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Expression of *ABSCISIC ACID INSENSITIVE 4* (*ABI4*) in developing Arabidopsis seedlings

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We have recently demonstrated that the transcription factor ABSCISIC ACID INSENSITIVE 4 (ABI4) mediates abscisic acid and cytokinin inhibition of lateral root formation by reducing polar auxin transport in *Arabidopsis thaliana*.[1] In that study, we provided a direct demonstration of *ABI4* expression in phloem companion cells and parenchyma of the vascular system in the mature regions of the roots. Although also studied in mature plants, *ABI4* has been studied primarily in germinating seedlings and its expression has been assumed by some researchers to be restricted to early germination stages. We thus constructed transgenic Arabidopsis plants expressing an *ABI4:GUS* construct, and followed *ABI4* promoter activity during seedling development, focusing on the roots.

Abscisic Acid Insensitive Mutants

Abscisic acid insensitive (*abi*) mutants were identified from a selection of germinating seedlings in the presence of abscisic acid (ABA), a germination-inhibiting phytohormone.[2,3] Further studies of these mutants revealed that *ABI1* and *ABI2* encode protein phosphatases,[4,5] *ABI3*, *ABI4* and *ABI5* encode transcription factors,[6-8] and *ABI8* encodes a novel protein whose function has yet to be determined.[9]

Isolation of Different Mutant Alleles of *abi4*

The first *abi4* mutant was selected by germination in the presence of ABA.[3] This mutant also displayed reduced seed dormancy. A number of mutant alleles of *abi4* were also isolated by screening mutants that germinated in the presence of a high concentration of NaCl (*salobreno 5*, *san5*),[10] sucrose (*sucrose insensitive 5*, *sis5*),[11] or glucose (*glucose insensitive 6*, *gin6*).[12] Other screens of germinating seeds for mutants with reduced ability to respond to elevated levels of sucrose (sucrose-uncoupled *6*, *sun6*),[13] or for mutants with impaired sucrose induction of starch biosynthesis (*impaired sucrose induction 3*, *sis3*),[14] resulted in the isolation of additional *abi4* mutants.

ABI4 Expression Data

Expression analyses of the *ABI4* gene showed that it is highly expressed in seeds[15] and at seed germination, and that its steady-state mRNA levels drop sharply a few days after germination. In germinating seedlings, *ABI4* was shown to be induced by external application of glucose[16-19] and mannitol[17] *ABI4* was induced in ABA-treated imbibed seeds,[20] but not in germinating seedlings.[16] It was argued that *ABI4* expression is limited to seed maturation and to a few days following germination.[15,18,20]

On the other hand, *ABI4* was reported to be expressed in shoots and roots of three-week-old plants.[7] Furthermore, we recently showed, both quantitatively and histologically, that *ABI4* is expressed in mature regions of the roots at later developmental stages and that its steady-state levels are enhanced by ABA and cytokinin and reduced by auxin.[1]

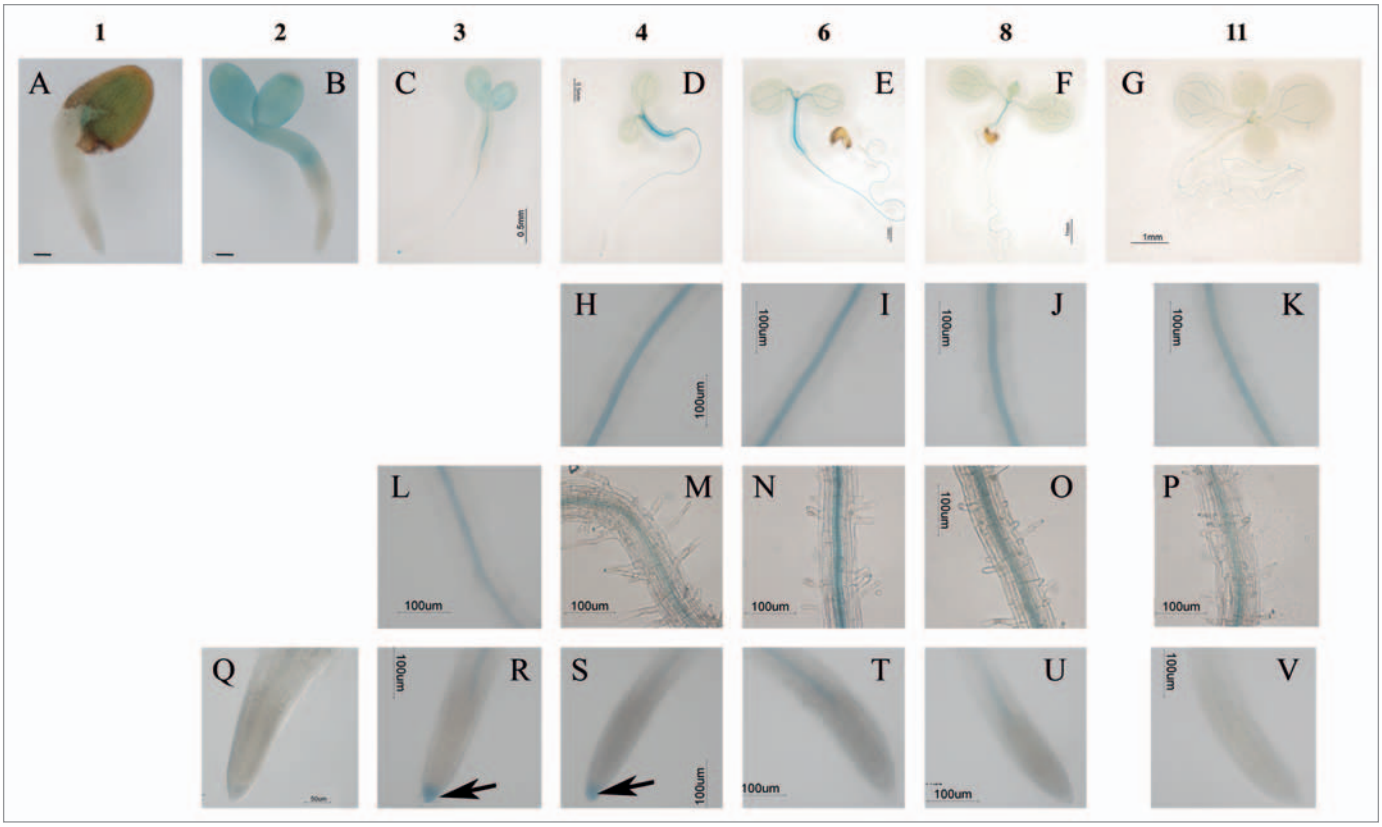

Figure 1. Expression of ABI4 in germinating seeds and developing seedlings. Seeds of *ABI4:GUS*-expressing Arabidopsis were surface-sterilized, cold-treated for 3 days, and sown on Petri dishes containing 0.5x MS, 0.5% sucrose and 0.4% (w/v) agar at 1-day intervals for 11 days. Plates were incubated at 22°C under a 12 h light/dark regime. All plants were harvested on day 11, fixed with acetone and simultaneously stained for GUS activity. Plants were cleared in 70% ethanol and photographed under a stereoscope (A–G) or microscope (H–V), using the same exposure. Time (in days) between sowing and harvest is indicated. Upper row (A–G), whole plant; 2nd row (H–K), upper root region; 3rd row (L–P), transition zone; lower row (Q–V), root tips. Arrow indicates GUS staining in the root tips. Bars: (A , B and H–V), 0.1 mm; (C–E) 0.5 mm; (F and G) 1 mm.

Proposed Biological Roles for *ABI4*

In accordance with the assumption that *ABI4* expression is restricted to early germination stages, most studies of this gene were carried out using germinating seeds. At these stages, *ABI4* was shown to be involved in glucose signaling,[12,14,16,17] sugar signaling and response pathways,[11,13,18] ABA signaling[3,11-13,15] and lipid mobilization in the embryo and germinating seeds,[21,22] as well as in chloroplast functioning and retrograde signaling.[20,23]

Other studies, using older plants, showed that *ABI4* is involved in nitrate-modulated root branching,[24] sugar insensitivity in fully expanded leaves,[25] responses to plant pathogens and tolerance to β-amino-butyric acid (BABA)-induced water stress,[26-29] mitochondrial retrograde signaling,[30] chloroplast photosynthesis,[31,32] and modulation of hormonal control of lateral root formation.[1] These studies clearly argue that *ABI4* is expressed at later developmental stages.

ABI4 Expression in Germinating and Developing Seedlings

For a more in-depth delineation of the expression of *ABI4* during seedling development, we followed *ABI4*-promoter activity during seed germination and seedling development, with a focus on its expression in the roots. Arabidopsis plants were transformed with an *ABI4:GUS* construct. Homozygous plants were selected and activity of the GUS reporter gene was assayed in several independent transformants. Cold-treated surface-sterilized seeds of *ABI4:GUS* plants were plated on Petri dishes containing 0.5x MS supplemented with 0.5% (w/v) sucrose.[1] Plates were incubated at 22°C under a 12 h light/dark regime. Seedlings were fixed in acetone and stained as described previously.[1] Although this procedure reduces staining intensity, it allows better histological localization of the signal. High GUS expression was already observed in cotyledons on day 1, even before they emerged from the seed coat (**Fig. 1A**), whereas the emerging radicle did not express GUS activity (**Fig. 1A**). On day 2, there was strong GUS staining of the cotyledons and hypocotyl, but not of the root tip (**Fig. 1B and Q**). Most cotyledons did not express GUS in the distal parts of their cotyledons (**Fig. 1B and C**). GUS levels were reduced on day 3, with staining observed primarily in the vascular system and extending to the differentiating root (**Fig. 1L**). The root tip was also stained (**Fig. 1R** and arrow). GUS expression in the cotyledons continued to decrease (**Fig. 1D–G**), with low expression observed in the plant leaves at older stages (**Fig. 1G**). In fact, under low magnification, the root signals might be overlooked

at this developmental stage. Expression in the hypocotyls also dropped with seedling development, but at a lower rate than the decrease observed in the cotyledons. Staining at the root tip was observed only on days 3 and 4 (**Fig. 1R and S**), suggesting transient expression at this site. Moreover, since GUS is a stable protein in plants with a long half life,[33] it is highly likely that the peak of promoter activity in the root tips was shorter than that detected histochemically. Upper root regions showed high GUS expression (**Fig. 1H–K**). Expression levels varied in the differentiation/root-hair zone (**Fig. 1M–P**). On day 3 (**Fig. 1L**), this zone is still young, with very few developing root hairs, and thus *ABI4* expression level was relatively lower than at later stages. *ABI4* expression peaked on day 6 (**Fig. 1N**) and slowly decreased thereafter. In roots of older plants, ABI4 faded in the root-hair zone, in accordance with ABI4's role in modulating lateral root formation.[1]

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