Original article

Flavonoids modify root growth and modulate expression of SHORT-ROOT and HD-ZIP III

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ABSTRACT

Flavonoids are a class of distinct compounds produced by plant secondary metabolism that inhibit or promote plant development and have a relationship with auxin transport. We showed that, in terms of root development, Copaifera langsdorffii leaf extracts have an inhibitory effect on most flavonoid components compared with the application of exogenous flavonoids (glycosides and aglycones). These compounds alter the pattern of expression of the SHORT-ROOT and HD-ZIP III transcription factor gene family and cause morpho-physiological alterations in sorghum roots. In addition, to examine the flavonoid auxin interaction in stress, we correlated the responses with the effects of exogenous application of auxin and an auxin transport inhibitor. The results show that exogenous flavonoids inhibit primary root growth and increase the development of lateral roots. Exogenous flavonoids also change the pattern of expression of specific genes associated with root tissue differentiation. These findings indicate that flavonoid glycosides can influence the polar transport of auxin, leading to stress responses that depend on auxin.
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1. Introduction

Some plant species can use strategies to compete with other species to increase reproductive success and tolerate environmental stress. Among the strategies used by different groups of plants is allelopathy, a chemical mechanism of interaction in which one plant inhibits or promotes another (Rice, 1984) for its own benefit by reducing the competitive pressure for resources (Belz, 2007; Kato-Noguchi et al., 2014). Allelochemicals inhibit the growth of primary roots (Rizvi and Rizvi, 1992; Soltys et al., 2014), and also increase the number of lateral roots in response to stress (Krishnamurthy and Rathinasabapathi, 2013). Higher ramification indexes in roots under different environmental pressures may be the consequence of structural changes in cells and constituent tissues (Gniadzowska and Bogatek, 2005) or due to hormone responses.

In addition to the action of plant hormones, the expression pattern of various genes is correlated with the adjustment of indeterminate root growth and has been identified in Arabidopsis thaliana. SHORT-ROOT (SHR) is an important component in the pathway of development, which regulates the specification of meristematic root cells (Benfey et al., 1993; Nakajima et al., 2001; Yruela, 2015). The expression of HD-ZIP III transcription factors guides the differentiation of xylem in the protoxylem or metaxylem (Carlsbecker et al., 2010; Petricka et al., 2012).

Several members of these gene families are subject to post-transcriptional regulation via small regulatory RNAs, specifically microRNAs (miRNAs) a group of small non-coding RNAs (21–22 nt) that post-transcriptionally regulate genes (Lee et al., 1993; Lagos-Quintana et al., 2001; Reinhart et al., 2002). For instance, the transcripts of the HD-ZIP III genes are targets of the microRNAs miR165 and miR166 (Juarez et al., 2004). Franco et al. (2015) showed that plant extract composed mainly of flavonoids can alter the expression of HD-ZIP III genes and miR166. Although flavonoids are major plant phenylpropanoid metabolites found throughout

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the plant kingdom (Buer et al., 2010), knowledge regarding to flavonoids and the expression pattern of microRNAs is scarce.

*Copaifera langsdorffii* is a species known for its medicinal properties and has a great diversity of flavonoids (Sousa et al., 2012). Aromatic and medicinal plants have biologically active substances with structures that resemble allelochemical substances and therefore have allelopathic potential (Mathela, 1991). However, due to advances in molecular biology, new studies are needed for a better understanding of allelochemical mechanisms of action, mainly associated with root development. Recent studies have reported the importance of the relationship between flavonoids and auxin signaling in plant development (Krishnamurthy and Rathinasabapathi, 2013).

The biological activities of flavonoids in root development have shown interaction with the acropetal pattern and lateral movement of auxins (Krishnamurthy and Rathinasabapathi, 2013; Maloney et al., 2014; Yin et al., 2014).

Roots growing in solutions rich in flavonoids shows morphophysiological responses that are dependent on the expression pattern of the SHR gene and members of the transcription factor family HD-ZIP III. In this study, we applied *C. langsdorffii* leaf extract and isolated flavonoids to *Sorghum bicolor* seeds and seedlings. Our objective was to answer questions about the morpho-anatomical and gene expression patterns of some molecular targets. We found that the *C. langsdorffii* leaf extract (with the chemical profile elucidated) and flavonoids (aglycone and glycoside) with allelopathic activity affect root development in a similar manner.

2. Materials and methods

2.1. Plant material and leaf extract

*C. langsdorffii* leaves were collected in September 2010 in *Cerrado stricto sensu* located around Botucatu, Brazil (22°42′07.82″S 48°20′28.65″O) at 511 m of altitude. Samples were deposited in the Herbarium Irina D. Gemchujnicov (BOTU) from UNESP, Botucatu with the identification number 28515 BOTU.

The leaves were dried in an oven at 40 °C for 48 h, and 2000 g was submitted to extraction with 5L of methanol 100% at room temperature by percolation over 5 days. The solvent was evaporated under reduced pressure in a rotary evaporator and subsequently lyophilized, resulting in 41 g of *Copaifera* leaf extract.

2.2. HPLC-PAD-ESI-MS analysis instrumentation

The HPLC-PAD-ESI-MS analysis was performed using Accela High Speed LC (Thermo Scientific, San Jose, CA, USA), with column model Luna 5 μm C18 100Å 250 mm × 4.6 mm I.D. and 4 mm × 3 mm guard column (Phenomenex, Torrance, CA, USA). The mass spectra were obtained on a mass spectrometer LCQ Fleet (Thermo Scientific) equipped with an Accela (Thermo Scientific) LCQ Fleet with an Ion Trap (IT) 3D and ionization by electrospray (ESI). The negative mode was chosen to generate and analyze the mass spectra in first-order under the following conditions: capillary voltage: 4 V, voltage of 5 kV, spray temperature of 280 °C, and capillary gas spray (N₂) flow 60 (arbitrary units). The range of acquisition was m/z 50-1000, with two or more events of scanning performed simultaneously on the mass spectrometer function. Xcalibur software version 1.0 (Thermo Finnigan, San Jose, CA, USA) was used during the acquisition and processing of the spectrometric data. The extract was analyzed at the final concentration of 10 mg mL⁻¹ solubilized in MeOH:H₂O (1:1) and filtered with PTFE membrane filter (0.45 μm pore).

The compounds were identified via HPLC-PAD-MS analysis by comparing the retention time and the UV and MS spectra of the peaks in the samples with those of authentic reference samples or isolated compounds and available literature (Sousa et al., 2012).

2.3. Conditions for plant growth and biological assays

Sorghum seeds were sterilized in 2% solution of sodium hypochlorite for 2 min and then were washed with distilled water. Stock solutions were prepared with leaf extract and diluted at a concentration of 400 mg L⁻¹, half of a lethal dose (LD50) to apply to the seeds. This dose was determined by prior studies of dose response (Figs. S1 and S2). Complementary treatments were performed with two flavonoids (quercetin and rutin) at a concentration of 1 mM, auxin indoleacetic acid (IAA) in a concentration of 1 μM, and the auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA) at a concentration of 10 μM. Experimental treatments and the control were placed with 20 seeds to germinate in 9 cm Petri dishes, in triplicates, with 10 mL of each stock solution of extract, flavonoids, and plant growth regulators. The control experiment was performed using deionized water. IAA and NPA were applied 48 h after the seeds were soaked in deionized water to avoid inhibiting germination. The seeds were kept in a growth chamber with a 12:12 h light/dark photoperiod at 25 °C. After five days in the growth chamber, the following parameters of the seeds were analyzed: germination speed index (GSI), root growth, number of lateral roots, relative quantification of gene expression and anatomical changes in roots. The GSI was calculated with the following equation:

\[
\text{GSI} = \left( \frac{G_1}{N_1} \right) + \left( \frac{G_2}{N_2} \right) + \left( \frac{G_3}{N_3} \right) + L + \left( \frac{G_n}{N_n} \right)
\]

G1, G2, G3, ..., Gn = number of seedlings computed in the first, second, third, and last count. N1, N2, N3, ..., Nn = number of days from seeding to first, second, third, and last count.

2.4. Gene expression analysis with qRT-PCR

Total RNA isolation was performed using the Spectrum Plant Total RNA Kit (Sigma–Aldrich, St. Louis, MO, USA). The roots were used for three repeated experiments using about 20 seedlings from each treatment and control. Genomic DNA was eliminated using the On-Column DNase 1 Digestion Set kit (Sigma–Aldrich). The first-strand cDNA was synthesized using pulsed stem-loop RT-PCR (Varkonyi-Gasic et al., 2007) with the Improm-II Reverse Transcription Kit (Promega, Madison, WI, USA). The 7300 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) and the GoTaq qPCR Master Mix kit (Promega) were used to determine gene expression (Supplemental Table 1). The expression relative to the sorghum reference gene *rRNA18S* and the control with plants treated with deionized water was calculated.

The S. bicolor Genome Initiative locus identifier numbers for the genes investigated in this study were as follows: *SHR* (Sb02g037890), *PHB* (Sb08g021350), *PHV* (Sb01g013710), *REV* (Sb01g013710), *rRNA18S* (Sb02g027580), and *miR166* (MIMAT0001394).

2.5. Light microscopy analysis

Samples were collected from root tips and from 1 cm above the root tips of five seedlings from each treatment (n = 5). The material was fixed in FAA 50 (Johansen, 1940), dehydrated in alcoholic series, and embedded in methacrylate resin (Leica Historesin) (Gerrits, 1991). The material was sectioned using a rotary microtome, and the sections (6 μm thick) were stained with Toluidine Blue 0.05%, pH 4.7 (O’Brien et al., 1964). Permanent slides were mounted with synthetic resin Entellan (Mercck Millipore, Darmstadt, Germany) and analyzed using an Olympus BX 41 light
microscope (Olympus Corporation, Tokyo, Japan) equipped with digital camera.

2.6. Statistical analysis

The results are presented as average ± standard deviation (SD). Statistical comparisons were performed with one-way analysis of variance (ANOVA) complemented by Tukey’s test, comparing each treatment with control. Statistical significance was set at $P<0.05$. Sigma Plot version 12.0 was used for graphic design and statistics.

3. Results

3.1. Flavonoids identified in C. langsdorffii leaf extract

The HPLC-PAD-ESI-MS analyses of the 100% MeOH extract (Fig. 1) from the C. langsdorffii leaves revealed peaks with retention time (RT) between 0 and 35 min feature spectra in the UV–vis region specific of the benzyl system, with the maximum in the region of 210–220 and 260–280 nm. This suggests the presence of derivative compounds of gallic acid (Sticher, 2008). The presence of two peaks with RT = 36.98 (Peak 1) and RT = 41.70 min (Peak 2) with maximum absorption in the spectral range of 240–290 nm assigned to ring A and 300–390 nm assigned to ring B, typical in flavonoids, glycosides (Merken and Beecher, 2000). Peak 1 revealed the presence of the precursor ion at $m/z$ 447 [M–H]$^-$ and $\lambda_{\text{max}}$ at 253 and 356 nm and was identified as quercetin-3-O-rhamnoside. Peak 2 revealed the presence of the precursor ion at $m/z$ 431 [M–H]$^-$ and $\lambda_{\text{max}}$ at 264 and 354 nm and was attributed to kaempferol-3-O-alpha-rhamnoside.

3.2. Influence of treatments on germination speed

The percentage of germinated sorghum seeds did not show changes after the extracts and flavonoids were applied. However, germination was slowed (the exception were the treatments with IAA and NPA applied after germination). Inhibition compared to the control was observed in the groups treated with leaf extract (41.9%), quercetin (42.6%), and rutin (38.1%) (Fig. 2).

3.3. Response of root development in plants treated with allelochemicals

As show in Fig. 3, root growth was inhibited in all groups compared to the control. The treated groups showed root growth inhibition of 44.2% (leaf extract), 36.2% (quercetin), 45% (rutin), 64.5% (IAA), and 60.6% (NPA).

The development of lateral roots was assessed with the average of the lateral roots and the total number of each experiment compared to the control. The group treated with quercetin showed no difference compared to the control but had a 6% increase in the number of lateral roots. The groups treated with leaf extract, rutin, and IAA had an increase of 87.8%, 78.7%, and 193.9%, respectively, in the number of lateral roots. The group treated with NPA showed a 60.6% decrease in the number of lateral roots compared to the control (Fig. 4).

3.4. miR166, HD-ZIP III and SHR gene expression is affected in sorghum roots growing under allelopathic and auxin treatments

The expression of the genes (Table 1) showed a correlation between the groups treated with IAA and rutin, and another corre-
Table 1
Relative expression of miR166, SHR, PHB, PHV and REV genes in sorghum root under the effect of different treatments. Gene expression is represented with ratio (relative expression) of absolute value of expression value of each gene-by-gene expression normalizer 18S ribosomal RNA.

<table>
<thead>
<tr>
<th>Selected genes</th>
<th>Leaves extract</th>
<th>Quercetin</th>
<th>Rutin</th>
<th>IAA</th>
<th>NPA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expression ratio</td>
<td>Expression ratio</td>
<td>Expression ratio</td>
<td>Expression ratio</td>
<td>Expression ratio</td>
</tr>
<tr>
<td>miR166</td>
<td>1.535&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.314&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.007&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.019&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.000&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SHR</td>
<td>12.000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.002&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.016&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PHB</td>
<td>11.672&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.379&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.008&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.936&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PHV</td>
<td>3.242&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.004&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.383&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.013&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.653&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>REV</td>
<td>1.382&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.176&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.286&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.429&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.427&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Indicates no difference to control.
<sup>b</sup> Indicates significant down-regulation.
<sup>c</sup> Indicates significant up-regulation.

Fig. 2. Germination speed index (GSI) percentage compared to control of sorghum seedlings of different treated groups. Level of significance *P<0.05 by analysis of variance followed by Tukey test.

Fig. 3. Sorghum roots growth percentage compared to control. Level of significance *P<0.05 by analysis of variance followed by Tukey test.

downregulation of miR166. Roots treated with leaf extract showed upregulation of the SHR, PHB, and PHV genes.

3.5. Root anatomy

Despite the treatments and gene expression results, all roots presented similar cell structure, independent of the treatments. In all samples, the roots had a uniseriate epidermis with elongated hairs (Fig. 5A–C, E and F), except under NPA treatment (Fig. 5D), where no root hairs were observed. The cortex consisted of 5–7 layers of parenchyma cells. Lignified cell wall thickenings were observed in the exodermis in all groups but were more evident in the leaf extract (Fig. 5B) and IAA (Fig. 5C) groups. In plants treated with leaf extract and quercetin, in addition to the outermost cortical cell layer, the subjacent parenchyma cells exhibited prominent lignified thickening in the walls (Fig. 6A and B). Most of the endodermal cells presented additional lignified “U” shape thickenings (Fig. 6A, C and D). One to two rows of thick-walled cells composed the pericycle (Fig. 6A, C and D); the lignified thickenings of the cell walls was more evident in the pericycle of the plants treated with IAA (Fig. 5C). The xylem presented 9–10 arches of protoxylem; 2–4 wide metaxylem vessels were present in the central region of the root intermixed with parenchyma cells in all groups (Fig. 5A–E). Lateral root emergence was observed in all cross sections of the plants growing in all treatments; however, visualization of the lateral roots in plants treated with quercetin was scarce (Fig. 5A–D and F).

4. Discussion

*C. longsdorffii* leaf extract contains flavonoid glycosides (quercetin-3-O-alpha-rhamnoside and kaempferol-3-O-alpha-
rutin (O-alpha-rhamnoside) as major compounds in its chemical profile, and inhibitory action is observed in germination speed and root growth with patterns similar to those of the flavonoid rutin. In this study, the *Copaifera* leaf extract was found to be rich in flavonoids with glycosides. The extract inhibits root development, similar to the results for isolated flavonoids. Endogenous flavonoids and exogenous flavonoids in different doses can influence auxin transport in roots and induce lateral root growth in stress situations (Maloney et al., 2014).

The delay in the germination speed is an indicator of allelochemical compound action that affects the elongation and cell division mechanisms. Despite the decrease in the GSI, the final number of germinated seeds is not affected. Hoagland and Williams (2004) observed that this can occur through the activation of cellular detoxification mechanisms. The same authors also observed that the time for activation of this mechanism slows germination, which can help plant survival in a competitive environment.

Primary root growth is even more sensitive to the effect of allelochemicals. The inhibition of the development of the root system of neighboring species leads to a reduction in competitive pressure on the plant favoring its development (Ferreira and Aquila, 2000; Prates et al., 2000; Kato-Noguchi et al., 2013). De Martino et al. (2012) studied the effect of various flavonoids on the germination and early development of the roots of *Raphanus sativus* and *Lepidium sativum*. The authors observed that germination was slightly affected and early root growth was affected even more.

In the present study, the development of lateral roots was influenced by the treatments. We observed a higher number of lateral roots in plants treated with leaf extract, rutin, and IAA indicating a positive correlation between the application of exogenous auxin and flavonoid glycosides. The results are similar for the group treated with rutin and the group treated with leaf extracts, containing the major compounds quercetin-3-O-alpha-rhamnoside and kaempferol-3-O-alpha-rhamnoside, in contrast quercetin, an aglycone, produced no difference relative to the control. Auxin affects primary root and lateral root development (Krishnamurthy and Rathinasabapathi, 2013), which can occur by alterations in genes involved in signaling in the context of apical meristem organization and cell type specialization (Yu et al., 2015). This interesting observation reinforces data by Peer and Murphy (2007), whom demonstrated that flavonoids can modulate auxin transport, affecting auxin-dependent tropic responses and possibly interfering with

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**Fig. 5.** Cross sections of *Sorghum bicolor* roots. (A) Control; (B) leaves extract; (C) Indoleacetic acid (IAA); (D) 1-N-naphthylphthalamic acid (NPA); (E) quercetin (QUER); (F) rutin (RUT). Scale bars = 150 μm.
the number of lateral roots. The presence of high concentrations of flavonoids can inhibit root growth, likely due to the breakage of cell homeostasis leading to allelopathic stress. Although root growth inhibition could be due to the flavonoids present in leaf extract and rutin (flavonoid glycoside), flavonoids can present an important function in signaling to auxin transport. This can be noted by the correlation shown between the root growing of plants treated with the extract, rutin and IAA, resulting in an increase in lateral roots. Recently, in tomato plant mutants with altered flavonoid biosynthesis, positive roles for flavonols were identified in the formation of lateral roots and negative roles in the formation of root hairs through auxin transport modulation (Maloney et al., 2014), while inhibitory activity of flavonol glycoside kaempferol 3-O-rhamnoside-7-O-rhamnoside was observed in polar auxin transport in A. thaliana shoots (Yin et al., 2014).

In addition to a higher number of lateral roots, another indicator of auxin effect is the increase in the expression levels of PHB and PHV in roots from plants treated with leaf extract, rutin, and IAA; the genes that encode for the transcription factors of the family HD-ZIP III genes responded to auxin treatments (Chapman and Estelle, 2009; Sunkar et al., 2012). Krishnamurthy and Rathinasabapathi (2013) showed that in A. thaliana plants treated with arsenite, auxin transport was affected, root growth was inhibited, the number of lateral roots increased, and lipid peroxidation levels were high, which indicated oxidative stress. However, in plants treated subsequently with exogenous auxin, the effects of oxidative stress were smaller, which shows the importance of auxin as an indicator of stress tolerance. Higher lateral development (lateral roots) and inhibition of primary root growth are morphological mechanisms of stress tolerance. This was observed in plants treated with IAA, rutin, and leaf extracts, but in the group treated with auxin transport inhibitor (NPA), the growth of the primary roots and the number of lateral roots were lower compared to control plants. According to reports on Hordeum vulgare, miR166 expression decreases in roots growing under stress (Sunkar et al., 2012), therefore the decrease in the expression of miR166 observed in roots of plants treated with quercetin and rutin is another indicator of stress.

In addition, miR166 expression did not change under IAA treatment, while the PHB were more expressed (Table 1). The increase in the expression of the PHB gene and in the number of lateral roots are also positively correlated in the treatment with IAA, rutin and leaf extracts groups, illustrating a possible role for auxin for signaling of stress. According to Carlsbecker et al. (2010), PHB expression is correlated with the determination of the xylem type. High levels of expression are associated with the formation of metaxylem, and low levels are associated with the formation of protoxylem arches. PHB is the only member of the HD-ZIP III family which is expressed outside the vascular cylinder, also occurring in the endodermis, and has activity in the ontogenesis of roots as the transcription factor in the pericycle. An increase was observed in PHB expression when the first morphological changes in the pericycle after the beginning of cell division occurred, and remained high until the lateral roots emerged (Hawker and Bowman, 2004). PHB seems to signals the resumption of pericycle meristematic activity, leading to the emergence of lateral roots. According to our results, C. lansdorffii leaf extract decreased primary root growth and stimulated lateral root growth. The same result was observed by Franco et al. (2015) with Myrcia guianenses, although no lateral root growth was observed. This response possibly occurs because M. guianenses leaf extract stimulates the expression of miR166, which negatively regulates the transcripts of the HD-ZIP III family, especially PHB gene, as a consequence of which cell differentiation did not occur in pericycle.

The data agree with the literature and may show that leaf extract, rutin, and IAA can increase the signaling by auxin influencing expression of the PHB.

The higher the activity in the pericycle, the greater the production of lateral roots. Thus, increase in the expression levels of
The PHB caused by allelopathic compounds might lead to a higher meristematic activity of the pericycle inducing new cellular divisions in the development of roots (Clark and Harris, 1981; Enstone et al., 2002).

The expression level of SHR showed distinct results among the groups. Treatments with leaf extract produced an increase in SHR expression level, but the root anatomy of the seedlings treated with leaf extract and IAA had endodermal cells with thicker walls. This result suggests that an alteration in only SHR expression is not sufficient to lead to differentiation in ground tissue modification but balances of the expression levels of SHR and other genes (Scheres et al., 1995; Wachsmann et al., 2015).

Thus, quercetin and rutin have similar allelopathic potential: delaying germination and inhibiting root growth. However, treatment with IAA and NPA showed that the differences in glycoside composition in flavonoid patterns and in the flavonoids present in the leaf extract can act as signal for different response mechanisms to abiotic stress. In the rutin and leaf extract treatments, characterized by the predominant presence of flavonoid glycosides, had results similar to the treatment with exogenous auxin were found. The treatment with aglycone quercetin showed similar results to those found in the control with NPA. The changes in the number of lateral roots and in the expression pattern of SHR and HD-ZIP III genes associated with early development of root tissue development showed that flavonoid glycosides can influence the polar transport of auxin, which leads to stress responses dependent on auxin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2015.09.009.

References


