

# Identification of gravitropic response indicator genes in *Arabidopsis* inflorescence stems

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**Keywords:** *Arabidopsis thaliana*, shoot gravitropism, inflorescence stem, *IAA5*, gene expression, gravitropic response indicator

**Abbreviations:** AuxRE, auxin responsive element; GUS,  $\beta$ -glucuronidase; GFP, green fluorescent protein; GRI, gravitropic response indicator; WT, wild type

Differential organ growth during gravitropic response is caused by differential accumulation of auxin, that is, relative higher auxin concentration in lower flanks than in upper flanks of responding organs. Auxin responsive reporter systems such as *DR5::GUS* and *DR5::GFP* have usually been used as indicators of gravitropic response in roots and hypocotyls of *Arabidopsis*. However, in the inflorescence stems, the reporter systems don't work well to monitor gravitropic response. Here, we aim to certify appropriate gravitropic response indicators (GRIs) in inflorescence stems. We performed microarray analysis comparing gene expression profiles between upper and lower flanks of *Arabidopsis* inflorescence stems after gravistimulation. Thirty genes showed > 2-fold differentially increased expression in lower flanks at 30 min, of which 19 were auxin response genes. We focused on *IAA5* and *IAA2* and verified whether they are appropriate GRIs by real-time qRT-PCR analyses. Transcript levels of *IAA5* and *IAA2* were remarkably higher in lower flanks than in upper flanks after gravistimulation. The biased *IAA5* or *IAA2* expression is disappeared in *sgr2-1* mutant which is defective in gravity perception, indicating that gravity perception process is essential for formation of the biased gene expression during gravitropism. *IAA5* expression was remarkably increased in lower flanks at 30 min after gravistimulation, whereas *IAA2* expression was gradually decreased in upper flanks in a time-dependent manner. Therefore, we conclude that *IAA5* is a sensitive GRI to monitor asymmetric auxin signaling caused by gravistimulation in *Arabidopsis* inflorescence stems.

## Introduction

Plants perceive gravity and change their growth direction as a survival strategy for adaptation to environment. The shoot shows negative gravitropism, and the root shows positive gravitropism. Gravitropism is thought to consist of 4 sequential processes: gravity perception, signal conversion, signal transduction, and asymmetric organ growth. Gravity is perceived in statocytes (gravity sensing cells), which are endodermal and columella cells in shoots and in roots, respectively.<sup>1,2</sup> The statocytes contain amyloplasts, which are starch accumulating plastids to work as statolith. When plants are reoriented, amyloplasts in statocytes sediment to new cell bottom according to the gravity vector. Amyloplast sedimentation, which is a physical signal, should be converted into biochemical signals in the statocytes. There are several candidates of the biochemical signals. Cytosolic alkalization observed in columella cells in *Arabidopsis* roots is a possible signal triggered by amyloplast sedimentation.<sup>3</sup> In addition, transient changes in concentration of inositol 1,4,5-triphosphate<sup>4</sup> and/or Ca<sup>2+</sup><sup>5,6</sup> observed in the

responding organs are also suggested as the signals, although it is still unclear whether such changes occur in the statocytes and how they are involved in gravitropic response. In any case, the signal is finally transmitted to the elongating organ to trigger asymmetric organ growth.

Cholodny-Went hypothesis suggests that auxin asymmetrically distributes in lower side of gravistimulated organ, and then higher auxin concentration, which promotes or inhibits cell elongation in shoots or in roots, respectively, results in organ curvature.<sup>7</sup> Indeed, it has been shown that indol-3-acetic acid, a major endogenous auxin, was asymmetrically distributed after gravistimulation in rice and corn coleoptile.<sup>8-10</sup> Furthermore, our knowledge about the molecular mechanism to cause asymmetric auxin distribution is growing. Intracellular polar localization of PIN3, an auxin efflux carrier protein mainly expressed in statocytes, is involved in generation of asymmetric auxin distribution in *Arabidopsis* roots.<sup>11</sup>

Transcriptome analyses during early gravitropic response in *Arabidopsis* roots have shown that numerous genes including auxin response genes such as *AUX/IAA* and *SAUR* family genes

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**Table 1.** The list of genes whose expressions were increased > 2-fold in lower flanks relative to the upper flanks of inflorescence stems at 30 min after gravistimulation

AGI	Annotation	Fold change
At2g22810	Aminocyclopropane-1-carboxylate synthase 4 (ACS4)	8.63
<b>At1g15580</b>	<b>Indole-3-acetic acid inducible 5 (IAA5)</b>	<b>6.60</b>
At5g66580	Unknown protein	4.70
At3g57260	Beta-1,3-glucanase 2 (BGL2)	4.58
<b>At4g13790</b>	<b>SAUR-like auxin responsive protein family</b>	<b>3.83</b>
<b>At5g18010</b>	<b>Small auxin up RNA 19 (SAUR19)</b>	<b>3.71</b>
<b>At5g18080</b>	<b>Small auxin up RNA 24 (SAUR24)</b>	<b>3.57</b>
<b>At5g18030</b>	<b>SAUR-like auxin responsive protein family</b>	<b>3.44</b>
<b>At5g18020</b>	<b>Small auxin up RNA 20 (SAUR20)</b>	<b>3.35</b>
<b>At4g38850</b>	<b>Small auxin upregulated 15 (SAUR15)</b>	<b>3.29</b>
<b>At5g18050</b>	<b>Small auxin up RNA 22 (SAUR22)</b>	<b>3.24</b>
<b>At5g18060</b>	<b>Small auxin up RNA 23 (SAUR23)</b>	<b>3.22</b>
<b>At1g29500</b>	<b>SAUR-like auxin responsive protein family</b>	<b>3.10</b>
<b>At1g29440</b>	<b>Small auxin up RNA 63 (SAUR63)</b>	<b>3.03</b>
<b>At1g29460</b>	<b>SAUR-like auxin responsive protein family</b>	<b>2.96</b>
At5g02760	<i>Arabidopsis</i> PP2C clade D 7 (APD7)	2.89
<b>At1g29450</b>	<b>SAUR-like auxin responsive protein family</b>	<b>2.84</b>
<b>At4g14560</b>	<b>Indole-3-acetic acid inducible 1 (IAA1)</b>	<b>2.83</b>
At5g52900	Membrane-associated kinase regulator 6 (MAKR6)	2.82
At5g47370	Homeobox-leucine zipper protein 2 (HAT2)	2.62
At4g25420	Gibberellin 20-oxidase 1 (GA20OX1)	2.37
At1g78100	Auxin upregulated F-box protein 1 (AUF1)	2.31
At5g39860	Paclobutrazol resistance 1 (PRE1)	2.26
<b>At1g04240</b>	<b>Short hypocotyl 2 (SHY2/IAA3)</b>	<b>2.25</b>
<b>At1g29510</b>	<b>Small auxin upregulated 68 (SAUR68)</b>	<b>2.22</b>
At5g10760	Eukaryotic aspartyl protease family protein	2.22
<b>At1g15550</b>	<b>Gibberellin 3-oxidase 1 (GA3OX)</b>	<b>2.10</b>
<b>At3g03820</b>	<b>Small auxin up RNA 29 (SAUR29)</b>	<b>2.10</b>
<b>At3g23030</b>	<b>Indole-3-acetic acid inducible 2 (IAA2)</b>	<b>2.06</b>
<b>At1g52830</b>	<b>Short hypocotyl 1 (SHY1/IAA6)</b>	<b>2.02</b>

AGI: gene identification number by the *Arabidopsis* Genome Initiative. The gene annotation was retrieved from TAIR (<http://arabidopsis.org/index.jsp>). The genes in bold are *AUX/IAA* or *SAUR* family genes.

are upregulated.<sup>12-15</sup> Comparison of gene expression profiles between lower and upper flanks of *Brassica oleracea* hypocotyls or rice shoot base has demonstrated that auxin response genes are asymmetrically induced in the lower flanks of organs after gravistimulation, probably reflecting that auxin distribution.<sup>14,15</sup>

DR5 is a synthetic auxin responsive promoter which has multiple copies of auxin responsive elements (AuxRE).<sup>16</sup> *DR5::GUS* and *DR5::GFP* reporters produce  $\beta$ -glucuronidase (*GUS*) and green fluorescent protein (*GFP*), respectively, from the DR5 promoter in response to auxin. Thus, histochemical staining and fluorescence of their transgenic plants can indicate local auxin signal intensity.<sup>17,18</sup> Asymmetric auxin signaling after gravistimulation in primary roots and hypocotyls is also detected

with *DR5::GUS* and *DR5::GFP* reporters in *Arabidopsis*.<sup>11,19-21</sup> In contrast, *DR5::GUS* does not work in inflorescence stems,<sup>22</sup> and there are no reports on the availability of *DR5::GFP* to monitor auxin signaling in gravitropic response of inflorescence stems. In addition, DII-VENUS, a fluorescent reporter, was recently developed as an auxin sensor.<sup>23</sup> Since DII-VENUS is degraded in response to auxin, its abundance inversely correlates with auxin activity. Thus, DII-VENUS allows rapid detection of dynamic changes in auxin distribution at the cellular level. Actually, DII-VENUS can reflect asymmetric auxin distribution after gravistimulation,<sup>24</sup> although it has not been known whether it is suitable for monitoring auxin distribution during gravitropic response in inflorescence stems. Therefore, an appropriate reporter system is essential to study the details of gravitropic signaling processes in inflorescence stems.

Here, we focused to find appropriate gene(s) as gravitropic response indicators (GRIs) to monitor gravitropic signaling in inflorescence stems of *Arabidopsis*. We expected that genes differentially induced in lower flanks of inflorescence stems in response to gravistimulation can be used as GRIs. In this study, we identified 2 GRIs among auxin response genes found by differential transcriptome analysis between upper and lower flanks of the stems. We also demonstrated that the asymmetric expression of the GRIs is largely dependent on the gravity perception.

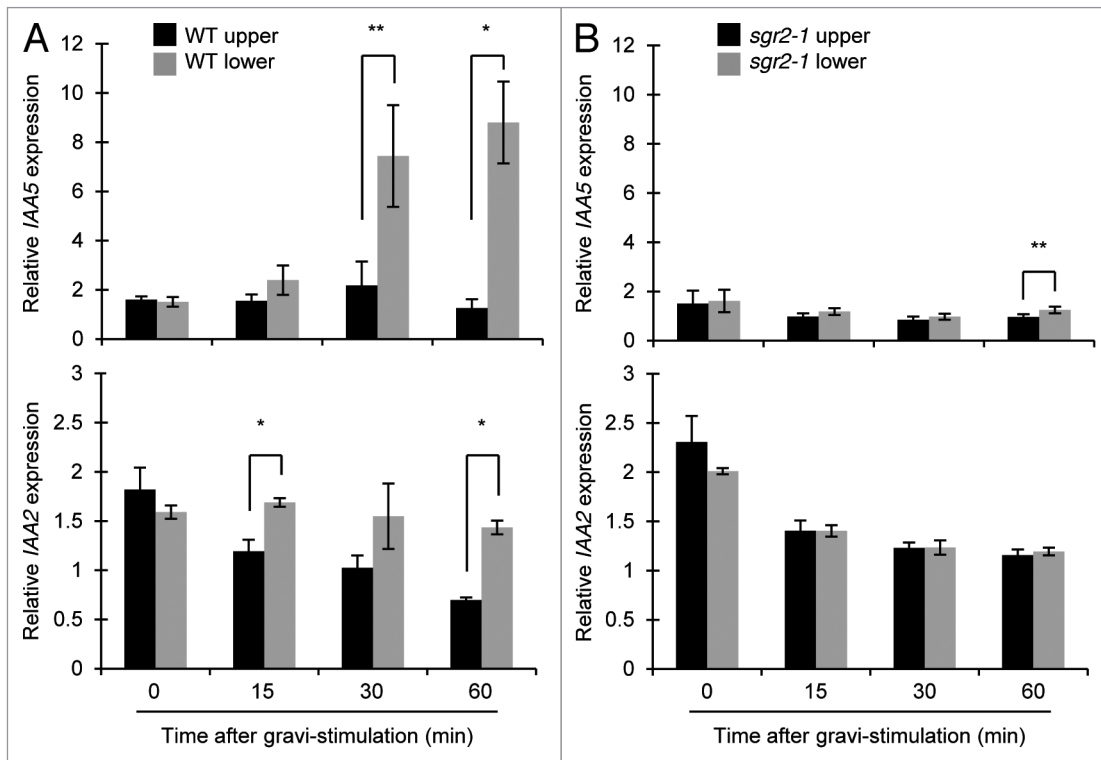
## Results

### Screening of GRI candidates in inflorescence stems of *Arabidopsis*

Inflorescence stems of *Arabidopsis thaliana* show the first visible bending at 20 min after gravistimulation by placing horizontally, and then the stems reached the vertical position in about 90 min.<sup>25</sup> To find genes, gravitropic response indicators (GRIs) which exhibit increased expression in the lower flanks relative to the upper flanks in inflorescence stems after gravistimulation, we performed comparative transcriptomic analyses between each flank at 10 min and 30 min with microarray. As a result, none of the characterized genes showed >2-fold differentially increased expression in lower flanks at 10 min (data not shown). In contrast, 30 genes were showed >2-fold differentially increased expression in the lower flanks relative to the upper flanks at 30 min (Table 1), indicating that differential gene expression between each flank becomes detectable from 10 to 30 min in our experimental condition. The 30 genes, which can be considered as GRI candidates, included 5 *Aux/IAA* family genes and 14 *SAUR* family genes, which are auxin response genes (Table 1).<sup>26</sup> The result implies that auxin asymmetric distribution may precede the differential expression of auxin response genes in inflorescence stems.

### Expression level of *IAA5* was increased in lower flanks of inflorescence stems after gravistimulation

Among 19 auxin response genes of GRI candidates, *IAA5* gene exhibits a 6.60-fold increased expression in the lower flanks relative to the upper flanks (Table 1). To validate this



**Figure 1.** Relative expressions of *IAA5* and *IAA2* after gravistimulation in upper and lower flanks of inflorescence stems. (A) wild type (WT), (B) *sgr2-1*. Relative expression level of *IAA5* and *IAA2* were normalized to expression of an actin gene (*ACT8*) in arbitrary units. Bars represent  $\pm$  SD of 3 biological replicates. The statistical significance was tested using a Student *t*-test between upper and lower flanks at same time (\* $P < 0.01$  and \*\* $P < 0.05$ ).

differential expression of *IAA5*, real-time qRT-PCR analysis was performed. Total RNA was extracted from upper and lower flanks of inflorescence stems after 0, 15, 30, and 60 min gravistimulation. Expression of *IAA5* was significantly increased in lower flanks of inflorescence stems 30 and 60 min after gravistimulation (Fig. 1A). On the other hand, the level of *IAA5* was stable in upper flanks from 0 min to 60 min (Fig. 1A).

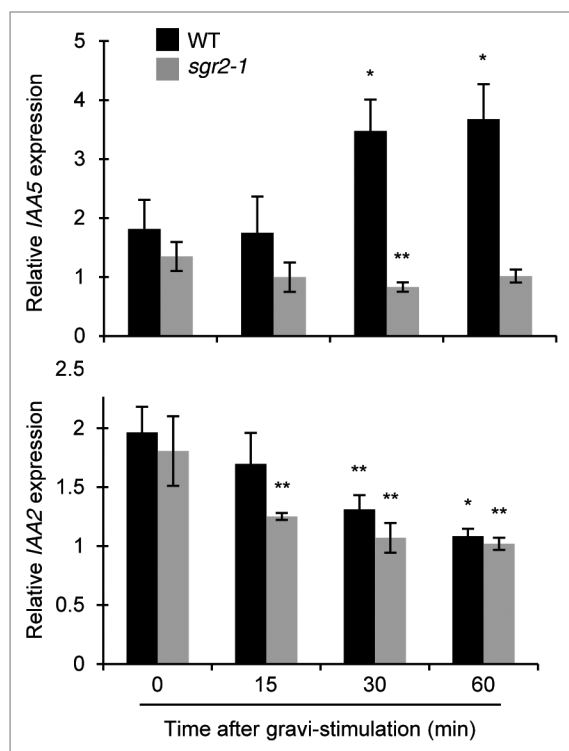
It has been reported that a reporter gene using the promoter of *IAA2* fused with *uidA* gene encoding GUS ( $\beta$ -glucuronidase) exhibits deeper staining in lower flanks than upper flanks of inflorescence stems 60 min after gravistimulation.<sup>22</sup> Our microarray analysis showed that *IAA2* exhibits a 2.06-fold increased expression in the lower flanks relative to the upper flanks (Table 1). Real-time qRT-PCR analysis showed that expression level of *IAA2* in upper flanks decreased at 15 min and 60 min although that in lower flanks was stable at all time-points (Fig. 1A). The results indicate that the differential expression of *IAA2* between upper and lower halves of stems was generated by a different manner from that of *IAA5*.

We also determined the change in expression levels of *IAA5* and *IAA2* genes in whole stems before and after gravistimulation. The expressions of both genes were remarkably changed at 30 and 60 min compared with those at 0 min (Fig. 2). However the patterns of those expressions were totally different, i.e., expression level of *IAA5* was increased, whereas that of *IAA2* was decreased in a time-dependent manner (Fig. 2). These results suggest that not all GRI candidate genes, which exhibit

relatively increased expression in lower flanks, are upregulated after gravistimulation in the whole stems.

#### Gravity perception was essential for differential expression of *IAA5* and *IAA2*

Since our gravistimulation, reorientation of plants, may contain mechanical stimulations, it is important to test whether the response of *GRI* gene expression is dependent on the gravity perception that happens in the statocytes. The *sgr2-1* mutant has a defect in gravity perception due to the abnormal amyloplast sedimentation in the shoot statocytes, resulting in little gravitropic response in inflorescence stems.<sup>27-29</sup> To examine whether expression changes in *IAA5* and *IAA2* upon gravistimulation are dependent on gravity perception, real-time qRT-PCR analyses were performed with *sgr2-1* mutant stems. Neither increase of *IAA5* expression in the lower flanks nor decrease of *IAA2* expression in the upper flanks was detected in the *sgr2-1* inflorescence stems upon gravistimulation (Fig. 1B). In addition, there is no significant difference in the expression levels of *IAA5* and *IAA2* between upper and lower flanks at any time-points except for small difference for *IAA5* at 60 min (Fig. 1B). In whole stems, expression level of *IAA5* or *IAA2* is almost constant or gradually decreased, respectively, after gravistimulation in *sgr2-1* (Fig. 2). The decreased expression of *IAA2* after gravistimulation was observed both in wild type and in *sgr2-1*, though the mechanism of the phenomena is unclear. These results indicated that intact gravity perception process was necessary for the differential expression of *IAA5* and *IAA2*,



**Figure 2.** Relative expressions of *IAA5* and *IAA2* after gravistimulation in whole inflorescence stems. Relative expression level of *IAA5* and *IAA2* were normalized to expression of an actin gene (*ACT8*) in arbitrary units. Bars represent  $\pm$  SD of 3 biological replicates. The statistical significance was tested using a Student *t*-test against relative expression at 0 min of WT ( $*P < 0.05$ ) or *sgr2-1* ( $**P < 0.05$ ).

and also suggested that the differential expression of *IAA5* and *IAA2* might mainly reflect asymmetric auxin distribution during gravitropism.

## Discussion

We performed comprehensive screening of differentially expressed genes in upper and lower flanks of inflorescence stems after gravistimulation (Table 1). The majority of 30 GRI candidate genes found in microarray screening is auxin response gene, which show expression biased to lower flanks, implying formation of differential distribution of auxin in inflorescence stems. We identified 2 auxin response genes, *IAA5* and *IAA2*, as GRIs to monitor gravitropic response 30 min after gravistimulation. However, the mechanisms producing the differentially increased expression in lower flanks seem to differ from one another (Fig. 1, 2, discussed below). Furthermore, intact gravitropic perception process was essential for their expression changes, because the changes in expression of both genes disappeared in *sgr2-1*.

The expression of *IAA5* was remarkably induced in lower flanks at 30 min after gravistimulation. In contrast, the expression of *IAA2* was reduced in upper flanks from 15 to 60 min after gravistimulation. In the previous report by Nadella et al.,

histochemical analysis of *GUS* reporter activity driven by *IAA2* promoter after gravistimulation showed that *GUS* staining seemed to be deeper in lower side than in upper side of WT inflorescence stems from 20 to 60 min.<sup>22</sup> Assuming that the *IAA2* promoter activity is decreased in the upper flanks, the result of staining pattern could not be formed because *GUS* protein is hardly degraded in such short time-span due to its high stability. One possible explanation is that the *IAA2* promoter activity could be upregulated by gravistimulation but it cannot operate the level of *IAA2* transcripts (Fig. 2) due to a post-transcriptional regulation, while the *IAA2* mRNA might be more severely degraded in upper flanks than lower flanks in a post-transcriptional manner (Fig. 1A).

The difference in expression level between upper and lower flanks after gravistimulation of *IAA5* is remarkably larger than that of *IAA2*. In addition, real-time qRT-PCR analyses using split or whole stems demonstrate that *IAA5* transcript is increased specifically in lower flanks at 30 min after gravistimulation without decrease of the *IAA5* transcript in upper flanks. The promoter of *IAA5* contains AuxRE and *IAA5* has been recognized as one of early/primary auxin-responsive gene.<sup>30,31</sup> The characteristics of *IAA5* expression makes it easier to understand the relationship between gene expression behavior and gravitropic response than that of *IAA2*. These facts indicate that *IAA5* is a rapid and sensitive GRI to monitor asymmetric auxin signaling caused by gravistimulation in *Arabidopsis* inflorescence stems. The measurement of *IAA5* asymmetric expression would be a new available method to quantify gravitropic response of inflorescence stems in addition to measuring the organ curvature. *IAA5* as a GRI will contribute to research on molecular mechanism of signaling process during gravitropic response in inflorescence stems.

## Materials and Methods

### Plant materials and growth conditions

The Columbia accession of *Arabidopsis thaliana* was used as the wild type (WT). The *sgr2-1* mutant was described previously.<sup>27,28</sup> Seeds were sterilized and plated on Murashige and Skoog (MS) plates followed by treatment at 4 °C for 3 days. Seedlings were germinated under constant light at 23 °C for 10 d. Plants were transplanted and grown on soil under constant white light at 23 °C.

### Total RNA extraction from gravistimulated inflorescence stem

To gravistimulate the inflorescence stems, plants harboring primary stems 4–8 cm in length were placed horizontally under non-directional dim light at 23 °C. Gravistimulated stems were decapitated 5 mm from the top of inflorescence, and segments 3 cm and 3.5 cm in length from the cutting position were used for whole stem analysis of real-time qRT-PCR and microarray analysis, respectively. For upper and lower flank analysis, these segments were sectioned longitudinally and manually. The segments were rapidly frozen by liquid nitrogen, and then total RNA was extracted with RNeasy Plant Mini kit (Qiagen,



Venlo, Netherland). Six whole stem segments or 10 upper and lower half segments were used for RNA isolation as one sample of real-time qRT-PCR analyses.

#### Microarray analysis

Prior to experiment, total RNA was examined for purity using an Agilent 2100 Bioanalyzer (Agilent, California USA). 0.5 µg of total RNA was used for cDNA synthesis and cRNA labeling reaction with Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent, California USA). In this reaction, each cRNA was labeled both cyanine-3 and cyanine-5 in separate reactions. These labeled RNA were hybridized to Agilent *Arabidopsis* 2 Oligo Microarray (Agilent, California USA) and were washed. Signals were detected with Agilent technologies Microarray Scanner (Agilent, California USA). Two hybridization including dye swap was performed for each experiments.

#### Real-time qRT-PCR

cDNA was synthesized with ReverTra Ace<sup>®</sup> qPCR RT Master Mix with gDNA Remover (TOYOBO, Tokyo, Japan) from 0.5 µg of total RNA. Real-time qPCR was performed using the LightCycler<sup>®</sup> 96 Real-Time PCR System (Roch Applied Science, Upper Bavaria, Germany) and KAPA SYBER<sup>®</sup> FAST qPCR Kit (KAPABIOSYSTEMS, Massachusetts USA) was used for preparation of real-time qPCR reaction mix. Reactions were run in triplicate in 3 independent experiments. The results of real-time qPCR were analyzed with LightCycler<sup>®</sup> 480 Software (Roch Applied Science, Upper Bavaria, Germany), and mRNA

relative expression levels (arbitrary units) were determined using the relative standard curve for *IAA5*, *IAA2*, and *ACT8*, which were generated by serial dilutions of cDNA.<sup>32</sup> *ACT8* was used as an internal control for normalization. The following primers used for amplification were designed at a specific region for each genes: At1g15580-IAA5-qPCR-F1 (5'-TGAAGGAAAG TGAATGTGTA CCAA-3'), AT1g15580-IAA5-qPCR-R1 (5'-GCACGATCCA AGGAACATTT-3'), At3g23030-IAA2-qPCR-F1 (5'-GAAGAATCTA CACCTCCTAC CAAAA-3'), At3g23030-IAA2-qPCR-R1 (5'-CACGTAGCTC AACTGTTGT TG-3'), At1g49240\_qPCR-F (5'-TCAGCACTTT CCAGCAGATG-3') and At1g49240\_qPCR-R (5'-CTGTGGACAA TGCCTGGAC-3').

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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