml of 1% potassium hydroxide in 70% methanol and incubated at room temperature for 2 min. Then, 1 ml of the mixture was mixed with 5 ml of methanol and centrifuged at 3000 rpm for 10 min to remove the precipitate. The supernatant was collected and adjusted to 25 ml. The absorbance was read at 495 nm with UV-Vis spectrophotometer.

Measurement of free IAA

Free IAA was estimated by the method described earlier with little modifications (Ernstsen *et al*. 1987). For extraction, 1 g fresh sample was crushed in liquid nitrogen and extracted with chilled 80% methanol by keeping for 24 h under dark at 4°C. The methanolic extract was centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was reduced to dryness in vacuum with a rotary evaporator. The residue was dissolved in 2 ml of 1M phosphate buffer (pH 7.8) and passed through the column packed with polyvinyl pyrrolidine phosphate (PVPP; MW 40,000). Column was eluted with 80 ml of 1M phosphate buffer. Filtrate was mixed with 150 ml of diethyl ether and poured in a separating funnel. The lower phase was collected and its pH was adjusted to 3. The diethyl ether extract was then filtered through sodium sulphate. The filtrate was dried in vacuum evaporator and powder was dissolved in 2 ml of methanol (HPLC grade). Resulting solution was used for estimation of endogenous auxin content using Salkowski's reagent (50 ml 35% $HClO_4$ and 1 ml $FeCl_3$). The absorbance was read with UV-Vis spectrophotometer at 530 nm. The content was calculated from the standard curve prepared from pure IAA (Sigma).

RNA isolation and RT-PCR

Total RNA was isolated from 100 mg of treated and untreated tissues by using TRIzol reagent (Invitrogen). cDNA was synthesized using 1µg of RNA in the presence of 200 U reverse transcriptase Superscript III (Invitrogen, USA), 1 µl of 10 mM dNTPs and 250 ng oligo (dT)12–18. Resulting cDNA was used to carry out the PCR reactions with gene specific primers: phenylalanine ammonia lyase (PAL) (forward primer 5′-CAAGAACGGTGGTGCTCTTC-3′ and reverse primer 5′-CCAGAACCAACTGCAGTACC-3′), chalcone synthase (CHS) (forward primer 5′-GTACAACTAGTG GTGTAGACA-3′ and reverse primer 5′-CCAACTTCACGAAGG TGAC-3′), chalcone isomerase (CHI) (forward primer 5′-CGA GTGACTATGATCTTGCC-3′ and reverse primer 5′-CTGACG CGTCGGCATAGC-3′), flavanone 3-hydroxylase (F3H) (forward primer 5′-GGTAGTTGATCATGGTGTTGA-3′ and reverse primer 5′-GTTCCTGGATCAGTGTCTCG-3′) and flavonol synthase (FLS) (forward primer 5′-GTCCACAACGTTGCATGGTG-3′ and reverse primer 5′-CACAACTTCTCGCAGCCTC-3′). Linearity between the amount of input RNA and the final RT-PCR products was verified and confirmed. After standardizing the optimal amplification at exponential phase, PCR was carried out under the conditions of 94°C-4 min, 94°C-30s, 50-60°C-40s, 72°C-50s for 25 cycles. The 26S rRNA-based gene primers were used as internal control for expression studies (Singh *et al*. 2004).

Microscopic analysis

To check the effect of quercetin and epicatechin on cell size, first nodal leaf of the treated tobacco seedlings was fixed overnight in formaldehyde: acetic acid: ethanol (FAA; 1:1:18). Fixed leaf was dehydrated in an alcohol series and was embedded in paraffin wax. Thin sections were cut using ultramicrotome and examined under light microscope attached with Nikon digital camera (Dxm 1200).

Microscopic study was also conducted to see the effect of quercetin and epicatechin on vascular system. For this, first nodal leaf and root of the treated tobacco seedling was used. The cell content of the tissues was removed by overnight incubation with 5% NaOH and 0.1% SDS at 37°C, followed by several washes in

distilled water. Thereafter, tissue was stained with Schiff's reagent (Sigma) and cleared as described earlier (Vollbrecht and Hake 1995). The cleared tissues were examined under light microscope attached with Nikon digital camera (Dxm 1200) for vascular patterning.

Table 1 Total flavonoid content in shoot and root of tobacco seedlings under untreated and quercetin/epicatechin treated condition. Seven days old tobacco seedlings treated with various concentrations of quercetin/epicatechin for 21 days were used for different flavonoids estimations as described in materials and methods.

Tissue and treatment	Flavonoid content (mg/g fresh weight)*		
	Flavones+ flavonols**	Flavanones***	Total flavonoids
Shoot			
Untreated control	$^{a}0.52 \pm 0.01$	$^{a}0.72 \pm 0.066$	$^{a}1.24 \pm 0.076$
EC-50	$^{a}0.57 \pm 0.01$	$^{a}0.74 \pm 0.04$	$^{a}1.31 \pm 0.05$
EC-100	$^{b}0.65 \pm 0.03$	$^{b}0.97 \pm 0.005$	$^{b}1.62 \pm 0.035$
Quercetin-50	$^{a}0.56 \pm 0.02$	$^{a}0.75 \pm 0.05$	$^{a}1.31 \pm 0.07$
Quercetin-100	$^{c}0.77 \pm 0.01$	$^{b}1.00 \pm 0.02$	$^{b}1.77 \pm 0.03$
Root			
Untreated control	$^{a}0.004 \pm 0.0002$	$^{a}0.97 \pm 0.08$	$^{a}0.974 \pm 0.080$
EC-50	$^{b}0.005 \pm 0.0005$	$^{a}0.96 \pm 0.00$	$^{a}0.965 \pm 0.0005$
EC-100	$^{d}0.009 \pm 0.0007$	$^{b}0.85 \pm 0.05$	$^{b}0.859 \pm 0.050$
Quercetin-50	$^{c}0.007 \pm 0.0004$	$^{a}0.96 \pm 0.03$	$^{a}0.967 \pm 0.030$
Quercetin-100	$^{c}0.01 \pm 0.0009$	$^{b}0.80 \pm 0.02$	$^{b}0.81 \pm 0.020$

*All results are presented as mean ± SD (n=3)
**Levels calculated as quercetin equivalents
***Levels calculated as naringenin equivalents
Different letters of the alphabet superscript to the values represents the significant difference in the mean values of estimates at 5% level

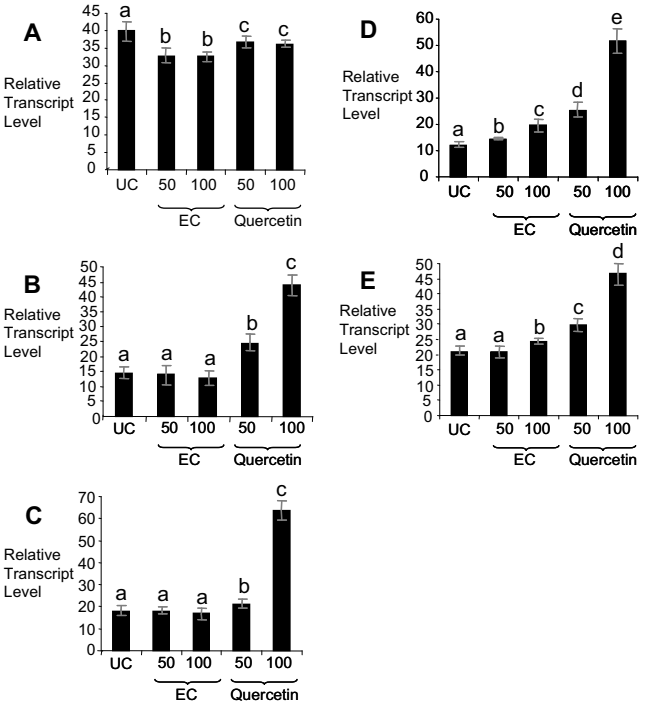

Fig. 1 Changes in the relative transcript level of (**A**) Phenylalanine ammonia lyase (PAL), (**B**) Chalcone synthase (CHS), (**C**) Chalcone isomerase (CHI), (**D**) Flavanone 3-hydroxylase (F3H) and (**E**) Flavonol synthase (FLS) in tobacco shoots in response to 50 and 100 µM treatments of epicatechin and quercetin. Values are the mean of three repeats of expression levels. Least significance difference was determined at 5 and 1% level. Different letters of the alphabet at the bar represent significant differences at 5% and same letters with an asterisk represent significant difference at 1%. Similar letters of the alphabet indicate insignificant differences.

RESULTS

Effect of epicatechin and quercetin application on flavonoid contents of shoot and root of tobacco seedlings

To check whether exogenously applied quercetin and epicatechin has any influence on the endogenous level of flavonoids in tobacco seedlings, total flavonoids content was estimated in shoot and root tissues. Total flavonoid content was increased with the exogenous application of both quercetin and epicatechin in tobacco shoots. The content was further increased with the increase in dose of epicatechin and quercetin exposure. The maximum flavonoid content of 1.77 ± 0.03 mg g^{-1} fresh weight was observed in the 100 µM quercetin exposed shoot followed by 1.62 ± 0.035 mg g^{-1} fresh weight with 100 µM epicatechin (**Table 1**).

On the other hand, total flavonoid content was decreased with exogenous application of epicatechin and quercetin in tobacco roots as compared to untreated roots. The response of root for flavonoid content was exactly reverse to that of shoot upon exogenous application of both epicatechin and quercetin. Hence, total flavonoid content was lowest in 100 µM epicatechin and quercetin exposed tobacco roots compared to untreated roots (**Table 1**).

Effect of epicatechin and quercetin on the expression of flavonoid biosynthesis pathway genes

Since shoot and root tissue showed opposite behavior for flavonoid content accumulation upon exogenous application of both quercetin and epicatechin, the influence of exogenously applied constituents was expected on the regulation of flavonoid biosynthesis pathway. In view of this, expression of flavonoid biosynthesis pathway genes was moni-

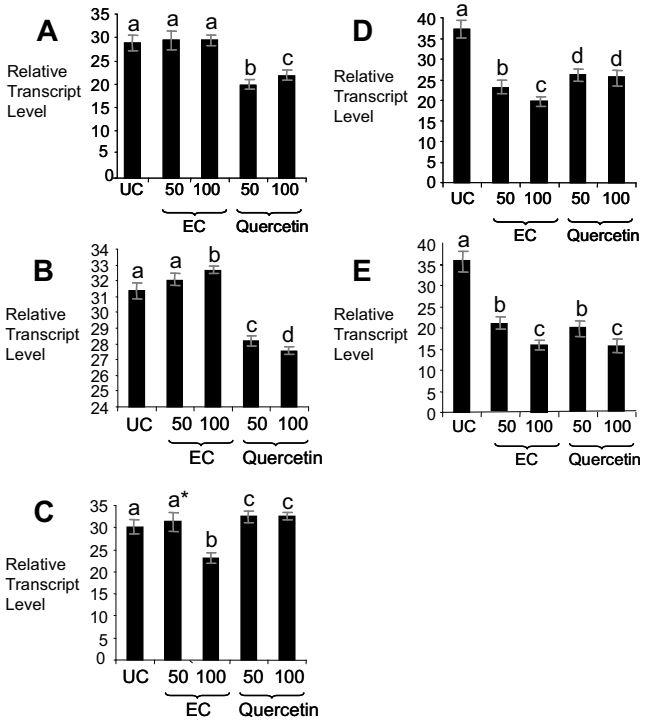

Fig. 2 Changes in the relative transcript level of (**A**) Phenylalanine ammonia lyase (PAL), (**B**) Chalcone synthase (CHS), (**C**) Chalcone isomerase (CHI), (**D**) Flavanone 3- hydroxylase (F3H) and (**E**) Flavonol synthase (FLS) in tobacco roots in response to 50 and 100 µM treatments of epicatechin and quercetin. Values are the mean of three repeats of expression levels. Least significance difference was determined at the 5 and 1% level. Different letters of the alphabet at the bar represent significant difference at 5% and same letters with an asterisk represent significant difference at 1%. Similar letters of the alphabet indicate insignificant differences.

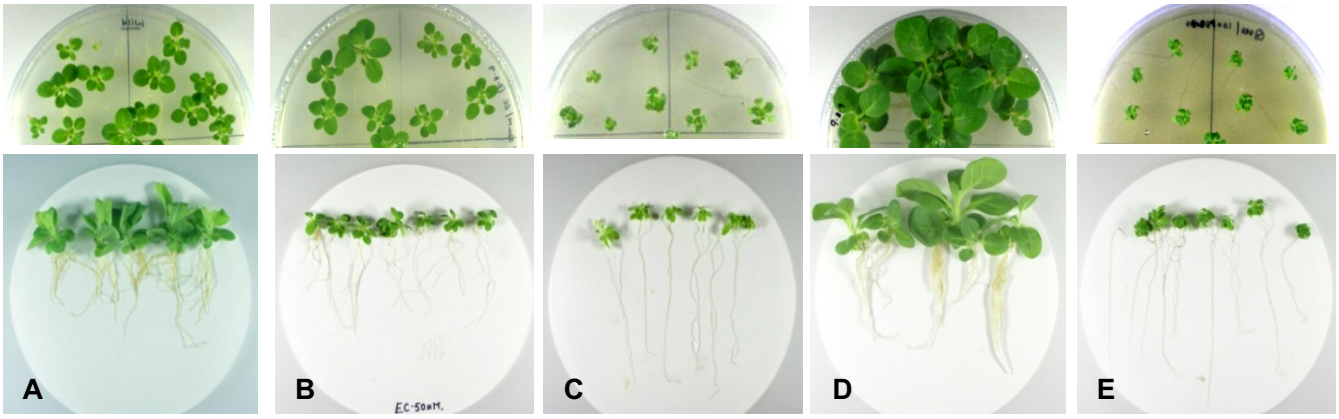

Fig. 3 The effects of different concentrations of flavonoids application on tobacco shoot and root development. Photographs show 7-days-old seedlings grown for 21 days on (**A**) 0.1% DMSO, (**B**) 50 µM epicatechin, (**C**) 100 µM epicatechin, (**D**) 50 µM quercetin and (**E**) 100 µM quercetin. Upper panel is the top view of seedlings and lower panel is the vertical view of seedlings in petri plates.

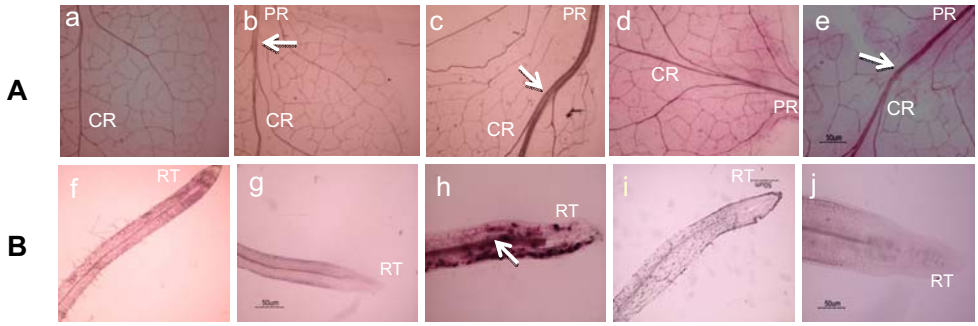

Fig. 4 Vascular organization in flavonoid treated and control plants. (**A**) Whole mount preparations of cleared leaf from tobacco seedlings grown on medium a) without flavonoids, b) 50 µM epicatechin, c) 100 µM epicatechin, d) 50 µM quercetin and e) 100 µM quercetin. Numerous parallel vessels were seen in the central and petiolar region of flavonoid treated plants. (**B**) Whole mount preparations of cleared primary root tips from tobacco seedlings grown on medium f) without flavonoids, g) 50 µM epicatechin, h) 100 µM epicatechin, i) 50 µM quercetin and j) 100 µM quercetin. Arrows indicate numerous parallel vessels extended towards the root apex in 100 µM epicatechin treated tobacco seedlings. PR, petiolar region; CR, central region, RT, root tip.

tored in tobacco shoot and root upon exposure to quercetin and epicatechin. The transcript levels of PAL, CHS, CHI, F3H and FLS was monitored. In tobacco shoot, epicatechin exposure influenced the transcript level of various enzymes in a concentration dependent manner. Upon exposure to 50 µM epicatechin, the expression of CHI and F3H was up-regulated while the expression of other three enzymes was not affected. Application of 100 µM epicatechin has significantly increased the transcript expression level of PAL, CHI, F3H and FLS. Interestingly, CHS expression was downregulated with 100 µM epicatechin (**Fig. 1**). The 50 µM quercetin application was observed to increase transcript expression level of all the five enzymes. The expression was further enhanced with 100 µM quercetin application (**Fig. 1**).

Influence of these two flavonoids was also seen on the expression of genes encoding flavonoid biosynthetic enzymes in tobacco roots. In epicatechin exposed tobacco roots, transcript expression of CHI, F3H and FLS was decreased. While PAL expression was unaffected with both 50 and 100 µM epicatechin exposure compared to untreated control. Only the expression of CHS was found to be upregulated with 100 µM epicatechin applications (**Fig. 2**). Similarly, quercetin exposure was also found to decrease the expression of PAL in addition to F3H and FLS. However, CHS and CHI expression was found to be increased with 100 µM quercetin compared to untreated control (**Fig. 2**). In general, results suggest that epicatechin and quercetin exposure enhanced the expression of genes encoding flavonoid pathway enzymes in shoot and decreased in root of tobacco seedlings. Interestingly, CHS showed reverse response in two tissues compared to other four enzymes.

Morphological responses of epicatechin and quercetin application on tobacco seedlings

Tobacco seedlings grown on media supplemented with 100 µM epicatechin and quercetin exhibited larger primary roots with no lateral and adventitious roots. Higher concentration of flavonoids exposure also resulted in smaller leaves and laminae (**Fig. 3**). The inhibition in lateral and adventitious root formation was also observed with 50 µM epicatechin but extent was lesser as compared to its higher dose. On the other hand, tobacco seedlings grown in the medium with 50 µM quercetin produced no difference in their root system as well as leaves and laminae. However, higher dose of epicatechin and quercetin application retarded the plant growth significantly (**Fig. 3**).

Effect of epicatechin and quercetin application on vascular system of tobacco seedlings

To assess the influence of these two flavonoids on plant vascular patterning, development of vascular systems was analyzed during their exogenous application. For this, roots and leaves were analyzed through histological sections. The first nodal leaf of the seedlings grown in the presence of 100 µM epicatechin and quercetin show increase in number of parallel vascular strands in central and petiolar region of the leaf as compared to that of untreated control. Also, these parallel vascular strands were separated from each other. The 50 µM epicatechin and quercetin exposure also enhanced the number of parallel vessels compared to unexposed plant; however, the increase was to a lesser extent compared to 100 µM exposure (**Fig. 4A**). Similarly, the effect of these flavonoids was also observed on vascular

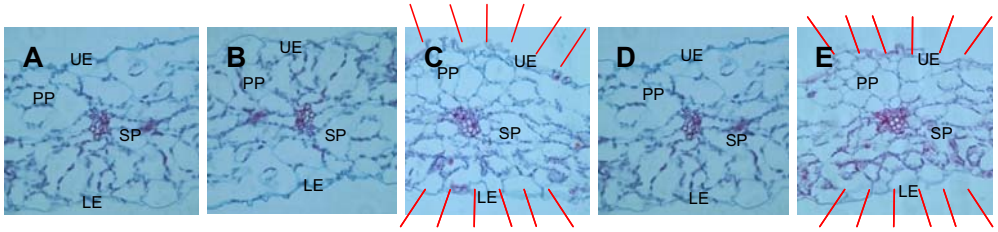

Fig. 5 Cell size comparisons in leaf cross-sections centered with central vein. (**A**) leaf cross-section of flavonoids untreated control plant and flavonoids treated plants such as (**B**) 50 µM epicatechin, (**C**) 100 µM epicatechin, (**D**) 50 µM quercetin and (**E**) 100 µM quercetin. Red bars indicate a shift towards the smaller cell size that was observed with higher concentrations of epicatechin and quercetin. UE, upper epidermis, LE, lower epidermis; PP, palisade parenchyma; SP, spongy parenchyma.

Table 2 Free IAA content in shoot and root of tobacco seedlings under untreated and quercetin/epicatechin treated condition. Seven days old tobacco seedlings treated with various concentrations of quercetin/epicatechin for 21 days were used for free IAA estimation as described in materials and methods.

Samples	Conc. of auxin (IAA)* (ng/g fresh weight)				
	Untreated control	EC-50 µM	EC-100 µM	Quercetin 50 µM	Quercetin 100 µM
Shoot	$^{a}20.5 \pm 0.76$	$^{b}25.2 \pm 2.0$	$^{c}41.5 \pm 2.6$	$^{b}28.0 \pm 2.2$	$^{c}44.0 \pm 2.5$
Root	$^{a}5.0 \pm 0.4$	$^{a}5.8 \pm 0.5$	$^{b}4.3 \pm 0.35$	$^{c}6.2 \pm 0.41$	$^{b}3.8 \pm 0.28$

*All results are presented as mean ± SD (n=3)
Different superscript letters next to the values represent significant differences in the mean values of estimates at the 5% level according to LSD

strands in the root tip. Numerous parallel vessels extended towards root apex were found only in the seedlings exposed to 100 µM epicatechin. Other treatments did not show any effect on vessels accumulation in the root tip region (**Fig. 4B**).

Effect of epicatechin and quercetin on leaf anatomy

To check the effect of flavonoids on cell size of leaf, microscopic analysis was conducted with tobacco seedlings exposed to 50 and 100 µM epicatechin and quercetin. For microscopic analysis, fixed leaves were cut with microtome and cross-sections centered with central vein were analyzed. Decrease in cell size was observed in the seedlings exposed to higher flavonoid concentrations compared to unexposed seedlings. Cell size of epidermis, palisade and spongy parenchyma revealed a shift to smaller size population upon exposure to 100 µM of both the flavonoids. However, no difference in cell size was observed with 50 µM exposure of both the flavonoids compared to unexposed control (**Fig. 5**).

Effect of epicatechin and quercetin on endogenous free IAA content of tobacco seedling

The similar morphological and anatomical responses as observed in this study have been documented by the use of synthetic auxin transport inhibitors. Therefore, endogenous free IAA content was determined in the shoot and root of tobacco seedlings to see the effect of quercetin and epicatechin. The endogenous free IAA content was increased in tobacco shoots upon exposure to both the flavonoids. The content was further increased with increase in dose of their treatment. The maximum free IAA content was observed in shoots exposed to 100 µM quercetin followed by 100 µM epicatechin treated shoots (**Table 2**). In tobacco roots, the endogenous free IAA was also increased upon the exposure to 50 µM epicatechin and quercetin (**Table 2**). However, 100 µM exposures of both the flavonoids decreased the free IAA content. The 50 µM quercetin treated roots showed the maximum free IAA content.

DISCUSSION

The goal of the present work was to check the influence of exogenous application of two flavonoids on growth and development of tobacco seedlings under *in-vitro*. For this, tobacco seedlings were grown in the presence of 50 and 100 µM of epicatechin and quercetin. The treated seedlings were analyzed for flavonoid content, flavonoid biosynthetic pathway genes expression, morphological changes and vascular patterning. The exogenous application of flavonoids generally upregulated the expression of genes encoding flavonoid biosynthetic pathway enzymes such as PAL, CHI, F3H and FLS in tobacco shoots compared to untreated control shoots (**Fig. 1**). The upregulation in gene expression of some of these enzymes was also found with their lower dose of flavonoid exposure. The expression of genes influenced by lower dose of flavonoid exposure can be categorized as early responsive genes and other can be late responsive. The decrease in expression of CHS at 100 µM epicatechin exposure in tobacco shoots might be due to negative regulation at higher dose (Pelletier *et al*. 1999). In contrast to this, the expression of these genes were either decreased or not affected with epicatechin and quercetin treatments in tobacco roots. Only the CHS expression was found to be upregulated with 100 µM epicatechin and quercetin exposure in tobacco roots (**Fig. 2**). This has suggested the reverse behaviour of CHS in two tissues compared to other four enzymes upon exogenous supply of quercetin and epicatechin.

This increase in expression of flavonoid biosynthetic enzymes with epicatechin and quercetin exposure in tobacco shoot than that in root, suggested higher accumulation of flavonoids in the aerial part of the seedlings. Our results support this and found higher levels of total flavonoid contents in the shoot than root (**Table 1**). Higher accumulation of flavonoids in shoot part of tobacco seedlings during the exposure of epicatechin and quercetin suggested free IAA accumulation in this region through the flavonoid-mediated inhibition of free IAA transport. While decrease in flavonoid content documented for lesser free IAA in the roots compared with the untreated control (**Table 2**). This result is supported by the fact that flavonoids act as endogenous regulators of auxin transport (Jacobs and Rubery 1988; Brown *et al*. 2001; Taylor and Grotewold 2005). Aglycone flavonols like quercetin and kaemferol have been reported as highly active inhibitors of auxin transport than glycosides (Mathesius *et al*. 1998b). Hence, free IAA retention in shoot portion of tobacco seedlings might be responsible for various observed morphological and developmental events.

Auxin is required at several developmental stages to facilitate lateral root formation. Lateral root primordial that are unable to divide if excised from the primary root, can be rescued if supplemented with exogenous auxin (Dubrovsky *et al*. 2001). Further it has been reported that higher levels of auxin (IAA) in the aerial part or shoot of a plant resulted in inhibition of lateral and adventitious roots formation (Reed *et al*. 1998). Similar inhibition in lateral and adventitious root formation was also observed in the exogenously exposed tobacco seedlings (**Fig. 3**). Application of IAA to

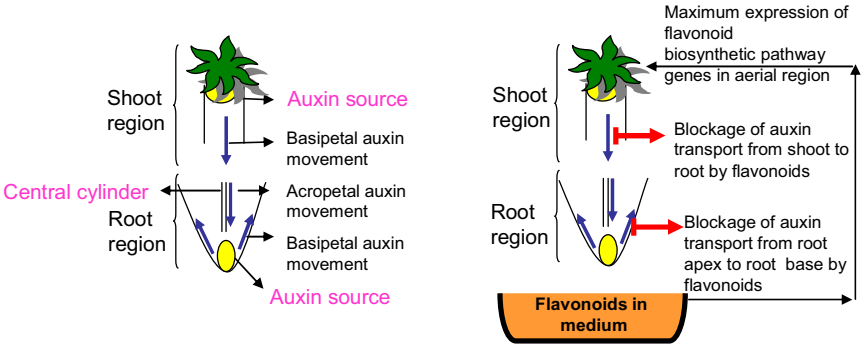

Fig. 6 Representative model depicting the transport and accumulation of auxin in tobacco seedlings during flavonoids application. (**Left**) Important sources of auxin required for the developing roots in plant. The *de-novo* synthesized auxin is transported basipetally from aerial parts of plant to the root tip. Auxin is also synthesized locally in the primary and lateral root tips. From roots, auxin can be transported acropetally within the root stele and basipetally from the root apex to other tissues with in the root tip. (**Right**) Applications of higher concentrations of flavonoids to tobacco seedlings through the medium resulted into flavonoid accumulation in the aerial region. Large numbers of developmental defects were produced as described in this study by the inhibition of basipetal auxin transport resulting from flavonoid accumulation.

growing plants stimulates lateral root development (Blakely *et al*. 1982; Muday and Haworth 1994). Conversely, growth of tomato roots on agar containing auxin-transport inhibitors, including NPA, decreased the number of lateral roots (Muday and Haworth 1994). A negative correlation was found between the degree of branching in root systems and the amount of NPA-binding activity present in roots in different species of plants (Lomax *et al*. 1995). Thus these evidences support the fact that free IAA is necessary for lateral root formation and inhibition of these lateral and adventitious roots is mediated by the blockage of free IAA transport resulted from flavonoid accumulation.

We have further studied the vascular system of plants grown in the presence of flavonoids. Variation in dosage of flavonoid applications altered the vascular system of a leaf in a predictable fashion. The number of parallel vascular strands in the central and the petiolar region of the leaf increased with increase in dosage of flavonoids (**Fig. 4A**). Secondary veins approached the primary vein at a more acute angle, and often did not fuse with the primary vein. As a result, several parallel veins could frequently be seen in the central and petiolar region. This could be because of the free IAA retention in aerial portion of the seedlings induced by maximum flavonoid accumulation. Numerous parallel vessels extended towards the root apex were found only with 100 µM epicatechin (**Fig. 4B**). This might be due to the inhibition of basipetal free IAA transport from root apex towards the root base. This could be resulted from more flavonoid accumulation in the root tip only and lesser in the root as a whole. Similar responses were evoked by three synthetic auxin transport inhibitors TIBA, NPA and HFCA in *Arabidopsis.* This has revealed that auxin transport is required for vascular tissue continuity and the restriction of vascular differentiation to narrow strands (Mattson *et al*. 1999).

Further, higher dose of epicatechin and quercetin inhibited plant growth to much higher level as compared to their lower dose and untreated control. This finding is supported by the fact that NPA restricted leaf auxin translocation and concurrently reduced leaf size in *Arabidopsis* and bean (Keller *et al*. 2004). Although, the exact mechanism of auxin in controlling leaf expansion remains unclear, increase in auxin level has been reported to have negative effects on leaf expansion. In this study, decrease in cell size of leaf epidermis, palisade and spongy parenchyma during exposure to 100 µM epicatechin and quercetin was observed (**Fig. 5**). This decrease in cell size could be due to flavonoid-mediated free IAA retention in the leaf of seedlings treated with higher doses of epicatechin and quercetin. Such changes in leaf cell size have earlier been reported through following different studies. Transgenic petunia (*Petunia hybrida*) overproducing auxin developed epinastic, smaller and narrower leaves than non-transgenic control plant (Klee *et al*. 1987). Similarly, *Arabidopsis* mutants *sur1* and *sur2* overproducing auxin resulted in less expanded leaf (Boerjan *et al*. 1995). Application of exogenous auxin to bean (*Phaseolus vulgaris*) and *Arabidopsis* leaf blades inhibited long-term blade elongation (Keller *et al*. 2004). On the other hand, a low auxin concentration has been documented to drive cell elongation and cell enlargement in tobacco cell culture (Zazimalova *et al*. 1995; Winicur *et al*. 1998).

Overall conclusion of this study has been documented through a model. The model depicts the two important sources of IAA for the developing roots. The primary one is the basipetal transport of *de novo* synthesized IAA from aerial parts of the plant to root tip and the secondary one is the locally synthesized IAA in the primary and lateral root tips. Within the root, IAA can be transported acropetally in the root stele and basipetally from the root apex to other tissues (**Fig. 6A**). The exogenous application of higher doses of epicatechin and quercetin resulted into flavonoid accumulation in aerial region of tobacco seedlings through coordinated upregulation of key regulatory enzymes of flavonoid biosynthetic pathway. In contrast to this, the roots showed lesser flavonoid accumulation. Higher level of flavonoid in the aerial region resulted into inhibition of basipetal IAA transport from aerial region to root of the tobacco seedlings (**Fig. 6B**). This inhibition in free IAA transport was found to be responsible for non formation of lateral and adventitious roots. More accumulation of free IAA in the aerial portion of the seedlings resulted in several morphological and anatomical alterations in the leaves of tobacco.

ACKNOWLEDGEMENTS

This work was supported by NMITLI project grant from the Council of Scientific and Industrial Research, Govt. of India. MM and VK would like to thank Council of Scientific and Industrial Research, Govt. of India for providing fellowship as SRF. The authors are thankful to Dr. Paramvir Singh Ahuja, Director, CSIR-IHBT for his timely support and guidance.

REFERENCES

Benkova E, Hejatko J (2009) Hormone interactions at the root apical meristem. *Plant Molecular Biology* **69**, 383-396

Blakely LM, Durham M, Evans TA, Blakely RM (1982) Experimental studies on lateral root formation in radish seedling roots: I. General methods, developmental stages, and spontaneous formation of laterals. *Botanical Gazette* **143**, 341-352

Boerjan W, Cervera MT, Delarue M, Beeckman T, Dewitte W, Bellini C, Caboche M, Van Onckelen H, Van Montagu M, Inzé D (1995) *Superroot*, a recessive mutation in *Arabidopsis*, confers auxin overproduction. *The Plant Cell* **7**, 1405-1419

Brown DE, Rashotte AM, Murphy AS, Tague BW, Peer WA, Taiz L, Muday GK (2001) Flavonoids act as negative regulators of auxin transport *in vivo* in

Arabidopsis thaliana. Plant Physiology **126**, 524-535

Casimiro I, Beeckman T, Graham N, Bhalerao R, Zhang H, Casero P, Sandberg G, Bennett MJ (2003) Dissecting *Arabidopsis* lateral root development. *Trends in Plant Science* **4**, 165-171

Casimiro I, Marchant A, Bhalerao RP, Beeckman T, Dhooge S, Swarup R, Graham N, Inzé D, Sandberg G, Casero PJ, Bennett M (2001) Auxin transport promotes *Arabidopsis* lateral root initiation. *The Plant Cell* **13**, 843-852

Chen D, Zhao J (2008) Free IAA in stigmas and styles during pollen germination and pollen tube growth of *Nicotiana tabacum*. *Physiologia Plantarum* **134**, 202–215

Debeaujon I, Leon-Kloosterziel KM, Koornneef M (2000) Influence of the testa on seed dormancy, germination, and longevity in *Arabidopsis*. *Plant Physiology* **122**, 403-413

Dixon MW, Jacobson JA, Cady CT, Muday GK (1996) Cytoplasmic orientation of the naphthylphthalamic acid binding protein in zucchini plasma membrane vesicles. *Plant Physiology* **112**, 421-432

Dubrovsky JG, Rost TL, Colon-Carmona A, Doerner PW (2001) Early primordium morphogenesis during lateral root initiation in *Arabidopsis thaliana*. *Planta* **214**, 30-36

Feinbaum RL, Storz G, Ausubel FM (1991) High intensity and blue light regulated expression of chimeric chalcone synthase genes in transgenic *Arabidopsis thaliana* plants. *Molecular Gene and Genetics* **226**, 449-456

Fischer C, Speth V, Fleig-Eberenz S, Neuhaus G (1997) Induction of zygotic polyembryos in wheat: Influence of auxin polar transport. *The Plant Cell* **9**, 1767-1780

Friml J (2003) Auxin transport: Shaping the plant. *Current Opinion in Plant Biology* **6**, 7-12

Fukaki H, Tasaka M (2009) Hormone interactions during lateral root formation. *Plant Molecular Biology* **69**, 437-449

Grebe M (2004) Ups and downs of tissue and planar polarity in plants. *BioEssays* **26**, 719-729

Jacobs M, Rubery PH (1988) Naturally occurring auxin transport regulators. *Science* **241**, 346-349

Keller CP, Stahlberg R, Barkawi LS, Cohen JD (2004) Long-term inhibition by auxin of leaf blade expansion in bean and *Arabidopsis*. *Plant Physiology* **134**, 1217-1226

Klee HJ, Horsch RB, Hinchee MA, Hein MB, Hoffmann NL (1987) The effects of overproduction of two *Agrobacterium tumefaciens* T-DNA auxin biosynthetic gene products in transgenic petunia plants. *Genes and Development* **1**, 86-96

Koornneef M (1990) Mutations affecting the testa color in *Arabidopsis*. *Arabidopsis Information Service* **28**, 1-4

Kubasek WL, Shirley BW, McKillop A, Goodman HM, Briggs W, Ausubel FM (1992) Regulation of flavonoid biosynthetic genes in germinating *Arabidopsis* seedlings. *The Plant Cell* **4**, 1229-1236

Laffont C, Blanchet S, Lapierre C, Brocard L, Ratet P, Crespi M, Mathesius U, Frugier F (2010) The compact root architecture1 gene regulates lignification, flavonoid production, and polar auxin transport in *Medicago truncatula*. *Plant Physiology* **153**, 1597-1607

Li X, Bonawitz ND, Weng JK, Chapple C (2010) The growth reduction associated with repressed lignin biosynthesis in *Arabidopsis thaliana* is independent of flavonoids. *The Plant Cell* **22**, 1620-1632

Ljung K, Bhalerao RP, Sandberg G (2001) Sites and homeostatic control of auxin biosynthesis in *Arabidopsis* during vegetative growth. *The Plant Journal* **28**, 465-474

Ljung K, Hull AK, Celenza J, Yamada M, Estelle M, Normanly J, Sandberg G (2005) Sites and regulation of auxin biosynthesis in *Arabidopsis* roots. *The Plant Cell* **17**, 1090-1104

Lomax TL, Muday GK, Rubery PH (1995) Auxin transport. In: Davies PJ (Ed) *Plant Hormones: Physiology, Biochemistry, and Molecular Biology*, Kluwer Academic Publishers, Dordrecht, pp 509-530

Lyndon RF (1990) *Plant Development-The Cellular Basis*, Unwin Hyman, Boston, MA, 264 pp

Mathesius U, Bayliss C, Weinman JJ, Schlaman HRM, Spaink HP, Rolfe BG, McCully ME, Djordjevic MA (1998a) Flavonoids synthesized in cortical cells during nodule initiation are early developmental markers in white clover. *Molecular Plant-Microbe Interactions* **11**, 1223-1232

Mathesius U, Schlaman HRM, Spaink HP, Sautter C, Rolfe BG, Djordjevic MA (1998b) Auxin transport inhibition precedes root nodule formation in white clover roots and is regulated by flavonoids and derivatives of chitin oligosaccharides. *The Plant Journal* **14**, 23-34

Mattsson J, Sung ZR, Berleth T (1999) Responses of plant vascular systems to auxin transport inhibition. *Development* **126**, 2979-2991

Muday GK, Haworth P (1994) Tomato root growth, gravitropism, and lateral development: Correlation with auxin transport. *Plant Physiology and Biochemistry* **32**, 193-203

Muller A, Hillebrand H, Weiler EW (1998) Indole-3-acetic acid is synthesized from L-tryptophan in roots of *Arabidopsis thaliana*. *Planta* **206**, 362–369

Murashige T, Skoog FA (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum* **15**, 473-497

Murphy A, Peer W, Taiz L (2000) Regulation of auxin transport by aminopeptidases and endogenous flavonoids. *Planta* **211**, 315-324

Nelson T, Dengler N (1997) Leaf vascular pattern formation. *The Plant Cell* **9**, 1121-1135

Päsold S, Siegel I, Seidel C, Ludwig-Muller J (2010) Flavonoid accumulation in *Arabidopsis thaliana* root galls caused by the obligate biotrophic pathogen *Plasmodiophora brassicae*. *Molecular Plant Pathology* **11**, 545-562

Peer WA, Brown DE, Tague BW, Muday GK, Taiz L, Murphy AS (2001) Flavonoid accumulation patterns of transparent testa mutants of *Arabidopsis*. *Plant Physiology* **126**, 536-548

Pelletier MK, Burbulis IE, Winkel-Shirley B (1999) Disruption of specific flavonoid genes enhances the accumulation of flavonoid enzymes and end-products in *Arabidopsis* seedlings. *Plant Molecular Biology* **40**, 45-54

Reed RC, Brady SR, Muday GK (1998) Inhibition of auxin movement from the shoot into the root inhibits lateral root development in *Arabidopsis*. *Plant Physiology* **118**, 1369-1378

Rubery P, Jacobs M (1990) Auxin transport and its regulation by flavonoids. In: Pharis R, Rood S (Eds) *Plant Growth Substances*, Springer-Verlag, Berlin, pp 428-440

Sachs T (1991) Cell polarity and tissue patterning in plants. *Development Supplementary* **91**, 83-93

Sakuta M (2000) Transcriptional control of chalcone synthase by environmental stimuli. *Journal of Plant Research* **113**, 327-333

Saslowsky DE, Dana CD, Winkle-Shirley B (2000) An allelic series for the chalcone synthase locus in *Arabidopsis*. *Gene* **255**, 127-138

Shirley BW, Kubasek WL, Storz G, Bruggeman E, Koornneef M, Ausubel FM, Goodman HM (1995) Analysis of *Arabidopsis* mutants deficient in flavonoid biosynthesis. *The Plant Journal* **8**, 659-671

Singh K, Raizada J, Bhardwaj P, Ghawana S, Rani A, Singh H, Kaul K, Kumar S (2004) 26S rRNA-based internal control gene primer pair for reverse transcription-polymerase chain reaction based quantitative expression studies in diverse plant species. *Analytical Biochemistry* **335**, 330-333

Stafford HA (1990) *Flavonoid Metabolism*, CRC Press, Boca Raton, FL, 298 pp

Swarup R, Bennett M (2003) Auxin transport: The fountain of life in plants? *Developmental Cell* **5**, 824-826

Swarup R, Friml J, Marchant A, Ljung K, Sandberg G, Palme K, Bennett M (2001) Localization of the auxin permease AUX1 suggests two functionally distinct hormone transport pathways operate in the *Arabidopsis* root apex. *Genes and Development* **15**, 2648-2653

Taylor LP, Grotewold E (2005) Flavonoids as developmental regulators. *Current Opinion in Plant Biology* **8**, 317-323

Vollbrecht E, Hake S (1995) Deficiency analysis of female gametogenesis in maize. *Genetics* **16**, 44-63

Winicur ZM, Zhang GF, Staehelin LA (1998) Auxin deprivation induces synchronous golgi differentiation in suspension-cultured tobacco BY-2 cells. *Plant Physiology* **117**, 501-513

Yang WC, Cremers HCJ, Hogendijk P, Katinakis P, Wijffelman CA, Fransen H, Kammen AV, Bisseling T (1992) *In-situ* localization of chalcone synthase mRNA in pea root nodule development. *The Plant Journal* **2**, 143-151

Zazimalova E, Opatrny Z, Brezinova A (1995) The effect of auxin starvation on the growth of auxin-dependent tobacco cell culture dynamics of auxin binding activity and endogenous free IAA content. *Journal of Experimental Botany* **46**, 1205-1213