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Harlequin (*hlq*) and *short blue root* (*sbr*), two *Arabidopsis* mutants that ectopically express an abscisic acid- and auxin-inducible transgenic carrot promoter and have pleiotropic effects on morphogenesis

Senthil Subramanian[+], Balasubramanian Rajagopal[+] and Christopher D. Rock*
*Department of Biology, Hong Kong University of Science and Technology Clear Water Bay, Kowloon, Hong Kong, China (*author for correspondence; e-mail borock@ust.hk); [+]these authors contributed equally to this work*

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Abstract

Plant growth and development is regulated by complex interactions among different hormonal, developmental and environmental signalling pathways. Isolation of mutants in these processes is a powerful approach to dissect unknown mechanisms in regulatory networks. The plant hormones abscisic acid (ABA) and auxin are involved in vegetative, developmental and environmental growth responses, including cell division and elongation, vascular tissue differentiation and stress adaptation. The *uidA* (β-glucuronidase; GUS) reporter gene driven by the carrot (*Daucus carota*) late embryogenesis-abundant *Dc3* promoter in transgenic *Arabidopsis thaliana* seedlings is ABA-inducible in the root zone of elongation and vasculature. We show here that the *ABA-insensitive2-1* mutation (*abi2*) reduces ABA-inducible *Dc3-GUS* expression in these root tissues. *Dc3-GUS* expression is also induced in root cortex cells by indole-3-acetic acid. We mutagenized, with ethyl methane sulfonate, 5100 M_1 *abi2/abi2* homozygous plants of a line that carries two independent *Dc3-GUS* reporter genes and screened M_2 clonal lines for ABA-inducible *Dc3-GUS* expression in roots. We isolated two novel single-gene nuclear mutants, *harlequin* (*hlq*) and *short blue root* (*sbr*), that ectopically express *Dc3-GUS* in roots and have pleiotropic effects on morphogenesis. The *hlq* mutant expresses *Dc3-GUS* in a checkered pattern in epidermis of roots and hypocotyls, accumulates callose and has deformed and collapsed epidermal cells and abnormal and reduced root hairs and leaf trichomes. It (*hlq*) is also dwarfed, skotomorphogenic and sterile. The *sbr* mutant is a seedling-lethal dwarf that over-expresses *Dc3-GUS* in the root and has radially swollen epidermal cells in the root and hypocotyl, supernumerary cell number in the root cortex and epidermis, abnormal vasculature, and abnormal epidermal cell patterning in cotyledons and leaves. It (*sbr*) also exhibits a semidominant root phenotype of reduced growth and lateral root initiation. The *hlq* and *sbr* mutants are not rescued by exogenous application of plant growth regulators. The *hlq* and *sbr* mutants do not require the *abi2-1* mutant gene for their phenotypes and map to chromosome III and I, respectively. Further characterization of the *hlq* and *sbr* phenotypes and genes may provide insights into the relationship of hormone- and stress-regulated gene expression to morphogenesis and plant growth.

Abbreviations: ABA, abscisic acid; BR, brassinolide; CAPS, cleaved amplified polymorphic sequence; EMS, ethyl methane sulfonate; GA, gibberellic acid; GUS, β-glucuronidase; IAA, indole-3-acetic acid; LEA, late embryogenesis-abundant; SSLP, simple sequence length polymorphism

Introduction

Morphogenesis in plants is characterized by highly regulated cell division and enlargement. The mechanisms controlling and localizing regions of growth remain largely unknown, not least of which is the role of plant hormone action. Only a few identified plant growth regulators are known to interact and modulate myriad physiological processes. The plant hormones abscisic acid (ABA) and auxin are involved in a wide

variety of developmental processes throughout the life cycle of higher plants (Kende and Zeevaart, 1997). Recent genetic analyses of hormone signalling in *Arabidopsis* are providing increasing evidence that hormone signalling pathways are integrated into complex regulatory networks controlling growth, development and responses to environmental cues (Chory and Wu, 2001).

Most genetic screens for ABA responses are based on physiological assays (e.g. germination and dormancy) and have not identified novel mutants with altered vegetative-specific responses to hormones (reviewed by Leung and Giraudat, 1998; Rock, 2000). Genetic screens for mutants with altered transgenic reporter gene expression have been instrumental in dissecting auxin, ABA, biotic and abiotic environmental stress signalling pathways (Li *et al.*, 1994; Bowling *et al.*, 1994; Ishitani *et al.*, 1997; Oono *et al.*, 1998; Foster and Chua, 1999). It has also been shown that ABA and auxin signalling mechanisms are functionally conserved among species (Kovtun *et al.*, 1998; Gampala *et al.*, 2001). Gene expression screens enable the identification of mutations in tissue-specific regulators as well as genes that are otherwise not distinguishable by a visible phenotype. Conversely, genetic analysis of pleiotropic phenotypes can reveal genes involved in processes otherwise hidden and open new vistas of understanding, insight and experimentation.

In an effort to identify novel factors affecting vegetative ABA response pathways, we mutagenized *Arabidopsis abi2-1* plants (Leung *et al.*, 1997; Rodriguez *et al.*, 1998) expressing a chimeric construct (*Dc3-GUS*) containing a β-glucuronidase reporter driven by the carrot late embryogenesis-abundant (LEA) gene promoter *Dc3* (Siddiqui *et al.*, 1998). *Dc3-GUS* has been shown to be a useful marker in analysing ABA and drought responses in *Arabidopsis* guard cells (Chak *et al.*, 2000; Rock, 2000). The *cis*-acting elements for seed-specific and ABA-inducible *Dc3* gene expression have been characterized. Genes encoding *Dc3* promoter-binding basic leucine-zipper proteins including the bZIP factor *ABA-INSENSITIVE 5* (Finkelstein and Lynch, 2000) have been cloned from *Arabidopsis* and other species (Kim *et al.*, 1997). Here we show that *Dc3 GUS* also is a marker for ABA- and auxin-inducible gene expression in roots of *Arabidopsis* seedlings. We screened M_2 clonal lines from ethane methane sulfonate (EMS)-mutagenized *abi2-1/Dc3-GUS* transgenic *Arabidopsis* for altered *Dc3-GUS* expression patterns in the roots and isolated two mutants, *harlequin (hlq)* and *short blue root (sbr)*, that exhibit ectopic *Dc3-GUS* expression in roots and have pleiotropic effects on morphogenesis and development. Detailed molecular characterization of such mutants may lead to better understanding of the role of hormone-regulated gene expression in cell division, cell elongation, development and stress responses.

Materials and methods

Plant materials and growth conditions

The *Arabidopsis thaliana* (L.) Heynh. genotypes used in this study were Landsberg *erecta* (CS20), Columbia (CS907), *abi1-1*(CS22), and *abi2-1* (CS23; Koornneef *et al.*, 1984). (Numbers in parenthesis refer to the Arabidopsis Biological Resource Center catalogue number; Ohio State University, Columbus, OH). The selection and growth of transgenic lines *Dc3GUS/abi1-1, Dc3GUS/abi2-1* and *Dc3GUS/Ler* used in the present study has been described (Chak *et al.*, 2000).

Mutagenesis

Homozygous double-transgene *Dc3-GUS/abi2-1* seeds were mutagenized with 0.3% v/v EMS (Sigma, St. Louis, MO) according to Lightner and Caspar (1998). A total of 5100 mature M_1 plants were subsequently harvested individually and the M_2 seeds stored at -20 °C as pedigrees for mutant screens. About 13% of M_1 pedigrees segregated for cotyledon pigment phenotypes in the M_2 generation, indicating that the mutagenesis was successful.

GUS staining and genetic screen

A visual screen for ectopic ABA-inducible *Dc3-GUS* expression was performed by germinating 30 sterilized M_2 seeds from each pedigree on square minimal-medium petri plates (Scholl *et al.*, 1998) solidified with 1.2% Phytagel (Sigma) and growing seedlings vertically in a growth chamber for 6 days. The plates were then placed horizontally and overlaid for 16 h with 100 μM ABA (Sigma) in sterile distilled water to induce *Dc3-GUS* expression. The seedlings were then stained for GUS activity as described previously (Chak *et al.*, 2000). The seedlings were then visually screened using a dissecting microscope and pedigrees that segregated for ectopic GUS expression phenotypes were propagated. M_3 pedigrees were re-screened, selected for further study and backcrossed at least twice (Koorneef *et al.*, 1998) to the parental

Dc3-GUS/abi2 line to clear the genome of extraneous mutations. For *Dc3* expression studies in *Ler, abi1-1* or *abi2-1* genotypes, the appropriate transgenic seeds were sown and induced with water, 100 μM ABA or 10 μM IAA (Sigma) and stained for GUS as described above.

Microscopy

Light microscopy was performed using a Zeiss Stereomicroscope (Göttingen, Germany) and for micro measurements a calibrated microscale was used. For light microscopy of GUS-stained roots, the seedlings were briefly rinsed in 70% ethanol followed by sterile distilled water to remove excessive GUS developer and observed under the microscope.

For 'live and dead' cell staining, seedling roots were embedded by allowing to grow into 2% Phytagel minimal medium. Embedded roots in 1–2 mm thick slices of gel were transferred to glass slides, immersed in water containing 100 μg/ml each of fluorescence diacetate (FDA; Sigma) and propidium iodide (PI; Sigma), then incubated in the dark (15 min), rinsed twice in sterile distilled water and epifluorescence viewed under excitation with a blue filter (450–490 nm) by means of a Zeiss axiophot microscope. The sample was then flooded with GUS developer solution for 16 h and photographed in bright field with the same field in view as previously documented.

For scanning electron microscopy, the seedlings were aligned on the conductive paste thinly spread over the microscope stage and immediately frozen under liquid nitrogen. The stage was placed into the cryo-chamber of the microscope and the condensed water removed by vacuum pumping. Then the samples were coated with platinum and observed under a Leica S440 scanning electron microscope (Cambridge, UK) fitted with a Fisons LT7480 cryoprep cryo-stage (Fisons Instruments, UK).

For confocal optical sections, the seedlings were stained with a 10 μg/ml solution of PI in water for 6 h and destained overnight in sterile water. For staining callose, seedlings were bleached in 100% ethanol for several hours and stained with aniline blue (0.1%; Aldrich, Milwaukee, WI) in water for 30 min and rinsed twice in distilled water. The seedlings or hand sections (for cross-sections) were then optically sectioned with a BioRad (Hercules, CA) MRC600 Laser confocal microscope.

Genetic mapping

For mapping, *hlq*/+ and *sbr*/+ heterozygous plants of the Landsberg *erecta* ecotype were crossed with the Columbia ecotype. A single F_2 line that segregated for *hlq* or *sbr* mutations was used for mapping experiments. DNA was extracted from 2–3-week old mutant seedlings as described by Edwards *et al.* (1991). The *hlq* recombinants were allowed to grow for 3–4 weeks to maximize template DNA yield. The DNA was used in simple sequence length polymorphism (SSLP; Bell and Ecker, 1994) and cleaved amplified polymorphic sequence (CAPS) analyses (Konieczny and Ausubel, 1993). Information about the position and primer pairs for these markers was obtained from The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org) and the primer pairs were either purchased from Research Genetics or synthesized by GENSET Singapore Biotechnology (Singapore). The mapping data were analysed with Mapmaker 3.0 software (http://www-genome.wi.mit.edu/genome_software) and the distance in centimorgans between the markers and the mutation calculated.

Chemicals

Solutions of *cis*-(±) ABA (Sigma), gibberellic acid (GA; Janssen Chimica, Belgium), indole-3-acetic acid (IAA; Sigma), 24-epibrassinolide (24-epiBR; CIDtech Research, USA), kinetin (Sigma), 6-chloroindole (Sigma) and 5-fluoro indole (Sigma) were diluted from 100 mM stock solutions prepared in either 50% or 90% ethanol; equivalent volumes of 50% or 90% ethanol were included in all treatments.

Results

Dc3-GUS *expression phenotype in wild type and* abi1 *and* abi2 *mutants*

Rock (2000) characterized the root-specific expression of *Dc3-GUS* in transgenic *Arabidopsis* and obtained evidence that 'separate but overlapping' ABA and drought response pathways in part are a consequence of differential tissue-specific gene expression in response to separate stresses. We further characterized the tissue-specific pathways regulating *Dc3-GUS* expression in roots by comparing GUS activities in response to various hormone treatments (ABA, indole-3-acetic acid (IAA), kinetin, gibberellic

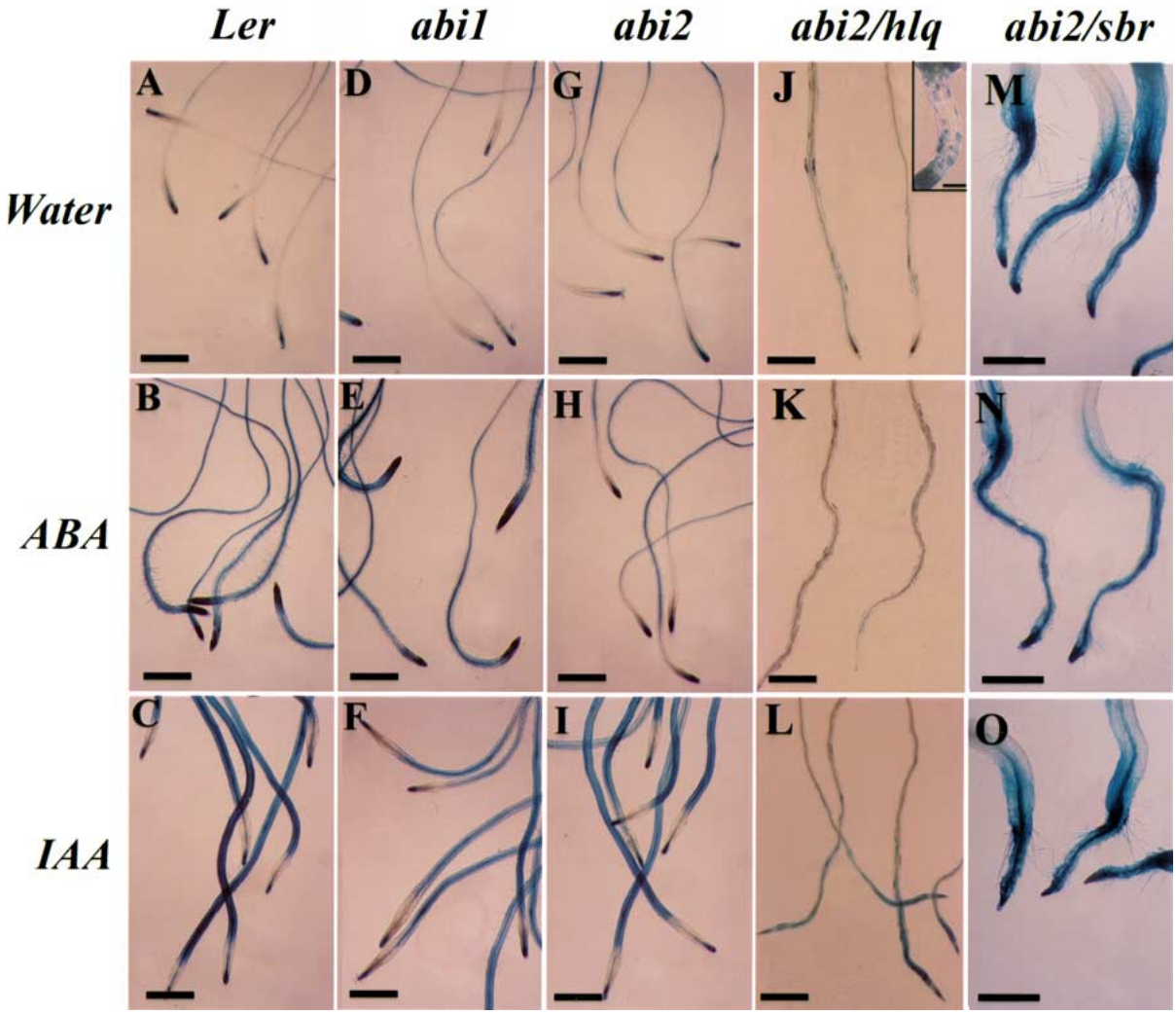

Figure 1. Induction of *Dc3-GUS* expression by ABA and IAA in 4-day old transgenic *Arabidopsis*, and tissue-specific effects of *abi2, hlq* and *sbr* mutations on *Dc3-GUS* expression. Wild-type *Ler*, *abi1*, *abi2*, *abi2/hlq* and *abi2/sbr Arabidopsis* seedlings were treated for 24 h with water, 100 μM ABA or 10 μM IAA and visualized by GUS staining. Constitutive *Dc3-GUS* activity in primary root meristems and, to a lesser extent, vasculature of wild-type *Ler* (A), *abi1* (D), and *abi2* (G). ABA induction of *Dc3-GUS* expression and swelling of root zone of elongation of wild type *Ler* (B) and *abi1* (E), but not *abi2* (H). IAA induction of *Dc3-GUS* in cortical cells of root zone of elongation of wild-type *Ler* (C), *abi1* (F), and *abi2* (I) and *hlq* (L). The *abi2/hlq* mutant exhibits ectopic *Dc3-GUS* expression in epidermis of hypocotyl (inset in J) and root zone of maturation under all treatments (J, K, L). The *abi2/sbr* mutant exhibits constitutive, ectopic expression of *Dc3-GUS* in cortical cells under all treatments (M, N, O). Scale bar = 500 μm.

acid (GA), ethylene) in wild-type (*Ler*) and *ABA-insensitive* (*abi1*, *abi2*) dominant-negative mutant genotypes which encode protein phosphatases type 2C (Rodriguez *et al.*, 1998; Gosti *et al.*, 1999). The results are shown in Figure 1A–I. As previously described (Rock, 2000), in the absence of ABA treatment the primary root meristems (and lateral root primordia) exhibited constitutive *Dc3-GUS* expression, with only faint GUS staining of mature root vasculature (Figure 1A). In response to 100 μM ABA treatment, primary roots swelled at the distal end of the zone of differentiation (Figure 1B), presumably due to the inhibitory effects of ABA on root cell growth (Himmelbach *et al.*, 1998). There was also moderate induction of GUS expression in the cortex and trichoblast (root hair) cells of the distal zone of differentiation and in the vascular tissue of the root (Figure 1B). Root hair growth was affected by exogenous ABA; the hairs grew shorter, broader and more bulbous, as has previously been observed in response to ABA or osmoticum

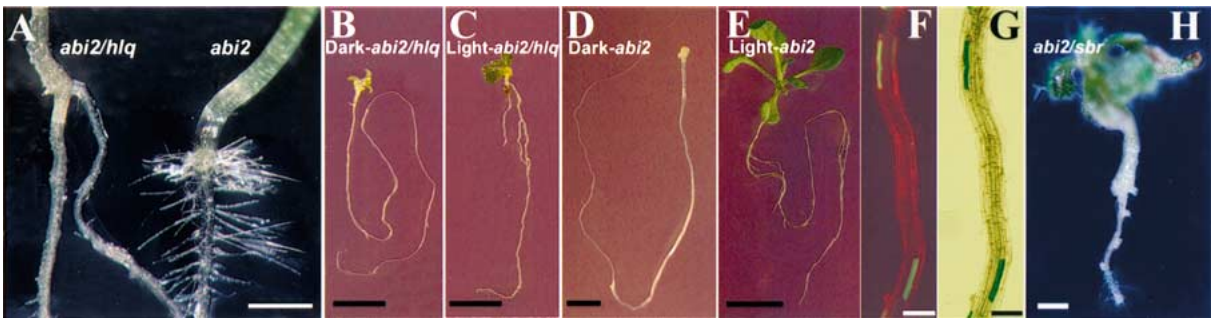

Figure 2. Pleiotropic phenotypes of *abi2/hlq* and *abi2/sbr* mutants. A. Root-shoot junction of 14-day old *abi2/hlq* seedling (left) showing rough epidermis and conspicuous absence of root hairs compared to *abi2* parental type (right). 14-day old *abi2/hlq* (B, C) and parental-type *abi2* (D, E) seedlings grown in dark (B, D) or light (C, E), respectively. F, G. 10-day old *hlq* root stained with FDA and PI (F) followed by GUS staining with X-Gluc (G). H. 21-day old *abi2/sbr* seedling showing dark green leaves and bulging structures in the root. Scale bar = 1 cm (A–E), 50 μm (F, G) or 1 mm (H).

(Schnall and Quatrano, 1992; Lew, 1996). Somewhat surprising was the observation that *Dc3-GUS* transgenic *Arabidopsis* responded to 10 μM auxin (IAA) by inducing GUS expression in the cortex of the root zone of maturation (Figure 1C). *Dc3-GUS* expression in roots in response to a logarithmic range (10^{-9} to 10^{-4} M) of exogenous IAA and synthetic auxin concentrations showed a typical log-linear relationship, with 10 μM IAA being near saturation for maximal *Dc3GUS* expression (X. Sun and C.D. Rock, unpublished observations). The other plant growth regulators tested (kinetin, GA, ethylene) did not have any effect on *Dc3-GUS* activity.

In the ABA-insensitive *abi1-1* mutant genotype, 100 μM ABA or 10 μM IAA treatment of 4-day old plants resulted in induction of *Dc3-GUS* activity in roots (Figure 1D–F) in a similar pattern and extent to that observed in wild-type plants. This suggests that the *ABI1* protein phosphatase is not required for ABA or IAA signalling in this tissue or at this stage of development. However, in the homologous *abi2-1* mutant genotype a reduced effect of 100 μM ABA on *Dc3-GUS* expression and swelling in the root zone of differentiation and trichoblasts was observed relative to *abi1-1* or wild type (Figure 1H). No significant qualitative or quantitative effect of the *abi2-1* mutation on IAA-inducible *Dc3-GUS* expression was observed (Figure 1I; data not shown). *Dc3-GUS* expression in response to sub-saturating concentrations of IAA was not obviously different between *abi1-1*, *abi2-1* or wild-type plants.

The harlequin (hlq) *mutant*

In an effort to identify genes involved in tissue-specific gene expression, we mutagenized seeds of the *abi2-1/Dc3-GUS* genotype and screened M_2 progeny of 5100 individually harvested M_1 lines for novel *Dc3-GUS* expression phenotypes. The *harlequin* (*hlq*) mutant was isolated based on the phenotype of a distinct checkered pattern of ectopic, strong *Dc3-GUS* expression in the hypocotyl, root epidermis, and zones of elongation and maturation in the absence of ABA inductive treatment (Figure 1J). The root epidermal cells that ectopically expressed GUS appeared to be atrichoblasts and were often spatially separated, both radially and vertically, from each other at a density that marked about 1 in 8 epidermal cells. The 6-day old *abi2/hlq* mutant seedlings had ABA and IAA induced *Dc3-GUS* expression phenotypes similar to that of parental type *abi2-1*, with the notable addition of ectopic *Dc3-GUS* expression in the epidermis (Figure 1K, L).

The epidermis of root and hypocotyl tissues had a superficially 'rough' appearance in *abi2/hlq* seedlings compared to *abi2* parental type (Figure 2A). The *abi2/hlq* mutants underwent skotomorphogenesis (promotion in the dark of photomorphogenesis, characterized by inhibition of hypocotyl elongation, promotion of apical hook opening and cotyledon expansion) that gave rise to dark-grown seedlings with de-etiolated, short hypocotyls, opened and expanded cotyledons and true leaves, in contrast to dark-grown parental type (compare Figure 2B with Figure 2D). Scanning electron microscopy of *abi2/hlq* mutants and *abi2* parental-type seedlings (Figure 3A, B; D, E) revealed that the *abi2/hlq* rough epidermis phenotype in both hypocotyls and roots was due to the conspicuous deformation and collapse of epidermal cells. Remarkably, the pattern of collapsed cells in the root epidermis mimicked the observed pattern of ectopic GUS

expression in *abi2/hlq* mutants (Figure 1J). Mutant *abi2/hlq* leaves had reduced numbers of trichomes, while stomatal and pavement cells in cotyledons were similar to *abi2* parental type (Figure 3G, H). Both the root hairs and trichomes of *abi2/hlq* mutants displayed abnormal morphology. The root hairs were fewer and shorter. The position of root hair emergence at the root apical pole of trichoblasts, according to the classification system of Masucci and Schiefelbein (1994), was normal in *abi2/hlq* mutants. The trichomes formed on *abi2/hlq* leaves were mostly unbranched (Figure 3K), in contrast to the normal three-pronged trichome morphology of *abi2* leaves (Figure 3J).

To further examine the relationship between the GUS-expressing epidermal cells and other observed root phenotypes, histochemical staining assays for live and dead cells and counter-staining for GUS in FDA and PI stained roots showed that the cells most strongly stained with FDA (Figure 2F) also expressed GUS (Figure 2H). Staining *abi2/hlq* mutants with aniline blue, which stains callose (Bougourd *et al.*, 2000), also showed a checkered pattern of callose deposition in the epidermal cells compared to the wild type (Figure 4J versus Figure 4I), and the checkered pattern superficially mimicked the GUS-staining patterns (Figures 1J and 2H).

Various physiological parameters of *abi2/hlq* mutants were measured and are presented in Table 1. The *abi2/hlq* mutant grew in the light as a severe dwarf, with shorter root and hypocotyl length, reduced hypocotyl and root diameters, and with chlorotic leaves. Mutant *abi2/hlq* seedlings were inviable when transferred to soil. Root length and root hair numbers were significantly reduced (>30-fold) in *abi2/hlq* seedlings compared to parental type (Table 1). Interestingly, the root length of the *abi2/hlq* mutants was slightly longer than that of the dark-grown *abi2* parental type (Table 1). The *abi2/hlq* mutant produced normal-looking flowers when grown on nutrient-rich media, and *abi2/hlq* pollen germinated *in vitro*, but no seeds were produced from numerous ($n = 18$) attempted out-crosses with *abi2/hlq* pollen as donor.

Because the dwarf growth and skotomorphogenic phenotypes of *abi2/hlq* mutants suggested a possible defect in hormone homeostasis or sensitivity, attempts were made to rescue the *hlq* dwarf phenotype by supplementing the nutrient agar growth medium with the plant growth regulators ABA, GA_3, IAA, 2,4-D, kinetin, 24-epiBR or treatment with ethylene. These treatments did not rescue the *abi2/hlq* mutant phenotypes, nor did they have effects that were qualitatively different from those observed in *abi2* parental type plants, suggesting that the *abi2/hlq* mutant is neither hormone-deficient nor hormone-insensitive.

The short blue root (sbr) *mutant*

The *sbr* mutant was identified in the same mutant screen as *hlq* by its phenotype of stunted roots that over-express *Dc3-GUS* throughout the roots in the absence of ABA (compare Figure 1A with M). *Dc3-GUS* expression was not altered upon treating *abi2/sbr* seedlings with 100 μM ABA or 10 μM IAA (Figure 1M–O), in contrast to wild-type *Ler* and the *abi2* parental type (Figure 1A–C and Figure 1G–I, respectively). Fluorometric GUS measurements indicated a two-fold increase in enzyme activity in *abi2/sbr* seedlings (50105 $\pm$ 6809 fluorescence units/h per μg protein) compared to *abi2* (25831 $\pm$ 5238 units/h per μg protein), supporting the visual observation that *abi2/sbr* roots over-express *Dc3-GUS*. ABA-inducible *Dc3-GUS* expression in guard cells of *abi2/sbr* cotyledons was similar to that observed in *abi2-1* (Chak *et al.*, 2000; and data not shown).

The *abi2/sbr* mutants had a severe dwarf growth phenotype. The primary root did not expand beyond a length of 3–4 mm or form lateral roots. Growth on nutrient agar resulted in a life span of up to 4 weeks before necrosis, during which time the seedlings produced few true leaves and appeared compressed in the apical-basal axis. The cotyledons and true leaves of the mutant seedlings were epinastic and darker green. The roots occasionally formed bulging structures and callus-like outgrowths after three weeks of growth (Figure 2H). Root growth of *abi2/sbr* seedlings was arrested as early as the 3rd day after germination. *abi2/sbr* seedlings often produced adventitious roots from the hypocotyl or the root-shoot junction. Scanning electron microscopy of *abi2/sbr* shoots and roots revealed deformation of epidermal cells in the mutant. Cells of the hypocotyl were often radially swollen and the cell files were disorganized (Figure 3C). Root epidermal cells also bulged radially (Figure 3F), giving rise to bulbous structures in 3-week old roots (Figure 2F). Root hairs appeared very close to the root tip indicating that the meristem and the zone of elongation are very short (Figure 3F). The pavement cells of adaxial cotyledon epidermis were larger in *abi2/sbr* mutants (Figure 3I) than those of *abi2* parental type (Figure 3G), with islands of small meristemoid cells associated with paired stomata (Figure 3I). Trichome

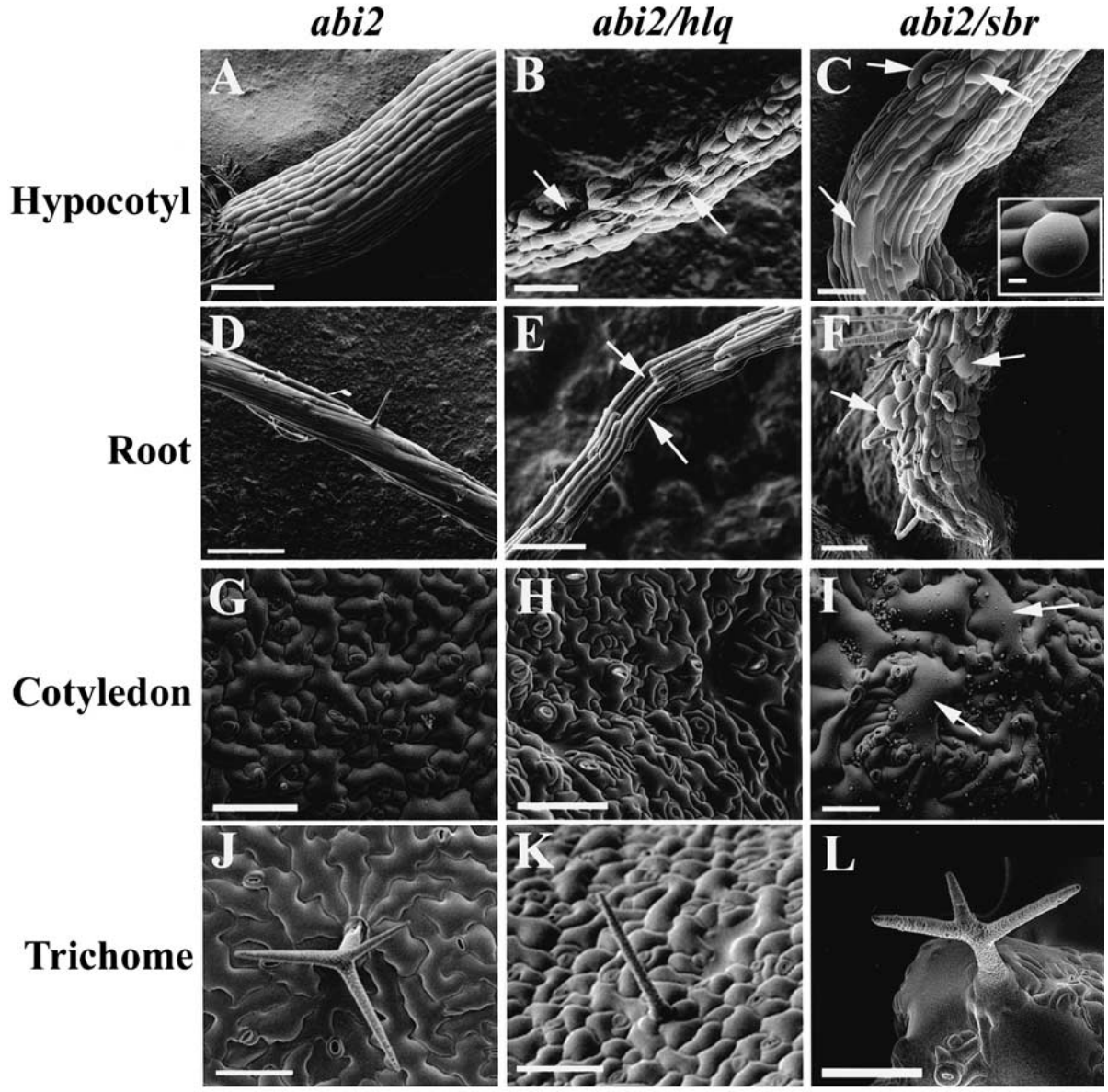

Figure 3. Abnormal epidermal cell morphology in *abi2/hlq* and *abi2/sbr* hypocotyl, root, and cotyledon. Scanning electron micrographs of hypocotyl surface (A–C), root epidermis (D–F), cotyledon surface (G–I) and trichomes (J–L) of parental-type *abi2*, *abi2/hlq* and *abi2/sbr* seedlings. The arrow-heads indicate either missing cells *(abi2/hlq)* or abnormally bulged cells *(abi2/sbr)*. Inset in C is a magnified image of a bulged epidermal cell. Scale bar = 100 μm in all panels except inset in C = 20 μm.

morphology was normal in the *abi2/sbr* mutant leaves (Figure 3L).

Germinating embryos of *sbr* occasionally accumulated anthocyanin in the shoot, similar to the *constitutive photomorphogenic/deetiolated/fusca* (*cop/det/fus*) mutants of *Arabidopsis* (Misera *et al.*, 1994). When germinated in the dark, mutant *abi2/sbr* seedlings were etiolated, but, the mutant hypocotyls did not elongate as much as the parental type (Table 1). The light-grown shoots of *abi2/sbr* mutants had higher chlorophyll levels than the parental type (Table 1). The mutant plants had more root hairs and thicker roots than the parental type (Table 1).

Confocal imaging of PI-stained *abi2/sbr* roots revealed that the number of cortical and epidermal cells visualized in the mature root zone (11.4 $\pm$ 0.3 and

Table 1. Measurements of growth parameters in light- and dark-grown *abi2* parental type, *abi2/hlq* and *abi2/sbr* mutant seedlings.

Parameter	Light			Dark		
	abi2	*abi2/hlq*	*abi2/sbr*	*abi2*	*abi2/hlq*	*abi2/sbr*
Root diameter (μm)	105 ± 8	86 ± 10	175 ± 24	89 ± 10	56 ± 12	112 ± 10
Hypocotyl diameter (μm)	304 ± 15	172 ± 8	335 ± 17	227 ± 21	160 ± 4	232 ± 16
Root hairs/mm root	18 ± 1	0.6 ± 0.2	54 ± 9	13 ± 1	0.2 ± 0.1	54 ± 7
Root length (mm)	66 ± 7	17 ± 3	3 ± 0.3	20 ± 2	26 ± 3	3 ± 0.2
Hypocotyl length (mm)	11 ± 1	3 ± 1	3 ± 0.5	17 ± 1	3 ± 1	10 ± 1.5
Chlorophyll content (μg per mg tissue)	0.27 ± 0.02	0.15 ± 0.03	0.34 ± 0.01	0.03 ± 0.01	0.10 ± 0.04	0.03 ± 0.05

Data represent mean (±SE) of measurements from 8–14 seedlings taken 14 days after sowing.

25.0 ± 3.0 respectively) were higher than those of *abi2* parental type (8 ± 0.5 and 17.5 ± 0.5 respectively; Figure 4A versus 4B). Furthermore, the epidermal cells in the mature parts of the *abi2/sbr* roots were shorter than the parental type (40 ± 4 μm vs. 111 ± 5 μm). Longitudinal optical sections through the root tip showed abnormally shaped cells in the *abi2/sbr* mutant roots. Whereas the parental-type root tip had typical meristemoid cells (Figure 4C), the *abi2/sbr* roots had flat cells at the root meristem (Figure 4D). The cells above the root meristem in the zone of elongation were isodiametric in shape and larger in size than those of the parental type. The root vascular tissue of *abi2/sbr* mutants was disorganized and the vessels were often discontinuous (Figure 4F) and twisted. The mutant cotyledons had incomplete venation. True leaves of the mutant developed with swollen primary veins that were often split and poorly branched. The tertiary vein network was largely incomplete and the marginal veins were usually absent (Figure 4H).

Addition of ABA, GA_3, NAA, 2,4-D, 24-*epi*-BR, auxin biosynthetic inhibitors 5-chloro-indole and 6-fluoro-indole, or kinetin to the growth medium failed to rescue the *sbr* phenotype indicating that homeostasis of these hormones is not affected in the mutant. The determinate nature of *sbr* root growth resembled in some respects the phenotype of *root meristemless1* (*rml1*), which encodes an enzyme of glutathione (GSH) biosynthesis (Vernoux *et al.*, 2000). Because *rml1* mutants can be rescued by exogenous GSH, we grew *abi2/sbr* seedlings on GSH-containing medium. However, the *abi2/sbr* mutants were not rescued by GSH addition.

Genetic characterization and mapping of hlq *and* sbr *mutants*

The *Dc3-GUS* phenotypic screen (and the pleiotropic nature of the *hlq* and *sbr* mutants) results in the death of individual seedlings. Hence we propagated the mutants by self-fertilization of heterozygous M_2 plants segregating for the *hlq* and *sbr* phenotypes and re-screened for the *Dc3-GUS* phenotype in M_3 lines ($P>0.6$ that deviations from expected 3:1 segregation ratios were due to chance; χ^2 test of 271 and 190 segregants for *hlq* and *sbr*, respectively). Heterozygous *hlq*/+ and *sbr*/+ lines were twice backcrossed to *abi2* parental type to remove extraneous mutations unlinked to *hlq* and *sbr* loci. Segregation analyses of self-fertilized *hlq*/+ and *sbr*/+ heterozygous stocks demonstrated that the *hlq* and *sbr* mutant alleles were inherited as single-gene, nuclear mutants. The *hlq* mutant allele is recessive in the *abi2* parental background, since F_1 heterozygous plants from backcrosses appeared phenotypically normal. It was observed in segregating *sbr* lines grown vertically on agar plants that about half of the seedlings had primary roots of intermediate length between *sbr/sbr* and putative *abi2* parental genotypes, suggesting that the *sbr* root growth phenotype may be semi-dominant. A test for a semi-dominant *sbr* root phenotype was performed with 20 individual plants from a segregating *sbr* stock. The seedlings were scored as putative *sbr*/+ heterozygotes or +/+ parental types, transplanted to soil, allowed to self-fertilize, and the F_3 lines scored for segregation of the *sbr* phenotype. Out of the 20 lines, only 3 were scored incorrectly in the previous generation by their root growth phenotype (data not shown). These results support the conclusion that *sbr* exhibits a semi-dominant root growth phenotype. Heterozygous *sbr*/+ plants otherwise appeared normal.

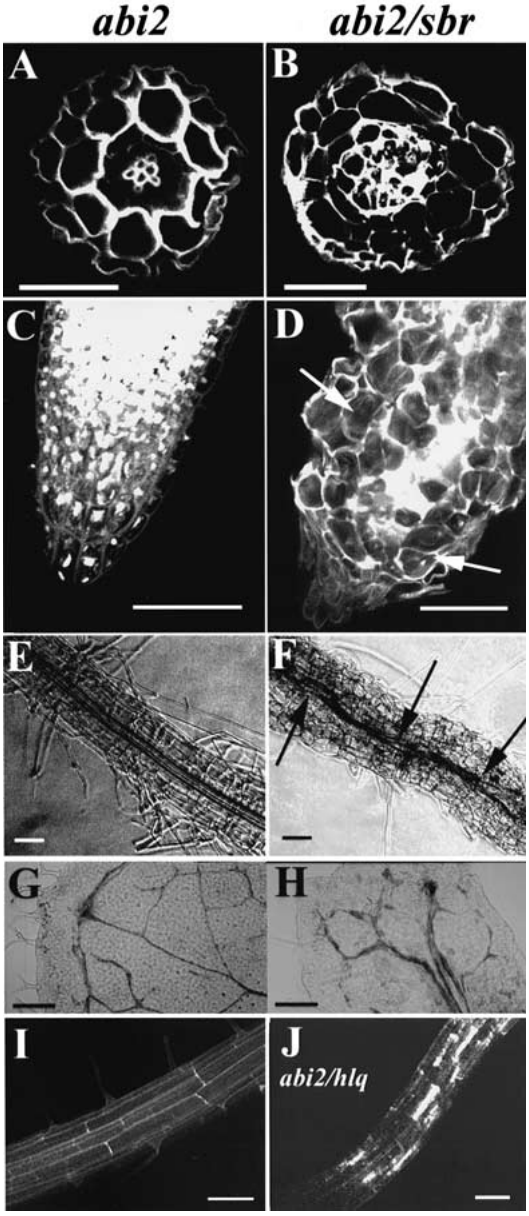

Figure 4. Anatomy of the *abi2* parental-type and *abi2/sbr* mutant roots. A–D. Optical sections of propidium iodide-stained *abi2* parental-type and *abi2/sbr* mutant roots obtained by confocal imaging. A, B. Cross-section of *abi2* parental-type root (A) and *abi2/sbr* root showing difference in the numbers of epidermal and cortical cells. C, D. Longitudinal optical sections through the root tip of *abi2* parental type (C) and *abi2/sbr* (D) showing differences in the size and shape of cells in the meristematic region. Arrows indicate two of the abnormally bulged cells in the *abi2/sbr* mutant root. E, F. Bright-field micrographs of whole-mount *abi2/sbr* (F) mature root zone showing discontinuous vessels (indicated by arrows) in the vasculature compared to the orderly and compact vasculature (E) *abi2* parental type. G, H. Cleared whole-mount *abi2/sbr* rosette leaf (H) showing swollen primary veins and poor branching compared to the normal reticulate venation in *abi2* parental-type leaf (G). I, J. Aniline blue-stained 10-day old *abi2/hlq* roots (J) showing ectopic deposition of callose compared to *abi2* (I). Scale bars = 50 μm.

In order to map the mutant loci, *hlq/+* and *sbr/+* heterozygous plants were crossed with the Columbia ecotype. The F_2 mutant recombinants from a single heterozygous F_1 parent were scored and DNA samples analysed by PCR for linkage to SSLP (Koniecnzy and Ausubel, 1993) and CAPS (Bell and Ecker, 1994) markers. The *hlq* locus maps ca. 6.6 cM south of the SSLP marker *nga162* on chromosome III (log of odds against linkage >59). The *sbr* locus maps ca. 3.1 cM south of the marker *T4O12_24* (vacuolar ATP synthase subunit B; 'ATPase') on chromosome I (log of odds against linkage >236).

Because *hlq* and *sbr* were derived from the *abi2* mutant background, it was possible that *hlq* and *sbr* mutant genes interacted genetically with *abi2*. In other words, the mutant phenotypes may be dependent on the presence of the *abi2-1* allele. Because the *ABI2* locus maps to chromosome V and the *abi2-1* mutant allele is easily genotyped by CAPS analysis (Leung *et al.*, 1997), we tested for genetic interaction of the *abi2* locus with the *hlq* and *sbr* mutant alleles by genotyping the *ABI2* locus in *hlq* and *sbr* recombinants in a segregating F_2 mapping population. Results of *abi2* CAPS analysis in 24 *hlq* and 28 *sbr* recombinants showed no evidence of 'linkage' (genetic interaction) with the *abi2-1* mutation (χ^2 test; $P>0.88$).

Discussion

Dc3-GUS *as a marker for ABA- and IAA-regulated gene expression*

The observation that auxins induce root-specific *Dc3-GUS* expression in the cortical cells of the zone of maturation, analogous to (but distinct from) the tissue-specific induction of *Dc3-GUS* by ABA, was unexpected. However, other stress-related gene promoters such as tobacco anionic peroxidase, alcohol dehydrogenase, and pyrroline-5-carboxylate synthetase (AtP5CS) are also coordinately regulated by ABA and auxins (Klotz and Lagrimini, 1996; Stritzhof *et al.*, 1997; Noguchi, 2000). Gaymard *et al.* (1998) and Rock (2000) have shown independently that root vascular tissue is sensitive at the transcriptional level to ABA. The constitutive expression of *Dc3-GUS* in lateral root primordia (Rock, 2000; X. Sun and C.D. Rock, unpublished observations), which are induced to proliferate by auxin, suggests *Dc3-GUS* can mark changes in endogenous auxin concentrations or cellular sensitivity to auxin, analogous to the *GH3* promoter of soybean in transgenic tobacco (Li *et al.*,

1999). The *Dc3* promoter has a *GH3*-like promoter palindrome TGTY. . . n13...GAGACA at position –231 (Kim *et al.*, 1997) that could bind ARF1, a transcription factor that binds to auxin-responsive promoters (Ulmasov *et al.*, 1997). ARF1 and the ABA transcription factors FUSCA3 and ABI3/VP1 share homology in the B3 DNA-binding domain (Ulmasov *et al.*, 1997; Rock, 2000). Conversely, the auxin-responsive element in the *GH3* promoter has an overlapping G-box (ABRE-like) element and binds a cognate bZIP factor (Hong *et al.*, 1995), analogous to ABA-inducible promoters. Although detailed molecular genetic analyses of the *Dc3* promoter and its interaction with specific ABA- and auxin-regulatory factors have yet to be performed, these observations support the possibility that TRAB1/ABI5-, ABI3/VP1- and/or ARF1-like transcription factors may interact to coordinate auxin-, ABA-, and stress-inducible *Dc3-GUS* expression. *Dc3-GUS* transgenic *Arabidopsis* is a useful tool for molecular genetic analysis of auxin-, ABA- and stress-regulated gene expression and their roles in plant growth, development, and response to the environment.

The pleiotropic nature of harlequin *and* short blue root *mutants*

The *sbr* and *hlq* mutants described here were isolated based on their ectopic *Dc3-GUS* reporter gene expression phenotypes. The pleiotropic nature of the *hlq* and *sbr* mutants raises questions of causality between the ectopic *Dc3-GUS* expression and other phenotypes. For example, a de-regulated signal transduction pathways marked by ectopic *Dc3-GUS* expression could result in the observed morphological defects in *hlq* and *sbr* plants. Alternatively, cellular stresses imposed by mutations in genes required for morphogenesis could indirectly cause ectopic *Dc3-GUS* gene expression. Ectopic expression of the auxin- and ABA-inducible *Dc3-GUS* promoter in root epidermal cells of *hlq* mutants and ABI2-dependent Dc3-GUS expression in root hairs may be related to a hypothetical link between root hair development and hormone homeostasis. The plant hormones auxin, ethylene and ABA have been implicated in root hair development and elongation (Schnall and Quatrano, 1992; Pitts *et al.*, 1998). The *sbr* and *hlq* mutants are novel genetic resources that may help understand the relationship between hormone-regulated gene expression and plant morphogenesis and stress responses.

The defects in root hair and trichome development in the *hlq* mutant resemble the *werewolf* (*wer*), *glabra2* (*gl2*), *caprice* (*cpc*) and *transparent testa-glabra* (*ttg*) mutants (Wada *et al.*, 1997; Hung *et al.*, 1998; Walker *et al.*, 1999; Lee and Schiefelbein, 1999). However unlike *ttg, wer, cpc* or *gl2* mutants, *hlq* seedlings display epidermal cell collapse, abnormal epidermal cell morphologies, and spatially distinct *Dc3-GUS* expression patterns that do not correlate with the atrichoblast-specific expression pattern of *GL2* and *WER* (Lee and Schiefelbein, 1999) suggesting a unique function for *HLQ*. Interestingly, in *wer* mutants the normal atrichoblast- and hypocotyl-specific expression of a GL2-promoter:GUS reporter gene is abolished, and instead *GL2:GUS* activity is seen in a few scattered trichoblast and atrichoblast cells (Lee and Schiefelbein, 1999), analogous to the *Dc3-GUS* phenotype of roots and hypocotyl seen in *hlq* mutants.

The morphological alterations in the *sbr* mutant suggests defects in cell elongation or specification which are reminiscent of, but yet distinct from, phenotypes of *short root* (Benfey *et al.*, 1994), *ectopic lignification 1* (Caño-Delgado *et al.*, 2000), *root meristemless 1* (Vernoux *et al.*, 2000) and *root radial swelling* (Baskin *et al.*, 1992) mutants. The abnormal vascular elements observed in the *abi2/sbr* mutants are similar to phenotypes seen in auxin signalling/transport and vascular differentiation mutants (Mattson *et al.*, 1999). Epinastic cotyledons and leaves, short hypocotyls, proliferation of cortical and epidermal cells and initiation of adventitious roots in the *abi2/sbr* mutant are consistent with a localized increased sensitivity to auxin signal. The semidominant phenotype of reduced lateral roots in *sbr*/+ heterozygous plants is reminiscent of the dominant gain-of-function alleles of Aux/IAA genes such as *shy2-2*/IAA3, *axr2-1*/IAA7, *axr3-1*/IAA17, *msg2*/IAA19, *slr*/IAA14, and *iar2*/IAA28 (Nagpal *et al.*, 2000). The Aux/IAA mutants exhibit related phenotypes to *sbr* such as short hypocotyls, altered lateral root abundance, and reduced root growth (Nagpal *et al.*, 2000; Worley *et al.*, 2000). It is plausible that the *abi2/sbr* mutants have a defect in auxin transport or signalling; however, the *abi2/sbr* mutant plants do not show some predictable auxin transport/signalling phenotypes such as agravitropism.

The cell wall and harlequin *and* short blue root *phenotypes*

The epidermis of plants, by virtue of its growth outside the constraints of the plant body, may exhibit cell expansion defects more readily. Cortical microtubules, cellulose microfibrils and other cell-wall components are important for normal morphogenesis and anisotropic epidermal growth (Wasteneys, 2000). Ectopic callose deposition and abnormal cell expansion observed in *hlq* and correlated with *Dc3-GUS* expression might be a consequence of disrupted cellulose synthesis (His *et al.*, 2001). Arabinogalactan proteins and hydroxyproline-rich glycoproteins (HRGPs) are major cell wall proteins thought to regulate normal morphogenesis (Majewska-Sawka and Nothnagel, 2000) and have also been implicated in ABA signalling (Wang *et al.*, 1995; Desikan *et al.*, 1999). It is possible that cortical microtubule activity, cellulose synthesis, or cell wall components are impaired in *sbr* and *hlq*.

In summary, we have described the root-specific expression patterns of an ABA- and auxin-inducible reporter construct, *Dc3-GUS,* in *Arabidopsis* and have shown that the *ABI2* protein phosphatase is important for ABA responsiveness of this tissue. We have isolated and characterized two novel single-gene nuclear mutants, *harlequin* (*hlq*) and *short blue root* (*sbr*), from a genetic screen on the basis of their altered *Dc3-GUS* expression in *abi2-1* roots. The complexity of the phenotypic alterations in the *abi2/hlq* and *abi2/sbr* mutants suggest a range of potential molecular defects such as factors controlling hormone- and stress-inducible gene expression, cell-cell communication, cell elongation, cell wall synthesis or an integrator of these processes. Map-based cloning of *HLQ* and *SBR* and characterization of the gene products will facilitate understanding of their function in regulation of stress-responsive gene expression affecting growth and development.

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