

Effects of shoot-applied gibberellin/gibberellin-biosynthesis inhibitors on root growth and expression of gibberellin biosynthesis genes in *Arabidopsis thaliana*

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Received on September 29, 2009; Accepted on December 14, 2009

Abstract: To elucidate the involvement of gibberellin (GA) in the growth regulation of *Arabidopsis* roots, effects of shoot-applied GA and GA-biosynthesis inhibitors on the root were examined. Applying GA to the shoot of *Arabidopsis* slightly enhanced the primary root elongation. Treating shoots with uniconazole, a GA biosynthesis inhibitor, also resulted in enhancement of primary root elongation, while shoots treated with uniconazole were stunted and bolting was delayed. Analysis of the expression of GA3ox and GA20ox confirmed the up-regulation of these genes in roots following the inhibitor application to shoots. The results suggest that the inhibition of GA-biosynthesis enhances the production of bioactive GAs in roots and promotes root elongation.

Keywords: *Arabidopsis*, branching, gibberellin, primary root elongation

Abbreviation: GUS, 1,5-bromo-4-chloro-3-indolyl- β -D-glucuronide; GA, Gibberellin; Uni, Uniconazole; Pacl, Paclobutrazole; QRT-PCR, quantitative reverse transcription PCR

Introduction

Plant hormones are important in regulating root growth. Early physiological work on different plant tissues demonstrated that, of the seven known plant hormones, gibberellic acid and brassinolide accelerate shoot growth, but do not always accelerate root growth. Auxin is the most extensively studied of the

plant hormones and plays a central role in regulation of root growth (Tanimoto 2005). Compared to auxins, GA functions are less remarkable. GAs are a class of diterpenoids, some of which act as hormones that control many aspects of plant growth and development. Cytokinin was also reported as one of important factors for the regulation of root growth, especially cell differentiation in elongation zone (Dello et al. 2007).

In the GA biosynthetic pathway, GA₁ and GA₄ act as bioactive hormones, while other GAs are either precursors of bioactive GAs or inactive forms. Recently, significant progress has been made in understanding GA biosynthesis and signaling pathways, including identification of genes encoding GA metabolic enzymes and components of the GA response pathway in model plants such as *Arabidopsis*, rice, pea, and tomato (Ross et al. 1997; Robbers et al. 1999; Yamaguchi and Kamiya 2000; Olszewski et al. 2002; Sun and Gulber 2004; Ueguchi-Tanaka et al. 2005). Knowledge of these GA genes allows investigation of possible biosynthesis regulation, namely through expression of the GA biosynthetic genes.

Our understanding of transport of GAs within the plant body is still limited. As a major route for transfer of materials between organs, the vascular system serves both as a long-distance communication network and a transport pathway for water and nutrients. It provides an important route for inter-organ communication that allows plants to integrate environmental inputs into physiological and developmental responses (Lough and Lucas 2006). Environmental inputs are sensed by mature organs, and signals generated are then transported to meristematic regions, where newly formed organs are

Bidadi H, Yamaguchi S, Asahina M, Satoh S 2010 Effects of shoot-applied gibberellin/gibberellin-biosynthesis inhibitors on root growth and expression of gibberellin biosynthesis genes in *Arabidopsis thaliana*. Plant Root 4: 4-11. doi:10.3117/plantroot.4.4

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influenced to better adapt to the environment in which they will develop and function. GAs can be transported from shoots to roots and vice versa; however, no specific transporter or carrier has been reported to date. Transfer of GAs and/or GA signals from leaves to roots to control gene expression of the xylem sap protein XSP30 in roots has been reported in cucumber (Oda et al. 2003). Moreover GA produced in the cotyledons is required for cell division during tissue regeneration in the cut cortex of cucumber and tomato hypocotyls (Asahina et al. 2002, 2007). These results suggest the involvement of shoot-produced GA and/or its precursor in maintenance of the physiological state of roots and hypocotyls. Previous studies using isotope-labeled GAs or grafting experiments with GA-deficient mutants have suggested that GAs or GA precursors may move around the plant body (Rudich et al. 1976; Reid et al. 1983; Yang et al. 1995; Eriksson et al. 2006). However, the necessity and physiological significance of GA movement during plant development and in environmental responses still remains unknown.

GAs are known to play an indispensable role in the normal development of roots, since artificial GA depletion causes abnormal expansion and suppression of root elongation (Tanimoto 1987, 1988, 2005). Investigations have implied that endogenous GAs control root growth also in *Lemna minor* (Inada and Shimmen et al. 2000). The role of GA in root growth of *Pisum sativum* has been elucidated also by using inhibitors of GA biosynthesis (Tanimoto 1988) and by analyzing GA-deficient mutants (Tanimoto 1994; Yaxley et al. 2001). Recent researches reported that gibberellin signaling in the root endodermis regulates *Arabidopsis* root growth by controlling cell elongation and root meristem size. (Ubeda-Tomas et al. 2008, 2009). However, studies on root elongation are few compared with those on shoot elongation, because promotion of root elongation by plant hormones is less obvious.

In this study, after morphological observations of the effect of shoot-applied GA and inhibitors on root growth, we analyzed the expression of several GA biosynthesis pathway genes using quantitative reverse transcription PCR (QRT-PCR) in both shoot and root tissues of 3-week-old *Arabidopsis* plants. We found that inhibitors of GA biosynthesis may be capable of up-regulating expression of several GA biosynthesis genes in roots, possibly to maintain normal root growth by a feedback regulation.

Materials and Methods

Plant material, growth conditions, germination, and root length measurements

Seeds of *Arabidopsis thaliana*, ecotype Columbia, were incubated in water at 4°C for 3 days prior to planting on half-strength MS medium (Murashige and Skoog Plant Salt Mixture, Wako, Japan) containing 1.5% agar and 0.5% sucrose.

Construction and transformation of *pAT-GA3ox1::GUS* gene fusion constructs were carried out as described previously (Mitchum et al. 2006; Yamaguchi et al. 2001). Kanamycin selection of *pGA3ox1::GUS* seeds was done on half-strength MS medium plates containing 1.5% agar, 0.5% sucrose, and 50 mg mL⁻¹ kanamycin. Plants having a single insertion were identified as those that showed 3:1 kanamycin resistance versus kanamycin-sensitive segregation patterns in the T₂ generation. Homozygous lines of the T₃ generation for each construct were used in observations.

For monitoring root growth, surface-sterilized seeds were spread on germination medium (MS medium containing 1.5% agar, 0.5% sucrose). Because seed storage time greatly impacts germination potential, the experiments were performed using seeds that had been stored for a similar length of time. To measure root length, seedlings were grown on vertically oriented MS medium in Petri dishes and incubated in a growth chamber under continuous light at 22°C. Images of plants were taken before and after treatments using a Leica camera (Leica Microsystems, Wetzlar, Germany). Root length was determined from digital images and measurements were performed using a digital curvimeter (Uchida, Japan).

Hormone and inhibitor treatments

GA₄ (Sigma, Germany), paclobutrazol (Wako, Japan), and uniconazole-P (Wako, Japan) were dissolved in ethanol and diluted 1000-fold in 0.1% (v/v) Tween 20 followed by filter sterilization. Shoot apices of 3-week-old *Arabidopsis* plants were treated with 5–10 μL 10⁻⁶ M GA₄ twice in 3 days, and primary root elongation and number of lateral roots were examined 3 days later. For uniconazole, 10⁻⁵ M was applied to shoots of 3-week-old *Arabidopsis* and measurements were done 3 days later.

Real-time quantitative RT-PCR

Total RNA was extracted from *Arabidopsis* roots using an RNAqueous RNA isolation kit with Plant RNA Isolation Aid (Ambion, Austin, TX). First-strand cDNA was synthesized from total RNA (1 mg) with the QuatiTect Reverse Transcription Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). QRT-PCR with Taq-Man technology (Holland et al. 1991) or SYBR Green I reagents (Qiagen) was performed using the first-strand cDNA

as template on a sequence detection system (ABI PRISM 7000, Applied Biosystems, Foster City, CA) as described in Yamauchi et al. (2004). For normalization across samples, 18S rRNA was used as an internal standard. To further confirm the reliability of data, measurements were repeated using two independent plant samples. Both independent experiments showed significant similar results. Nucleotide sequences of gene-specific primers were as follows:

GA20ox1 primer: 5'- GCCTGTAAGAAGCACGGT TTCT-3' and 5'- CTCGTGTATTCATGAGCG TCTGA-3'

GA20ox2 primer: 5'- CCAAGGCT TTCGTTGTC AA-3' and 5'- CCGCTCTATGCAAACAGC TCT-3'

GA20ox3 primer: 5'- TCGTGGACAACAAATGGC A-3' and 5'- TGAAGGTGTCGCTATGTTCA C-3'

GA3ox1 primer: 5'- TCCGAAGGTTTCACCATC ACT-3' and 5'- TCGCAGTAGTTGAGGTGA TGTTG-3'

GA3ox2 primer: 5'- GTTTCACCGTTATTGGCT CTCC-3' and 5'- TCACAGTATTTGAGGTGG TGGC-3'.

Histochemical analysis of GUS activity

For GUS staining, 3-week-old whole plants transformed by *pGA3ox1::GUS* were immersed in 1 mg mL⁻¹ 1,5-bromo-4-chloro-3-indolyl-β-D-glucuronide in 50 mM sodium phosphate, pH 7.0. Samples were then subjected to a vacuum for 5 min and incubated at 37°C for 8 h (Jefferson et al. 1987). X-gluc-stained tissues were stabilized by passage through a 70% ethanol series. Observations were made under a Leica camera (Leica Microsystems, Wetzlar, Germany). At least 10 independent lines were utilized for analysis.

Results and Discussion

Morphological effects of shoot-applied GA on roots

The first experiment measured primary root elongation and number of lateral roots after application of GA to shoots of *Arabidopsis*. Three-week-old *Arabidopsis* plant shoots were treated with 5–10 μL 10⁻⁶ M GA₄ twice over 3 days, and primary root elongation and number of lateral roots were examined 3 days after application. The extent of primary root elongation was slightly greater when GA was applied to the shoot compared with the control (Fig. 1A). This suggests that exogenous GA from shoots could bring about the promotion of primary root elongation.

Similarly, there was an increase in number of lateral roots after GA application to shoots of

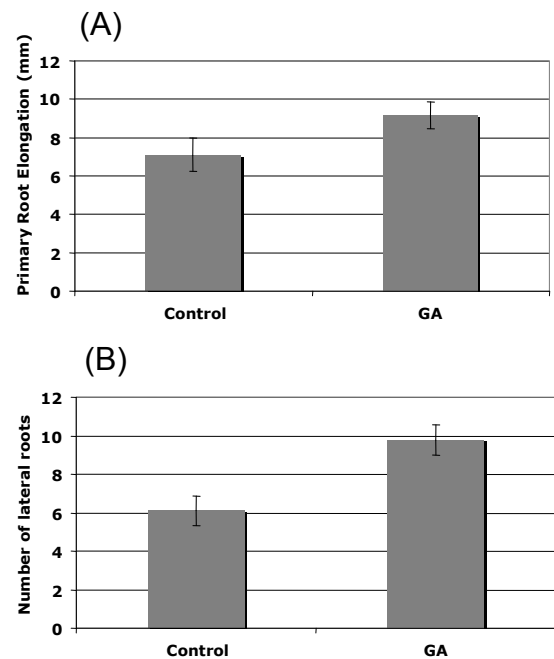


Fig. 1. Effect of shoot-applied GA₄ on primary root elongation (A) and number of lateral roots (B) of wild-type *Arabidopsis* roots. Three-week-old plants were treated with 5–10 μL 10⁻⁶ M GA₄ (GA) applied to the shoot area twice in 3 days, and primary root elongation (A) and number of lateral roots (B) were examined 3 days later. Error bars represent standard error ($n = 40$ plants), Student t test $P < 0.05$ for (A) and (B).

3-week-old *Arabidopsis* (Fig. 1B). The goal of this experiment was to examine the possible relationship between GA and lateral root formation after GA application to the shoot.

Although shoot-applied GA showed some promoting effects on primary root elongation and lateral root formation, further experiments of GA- biosynthesis inhibitors application, under identical conditions, might elucidate more clear influences of GA on roots.

Morphological effects of shoot-applied GA and Uni on seedling growth

As plant growth retardants are known to reduce shoot growth by inhibiting GA biosynthesis, we searched for specific chemical inhibitors of GA biosynthesis. Compounds with a nitrogen-containing heterocycle, such as triazoles and imidazoles, are known to act as inhibitors of various P450-dependent enzyme reactions. Among them, several compounds, including uniconazole and paclobutrazol, have been utilized as plant growth retardants. These inhibit P450 ent-kaurene oxidase, which catalyzes the oxidation of

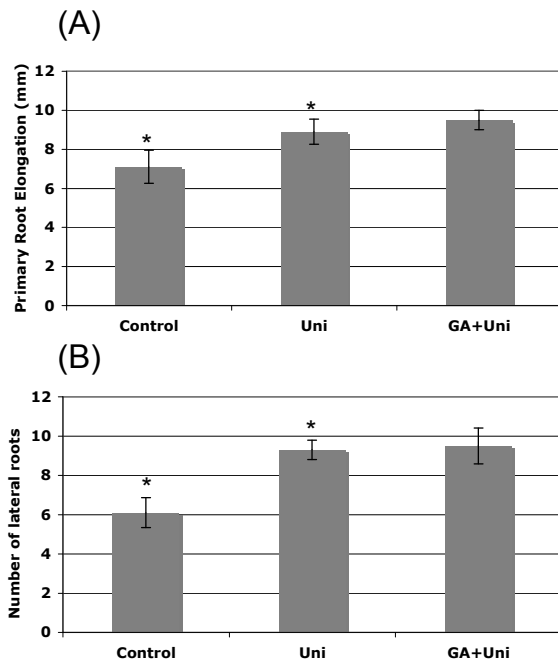


Fig. 2. Effect of shoot-applied uniconazole on primary root elongation (A) and number of lateral roots (B) in wild-type *Arabidopsis* roots. Three-week-old shoots were treated with 5–10 μL 10^{-5} M uniconazole, with (GAUni) or without (Uni) 10^{-6} M GA_4 , twice in 3 days, and primary root elongation (A) and number of lateral roots (B) were examined 3 days later. The duration was as in the GA application experiments. Error bars represent standard error ($n = 40$ plants), Student t test $P < 0.05$ for both (A) and (B).

ent-kaurene to ent-kaurenoic acid in GA biosynthesis. According to Izumi et al. (1989) and as far as it is known, uniconazole remains locally in its applied spot and barely moves to other parts, and paclobutrazol is considered to be more specific to GA biosynthesis enzymes.

To examine the long-distance effect of GA inhibitors on roots, we applied 5–10 μL 10^{-5} M uniconazole, twice in 3 days, to shoots of 3-week-old wild-type *Arabidopsis*. Primary root elongation and number of lateral roots were then examined 3 days later. The duration of the experiment was similar to that of the GA experiments described above. Surprisingly, 10^{-5} M uniconazole applied to shoots slightly promoted primary root elongation and number of lateral roots, comparing to the control (Fig. 2A, B). This effect was similar to the phenomenon previously found in *Lactuca sativa* (Tanimoto 1987). Co-application of GA and the inhibitor to shoots did not differ significantly. Parallel to root observations, we monitored morphological changes in the shoot

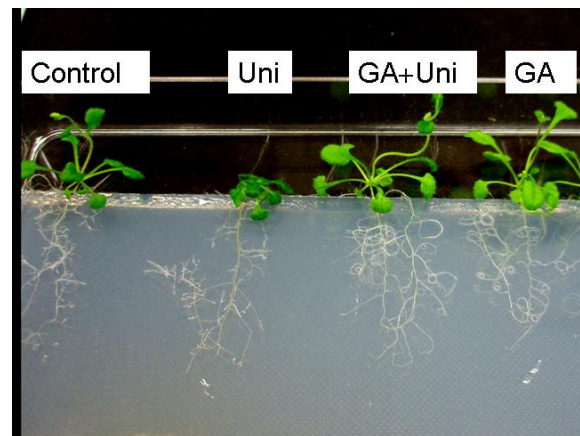


Fig. 3. Morphological effects of GA and uniconazole on seedlings of *Arabidopsis*. Shoots were treated with 10^{-6} M GA_4 (GA), 10^{-5} M uniconazole (Uni), or both (GAUni) and roots and shoots were observed 10 days later.

where uniconazole had been applied. As anticipated, 10 days after uniconazole application, the shoot exhibited severe growth defects; the morphology of the plants were similar to the phenotype of the *ga3ox1/2* mutant (data not shown), indicating that uniconazole suppressed GA biosynthesis in the shoot (Fig. 3). To elucidate the possible changes in the GA biosynthesis pathway after application of inhibitor or GA, we examined expression of GA biosynthesis-related genes using QRT-PCR.

Effect of shoot-applied GA biosynthesis inhibitors on expression of GA biosynthesis-related genes

The GA biosynthetic pathway has been elucidated using a combination of biochemical and genetic approaches. The final steps in the GA biosynthesis pathway, which produces active GAs, require activity of GA20- and GA3-oxidases; GA20ox catalyzes the conversion of GA_{12} to GA_9 to produce the precursor of active GA, and GA3ox catalyzes production of bioactive GA_4 from GA_9 in *Arabidopsis*. As far as we know, the level of endogenous active GA is governed by feedback regulation, where active GAs suppress expression of the *GA20ox* and *GA3ox* genes. Relative transcript levels of GA biosynthetic genes were therefore determined by QRT-PCR to examine the status of the GA biosynthesis pathway in shoots and roots 3 days after GA or inhibitor application to shoots (Fig. 4A–E). In most of the examined genes in Fig 4, paclobutrazol applications showed similar expression levels compared with uniconazole application.

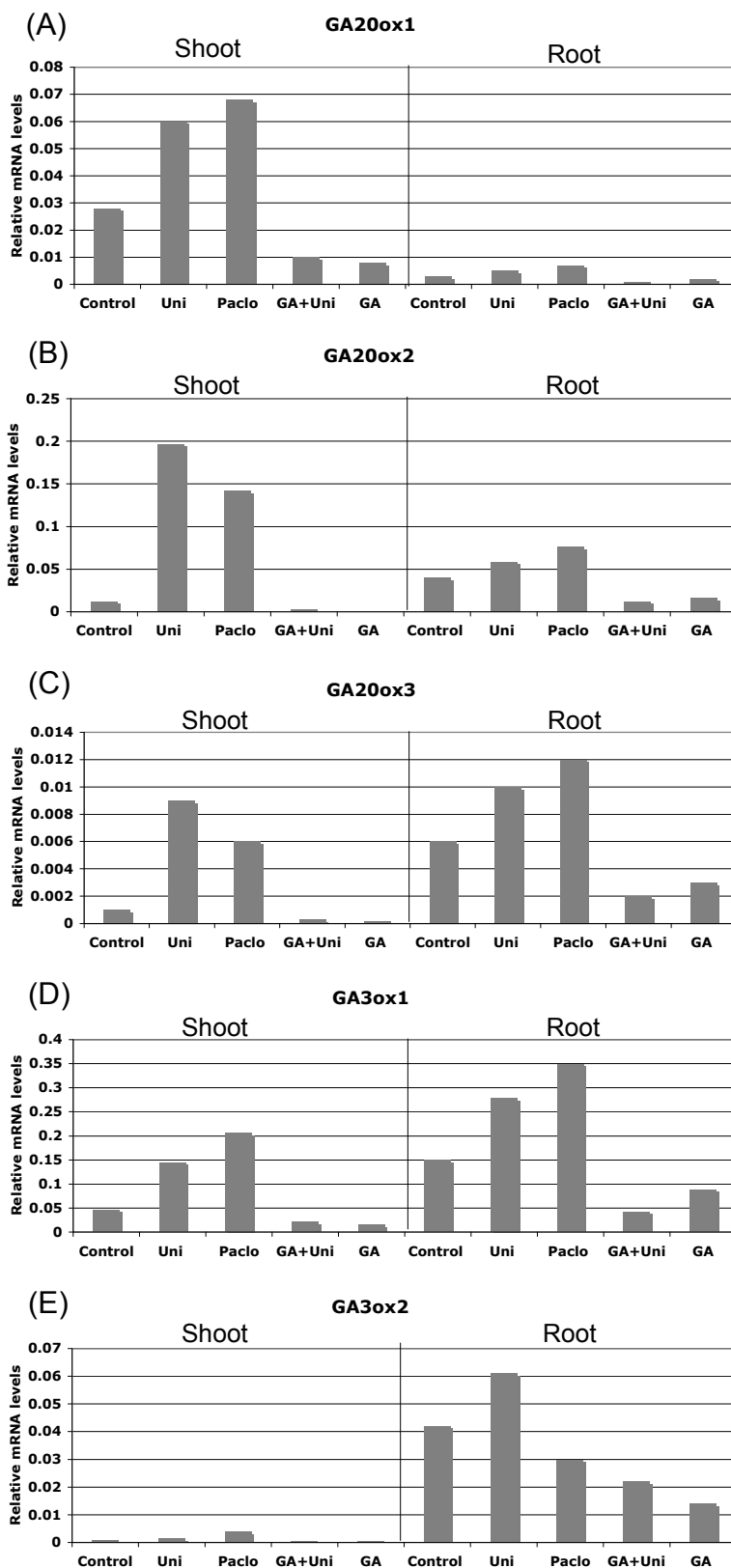


Fig. 4. Transcript levels of GA biosynthetic genes in shoots and roots of Arabidopsis. QRT-PCR was carried out in shoots and roots of 3-week-old plants after 10^{-6} M GA4 (GA) and 10^{-5} M uniconazole (Uni) application to shoots. Paclobutrazol was applied to the shoot apex at the concentration of 10^{-6} M. Experiments were performed twice with independent material and similar results were obtained. mRNA was prepared from shoots and roots, and transcription levels were quantified by QRT-PCR for GA20ox1 (A), GA20ox2 (B), GA20ox3 (C), GA3ox1 (D), and GA3ox2 (E). Results from one replicate are shown.

Real-time PCR data showed somewhat similar expression profiles for the *GA20ox1*, *GA20ox2*, and *GA20ox3* genes, although minute differences were

found (Fig. 4A-C). There was a remarkable effect of inhibitors on *GA20ox1* in shoots compared to roots (Fig. 4A). Moreover, there were similar up-regulation

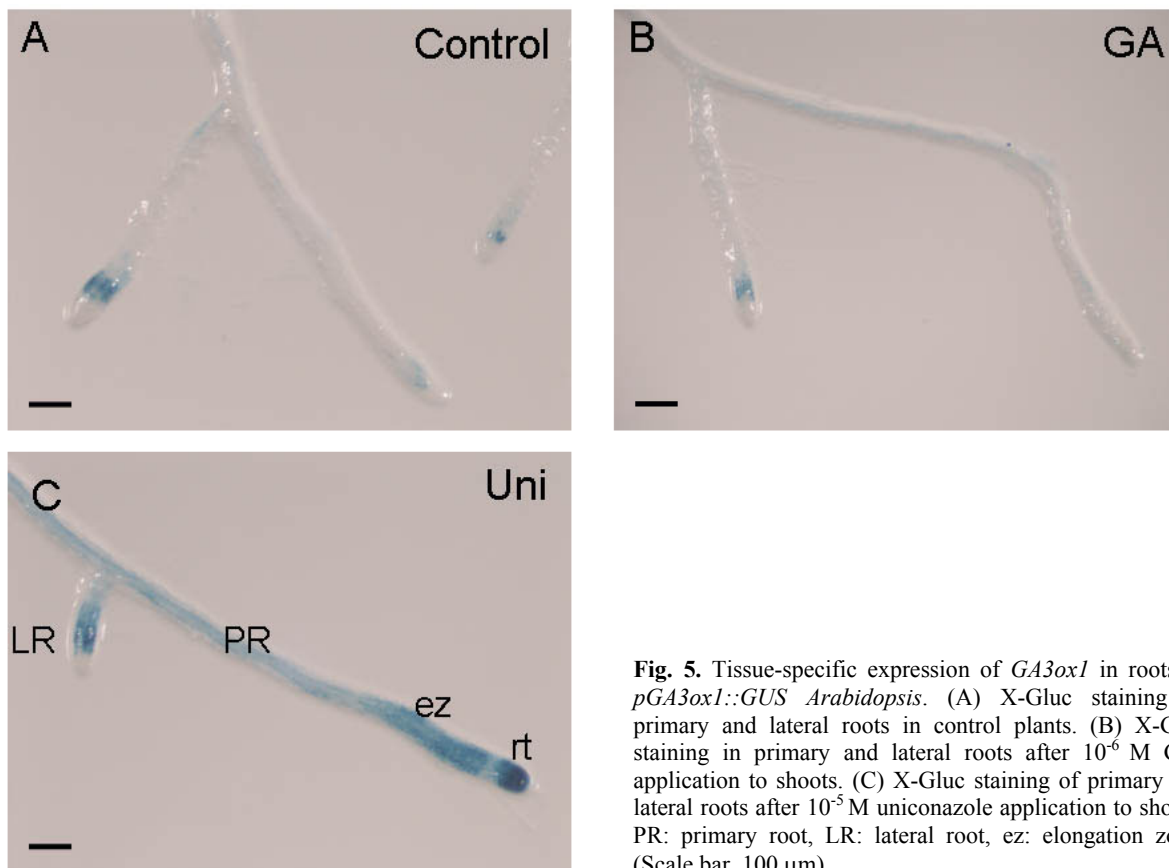


Fig. 5. Tissue-specific expression of *GA3ox1* in roots of *pGA3ox1::GUS Arabidopsis*. (A) X-Gluc staining of primary and lateral roots in control plants. (B) X-Gluc staining in primary and lateral roots after 10^{-6} M GA_4 application to shoots. (C) X-Gluc staining of primary and lateral roots after 10^{-5} M uniconazole application to shoots. PR: primary root, LR: lateral root, ez: elongation zone, rt: root tip. (Scale bar, 100 μ m).

patterns of *GA20ox* genes by the inhibitors in roots. The data also indicated that GA application suppresses expression of *GA20ox* genes in both roots and shoots.

Previous studies on *AtGA3ox* have shown that it is regulated by at least three factors: light (phytochrome), temperature, and GA activity (feedback inhibition) (Yamauchi et al. 2004). There is also evidence that GA deficiency elevates the level of *AtGA3ox1* transcripts to increase active GA (GA_4). To examine how GA applications determine the abundance of *AtGA3ox1* and *AtGA3ox2* mRNA over long distances, we examined whether transcript levels of *AtGA3ox1* and *AtGA3ox2* could be affected by GA or GA deficiency.

Fig. 4D and Fig. 4E respectively show expression levels of *GA3ox1* and *GA3ox2* in shoots and roots. *GA3ox1* and *GA3ox2* are the only GA3oxidases known to be expressed in high amounts in *Arabidopsis* roots, suggesting that they play a role in root development (Mitchum et al. 2006). Fig. 4D also reveals that application of uniconazole or paclobutrazol to shoots up-regulated expression of *GA3ox1* in shoots, while GA application to shoots suppressed *GA3ox1* expression. This result confirms the negative feedback

regulation of this gene, as shown by Chiang et al. (1995) and Yamaguchi et al. (1998). By comparing the *GA3ox1* and *GA3ox2* expression pattern, it can be concluded that *GA3ox1* shows higher expression and sensitivity to GA application to shoots. Fig. 4E shows that *GA3ox2* was expressed dominantly in root compared to shoot and up- and down-regulation by uniconazole and GA, respectively.

Changes in GUS-expression patterns of GA3ox1 in roots of GA or GA inhibitor-treated Arabidopsis

As mentioned previously, *GA3ox* and *GA20ox* control the level of bioactive GAs in many plant species. The morphological data from our GA-application experiments showed that GA-inhibitor application to shoots promoted primary root elongation, or at least did not show inhibitory effects on roots. Hence, feedback regulation of GA-levels after the inhibitor-application are suggested. Since *GA3ox1* is dominant in controlling bioactive GA levels, the expression pattern and diversity of this gene were assessed using a GUS reporter construct after GA and uniconazole treatments. *pGA3ox1::GUS* is expressed in the vasculature

of non-elongating regions of roots (Mitchum et al. 2006). After GA treatment, *pGA3ox1::GUS* expression in non-elongating regions with a rather faint expression pattern was observed (Fig. 5A, B). In contrast, uniconazole-treated shoots showed a strong pattern of expression in the elongation zone and root tip of both primary and lateral roots (Fig. 5C). Such enhancement of *GA3ox1* expression in the growing zones of roots in uniconazole-treated *Arabidopsis* may cause the enhancement of root elongation observed in Fig. 2.

Conclusion

Our morphological surveys (Figs. 2A, 2B, and Fig. 3) showed that uniconazole induced dwarfism and severe defects only in shoots, similar to results from GA-defective mutants, while primary roots continued to elongate and branch normally and were slightly promoted. These observations can be interpreted as follows. Under normal conditions, the shoot produces a GA precursor or active GA that is transported to roots where, in combination with active GA produced by the root itself, the root is supplied with enough GA for normal growth. After uniconazole application to shoots, the supply of GA or GA precursors from shoots decreases, causing activation of feedback regulation and a rise in expression of *GA3ox*, which consequently leads to the release of active GA into GA-starved roots. Subsequently, GA biosynthesis in roots is set in motion, and bioactive GA synthesized in elongation zone of primary and lateral roots promotes elongation in uniconazole-treated plants. However, the interference of other hormones after uniconazole treatment is less clear. Uniconazole is a plant growth retardant and evidently inhibits GA biosynthesis, ABA catabolism, and also BR biosynthesis to some extent (Saito et al. 2006; Yamamoto et al. 1997). According to Izumi et al. (1989) using C^{14} compound revealed that uniconazole can not move from shoot to root, however, the specificity of uniconazole is unclear, and it may affect the above-mentioned hormones, resulting in a number of possible combinations for regulation, which may contribute to the plant's ability to cope with temporarily or constantly changing environment (Brady and McCourt 2003). Therefore, further confirmation of the interactive effects of these hormones is required.

Acknowledgments

The authors appreciate assistance of Faezeh Taghizadeh-asl in measuring root length. Also authors thank Yuko Sato for her technical advice.

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