

Christopher D. Rock · Xin Sun

Crosstalk between ABA and auxin signaling pathways in roots of *Arabidopsis thaliana* (L.) Heynh.

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Abstract Studies of abscisic acid (ABA) and auxin have revealed that these pathways impinge on each other. The *Daucus carota* (L.) Dc3 promoter: *uidA* (β -glucuronidase: GUS) chimaeric reporter (ProDc3:GUS) is induced by ABA, osmoticum, and the auxin indole-3-acetic acid (IAA) in vegetative tissues of transgenic *Arabidopsis thaliana* (L.) Heynh. Here, we describe the root tissue-specific expression of ProDc3:GUS in the *ABA-insensitive-2* (*abi2-1*), *auxin-insensitive-1* (*aux1*), *auxin-resistant-4* (*axr4*), and *rooty* (*rty1*) mutants of *Arabidopsis* in response to ABA, IAA and synthetic auxins naphthalene acetic acid (NAA), and 2, 4-(dichlorophenoxy) acetic acid. Quantitative analysis of ProDc3:GUS expression showed that the *abi2-1* mutant had reduced GUS activity in response to ABA, IAA, or 2, 4-D, but not to NAA. Similarly, chromogenic staining of ProDc3:GUS activity showed that the *aux1* and *axr4* mutants gave predictable hypomorphic ProDc3:GUS expression phenotypes in roots treated with IAA or 2, 4-D, but not the diffusible auxin NAA. Likewise the *rty* mutant, which accumulates auxin, showed elevated ProDc3:GUS expression in the absence or presence of hormones relative to wild type. Interestingly, the *aux1* and *axr4* mutants showed a hypomorphic effect on ABA-inducible ProDc3:GUS expression, demonstrating that ABA and IAA signaling pathways interact in roots. Possible mechanisms of crosstalk between ABA and auxin signaling are discussed.

Keywords *Arabidopsis* · Abscisic acid (ABA) · Auxin (IAA) · *Daucus* · Gene expression ·

Late-Embryogenesis-Abundant protein · Mutant · Protein phosphatase · Root

Abbreviations ABA: Abscisic acid · ABI: ABA-insensitive · AREBs=ABFs: ABA-Response Element Binding Factors · ARFs: Auxin-responsive factors · GFP: Green fluorescent protein · IAA: Indole-3-acetic acid · IBA: Indole-3-butyric acid · GUS: β -glucuronidase (*uidA*) · 2, 4-D: 2, 4-(dichlorophenoxy)acetic acid · DPBFs: Dc3 Promoter Binding Factors · LEA: Late-Embryogenesis-Abundant · NAA: Naphthalene acetic acid · 4-MU: 4-methylumbelliferone · The 1.5 kbp promoter of *Dc3*: ProDc3

Introduction

Abscisic acid (ABA) and auxin, indole-3-acetic acid (IAA), are two of the “classic” plant hormones known to regulate myriad aspects of plant growth and development. Many physiological studies have suggested that these two hormones functionally interact in roots as regulators of growth, development, and tropisms (for reviews see Casimiro et al. 2003; Walton 1980). The 70-year-old Cholodny–Went hypothesis (see Trewavas 1992 for review) for tropic responses has recently been validated by two molecular genetic studies: expression of a green fluorescent protein (GFP)-based auxin biosensor (Ottenschlager et al. 2003) and gravity-dependent localization of an auxin-efflux carrier (Friml et al. 2002). Recent molecular genetic studies in *Arabidopsis* have revealed complex interactions and cell-specific expression of auxin transporter gene family members and elucidated the mechanisms of action of auxin in controlling embryogenesis (Friml et al. 2003) and phyllotaxis (Reinhardt et al. 2003). However, despite recent progress in understanding the intermediate and downstream steps in ABA and auxin signaling (see DeLong et al. 2002; Dharmasiri and Estelle 2004; Finkelstein and

C. D. Rock (✉)
Department of Biological Sciences, Texas Tech University,
Lubbock, TX 79409-3131, USA
E-mail: chris.rock@ttu.edu
Fax: +1-806-742-2963

Present address: X. Sun
The Graduate Department of Molecular and Medical Genetics,
University of Toronto, #4388 Medical Sciences Building,
1 King's College Circle, Toronto, M5S 1A8, Canada

Rock 2002; Hagen et al. 2004), the mechanisms of hormone action remain obscure. Some of the unknowns include the identities of the receptors, the substrates of kinases and phosphatases, the roles of secondary messengers as nodes to link with other pathways, whether gene products interact directly or indirectly, the roles and mechanisms of protein and mRNA instability, and the identities of signaling components linking the known elements into pathways or networks.

Hormone response mutants provide a means of dissecting the signal transduction pathways controlling physiological processes and elucidating molecular mechanisms (McCourt 1999). Genetic and/or molecular studies have identified approximately 50 ABA response and auxin response (and transport) loci, respectively, many of whose functions are conserved across species (for reviews see Boonsirichai et al. 2002; Finkelstein and Rock 2002; Hagen et al. 2004). Such studies have confirmed the roles of endogenous ABA in seed and lateral root development, dormancy versus germination, transpiration, and stress responses, and IAA in cell division, cell elongation, pattern formation, differentiation, and tropisms.

In Arabidopsis and probably all higher plants, ABA and IAA signaling are mediated by both redundant and independent mechanisms, some of which also appear to affect responses to other signals, including each others'. For example, when the maize ABA transcription factor VIVIPAROUS1 (the orthologue of ABI3) is expressed ectopically in Arabidopsis, a root-specific auxin-related phenotype is seen (Suzuki et al. 2001). Similarly, ABI3 has recently been shown to function in auxin-mediated lateral root development (Brady et al. 2003). In addition to mutants with pleiotropic defects in ABA response, many ABA mutants have been identified with defects in responses to auxin such as *auxin-resistant-2* (*axr2/IAA7*) (Nagpal et al. 2000; Wilson et al. 1990), *pleiotropic response1* (Bhalerao et al. 1999), *indole-3-butyric acid response-5* (*ibr5*) (Monroe-Augustus et al. 2003), and *sensitive to abscisic acid and auxin* (*sax1*) (Ephritikhine et al. 1999). The *hyponastic leaves 1* (*hyl1*) mutant affects the kinetics of root gravitropism and has pleiotropic phenotypes of shorter plant stature, increased branching, delayed flowering, leaf hyponasty, reduced fertility, increased root-growth resistance to auxin and cytokinin, and hypersensitivity of seed germination and root growth to ABA (Han et al. 2004; Lu and Fedoroff 2000; Vazquez et al. 2004). Most of these genes have been cloned and encode an auxin-induced transcription factor, an SNF1-like kinase-interacting factor, a putative dual specificity phosphatase, and a double-stranded RNA binding protein involved in microRNA metabolism, but their mechanisms of action are unknown. Possible mechanisms of crosstalk are discussed in many recent reviews (Casimiro et al. 2003; Chinnusamy et al. 2004; Finkelstein and Gibson 2002; Liscum and Reed 2002; McCarty and Chory 2000; Moller and Chua 1999).

The *AUX1* gene of *Arabidopsis thaliana* encodes a component of the auxin influx carrier and was isolated

through a screen for mutations that affect root-growth response to auxin and root gravitropism (Maher and Martindale 1980). Mutant seedlings of *aux1* display increased root-growth resistance to auxin and ethylene, altered root gravitropism, defects in lateral root formation, and altered auxin transport (Bennett et al. 1996; Marchant et al. 1999). The *reduced gravitropism 1* (*rgr1*)/*auxin resistance 4* (*axr4*) mutant affects root gravitropism and confers specific root-growth resistance to auxin and polar auxin-transport inhibitors (Hobbie and Estelle 1995; Simmons et al. 1995). *Rooty1* (*rty*) is allelic to *superroot1* (*sur*) and the ethylene response mutant *hookless3* (*hls*) (Boerjan et al. 1995; Gopalraj et al. 1996; King et al. 1995) and encodes an aminotransferase that may function in auxin redistribution or crosstalk between auxin and ethylene pathways.

The *Dc3* gene of carrot (*Daucus carota* L.) encodes a late-embryogenesis-abundant (LEA) gene inducible by ABA and stress in seeds and vegetative tissues (Siddiqui et al. 1998) and its promoter binds to the Arabidopsis basic leucine zipper transcription factor ABI5 and the closely related *Dc3* Promoter Binding Factors (DPBFs) and ABA-Response Element Binding Factors (AREBs, ABFs) (Finkelstein and Lynch 2000; Kim et al. 2002, 2004; Uno et al. 2000; for review see Finkelstein and Rock 2002). Sequence analysis suggests that the GH3-like element (Li et al. 1999) at position -231 in the *Dc3* promoter could be responsible for auxin-inducible expression. The ProDc3:GUS reporter is differentially regulated by the homologous *ABA-INSENSITIVE1* (*ABI1*) and *ABI2* protein phosphatase type 2C genes (Leung et al. 1997; Rodriguez et al. 1998) in guard cells and roots (Chak et al. 2000; Rock 2000) and is induced by IAA (Subramanian et al. 2002), making it a suitable molecular marker for tissue-specific analysis of ABA-, auxin and stress-regulated gene-expression pathways and their dissection by genetic analysis. Here, we report studies on the root-specific auxin signaling pathway(s) controlling ProDc3:GUS expression in the auxin transport/response mutants *aux1* and *axr4/rgr1* and the auxin over-producing mutant *rty1/sur1/hls3* (Sun 2003). We observed predictable effects of these mutations on natural and synthetic auxin induction of ProDc3:GUS expression in distinct root tissues. Unexpectedly, we observed that the *abi1-1* and *abi2-1* mutants had effects on auxin-inducible ProDc3:GUS expression, and likewise *aux1* and *axr4* had effects on ABA-inducible ProDc3:GUS expression, suggesting root-specific signaling crosstalk between ABA and auxin pathways.

Materials and methods

Plant materials and growth conditions

The *Arabidopsis thaliana* (L.) Heynh. genotypes used in this study were the Landsberg *erecta* wild type (Ler;

CS20), Columbia (CS907), *abi1-1* (CS22), *abi2-1* (CS23), *aux1-7* (CS3074), *axr4-2* (CS8019), and *rti1-1* (CS8156). CS numbers refer to the Arabidopsis Biological Resource Center catalogue number (Ohio State University, Columbus, OH, USA). ProDc3:GUS double insertion/*abi2-1* and Ler (P-series) recombinant lines used in this study were those described by Chak et al. (2000). The ProDc3:GUS 11-1 and 7-2 lines used to generate the double recombinant P-series were the kind gift of Prof. Terry Thomas, Texas A&M University, College Station, TX, USA. Arabidopsis growth conditions were as described in Chak et al. (2000). For isolation of homozygous *aux1* and *axr4* ProDc3:GUS recombinants, F₂ seeds from crosses were surface-sterilized and grown vertically on 0.1 μM 2, 4-D medium-containing plates. After 7 days, the seedlings with elongated roots were picked out as homozygous *aux1* and *axr4*, and then transplanted into soil and allowed to self-fertilize. Auxin-resistance scoring of the F₃ recombinant pedigrees was performed by germinating *aux1*, *axr4*, and wild-type Ler seeds along with recombinant lines on minimal medium plates containing 1 μM IAA or NAA or 0.1 μM 2, 4-D (Sigma, St. Louis, MO, USA). Homozygous *rti* recombinants were scored on minimal medium plates by their root growth phenotype.

Histochemical β-glucuronidase assays

ProDc3:GUS recombinant Arabidopsis seeds were surface sterilized by 30% commercial bleach for 4 min and washed three times with sterile distilled water. Surface-sterilized seeds were kept in 4°C for 3 days to break dormancy. Seeds were sowed on square minimal media Petri plates (Scholl et al. 1998) with 1.2% phytigel (Sigma) and grown vertically in a growth chamber for 6 days. The plates were placed horizontally and overlaid with water, ABA-containing or auxin-containing solutions for 24 h. Induced seedlings were then stained for GUS activity with 5-Bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) (Rose Scientific, Edmonton, AB, Canada) as described by Chak et al. (2000). Stained seedlings were visualized and photographed using a dissecting microscope (Carl Zeiss AG, Germany).

Quantitative fluorometric β-glucuronidase assays

Seedlings were grown vertically on minimal plates for 6 days as described above and transferred to hormone plates by forceps in a sterile hood and returned to the growth chamber for another 2 days. For quantitative GUS assay, roots of Ler and *abi2* ProDc3:GUS seedlings were dissected with a scalpel and collected in a 1.5 mL microfuge tube, with approximately ten roots per sample. In assays from exogenous IAA, NAA, and 2, 4-D treatments, seeds were germinated on minimal media plates containing 1 μM IAA or NAA or 0.1 μM 2, 4-D for 7 days, then seedling roots (approximately 10/

sample) were collected. The fluorometric GUS assay is based on 4-methylumbelliferone (4-MU; Sigma) fluorescence measured by a Cytofluor II microplate spectrofluorometer (Molecular Dynamics, Sunnyvale, CA, USA) calibrated with a standard curve of known concentrations of 4-MU. The GUS substrate was 4-methylumbelliferyl-β-D-glucuronide (Rose Scientific). Protein concentrations of root extracts (average of nine measurements per sample) were determined by the dye-binding method (Bio-Rad Protein Assay; Bio-Rad, Hercules, CA, USA). The GUS activities were calculated in relative units of fluorescence produced per-μg protein-per hour.

Results

Quantitative analysis of ABA-inducible and auxin-inducible ProDc3:GUS expression in *abi1-1* and *abi2-1* roots

Our previous studies demonstrated the dose-dependent induction of ProDc3:GUS in *Arabidopsis thaliana* roots and leaves by exogenous application of ABA (Chak et al. 2000; Rock 2000) or the naturally occurring auxin IAA (Subramanian et al. 2002), wherein it was reported that: (1) *abi2-1*, but not *abi1-1*, mutant roots were insensitive to ABA induction of ProDc3:GUS expression; and (2) there were no obvious qualitative effects of treatment with a saturating concentration of 10 μM IAA on ProDc3:GUS expression in 4-day-old *abi1-1* or *abi2-1* roots. However, upon close inspection and further dose-response analyses, a slight hypomorphic effect of *abi1-1* and *abi2-1* mutations on induction of ProDc3:GUS could be discerned and was further examined by quantitative fluorometric GUS assays of 6 or 7-day-old wild-type Ler, *abi1-1*, and *abi2-1*/ProDc3:GUS mutant roots subjected to sub-saturating concentrations (0.1–1.0 μM) of IAA or the synthetic auxins NAA and 2, 4-D. The results are shown in Fig. 1. Consistent with previous qualitative results (Subramanian et al. 2002), there was a 12-fold induction of ProDc3:GUS expression by 100 μM ABA treatment for 48 h; this induction was significantly reduced by 65% in *abi2-1* mutant roots relative to wild-type Ler, but not in *abi1-1* roots (Fig. 1a) ($P < 0.03$, Student's two-sided *t*-test, equal variance assumed). Treatment of wild-type seedlings with 1 μM 2, 4-D resulted in a 17-fold induction of ProDc3:GUS, which was significantly reduced >30% in the *abi1-1* and *abi2-1* mutants (Fig. 1a) ($P < 0.01$, Student's two-sided *t*-test; equal variance assumed). The fold-magnitude induction by 1 μM 2, 4-D of ProDc3:GUS was similar in magnitude to short-term (6 h) induction (in response to 20 μM IAA) of an artificial promoter containing a 4× multimer of the 183 bp auxin-responsive region of the *PS-IAA4/5* promoter (Oono et al. 1998). There was no inductive effect on ProDc3:GUS by 10 μM gibberellin GA₃ treatment (data not shown). Seedlings of wild-type Ler and *abi2-1* grown

for 7 days on different auxins were assayed for ProDc3:GUS expression and the results are shown in Fig. 1b. Growth on 0.1 μM 2, 4-D resulted in a >40-fold elevation in ProDc3:GUS activity in wild-type Ler roots, which was reduced by 27% in the *abi2-1* mutant background (Fig. 1b). Growth on 1 μM NAA resulted in a 76-fold induction of ProDc3:GUS activity over control wild-type, which was reduced only 16% in *abi2-1* mutant roots. Growth on 1 μM IAA induced ProDc3:GUS expression >50-fold, which was significantly reduced >60% in the *abi2-1* mutant roots (Fig. 1b) ($P < 0.04$, two-sided Student's *t*-test; equal variance assumed). Interestingly, *abi1* and *abi2* mutants were observed to germinate and grow better than wild type on 10 μM NAA or 1 μM 2,4-D (data not shown).

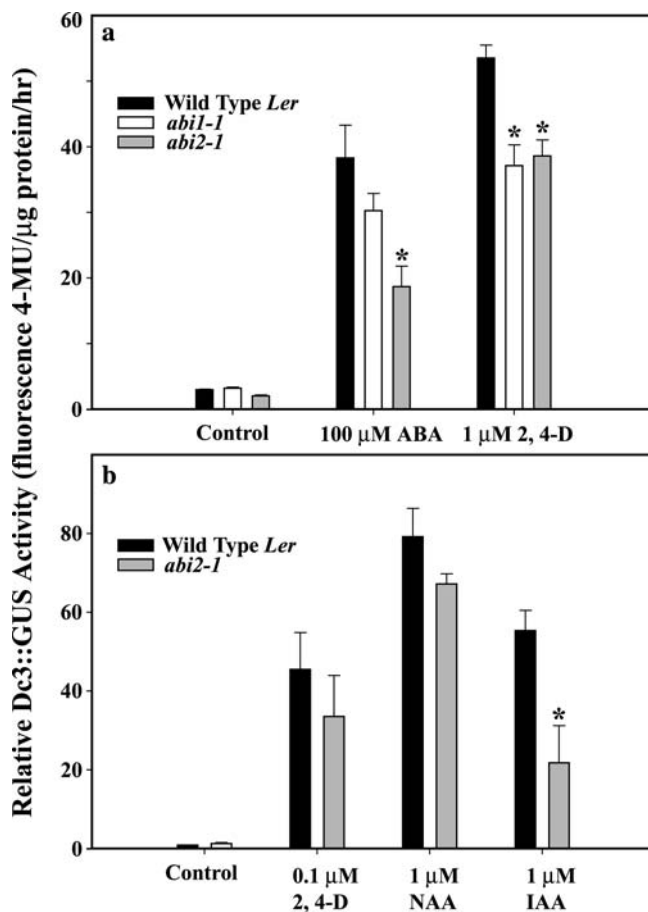


Fig. 1 Fluorometric assays of ProDc3:GUS activity in whole root extracts of wild-type Ler and *abi2-1* mutant treated with ABA or auxins. **a** Six-day-old seedlings (10 to 15 individuals per treatment) of the Ler and *abi2-1* homozygote genotypes harboring the ProDc3:GUS reporter gene were grown vertically on minimal media plates, transferred for 48 h to control plates or plates containing 100 μM ABA or 1 μM 2, 4-D, and roots assayed. **b** Seeds (10 to 15 per sample) were germinated and grown for 7 days on minimal media plates containing IAA, NAA or 2, 4-D and roots assayed. Results of both panels are the average (\pm SEM) of three replicates. Comparisons between wild-type Ler and *abi2* activities annotated with asterisks (*) are significantly different from each other ($P < 0.05$; two-sided Student's *t*-test, equal variance assumed)

Histochemical characterization of root tissue-specific auxin-inducible and ABA-inducible ProDc3:GUS expression in the auxin-insensitive *aux1*, *axr4*, and auxin-overexpressing *rtv* Arabidopsis mutants

Based on our quantitative results on reduced auxin induction of ProDc3:GUS in the *abi2-1* genotype and the well-known involvement of auxin in root growth and development, we undertook to characterize the root tissue-specific and hormone-inducible expression of the ProDc3:GUS reporter gene in the auxin response mutants *aux1*, *axr4*, and *rtv*. These mutants were chosen for study because they act further upstream in auxin signaling/homeostasis than auxin-regulated transcription factors such as AXR2 or AXR3 (cognates of a family of ~30 homologues in Arabidopsis) and therefore would not limit the scope for specific transcriptional effects on a heterologous promoter : reporter construct. The results of histochemical staining of ProDc3:GUS activity are shown in Fig. 2. When 6-day-old wild-type and *aux1* or *axr4* mutant seedlings were treated with water alone, constitutive ProDc3:GUS activity was restricted to the primary and lateral root meristems and root cap columella cells (Fig. 2a-c; data not shown), whereas *rtv* mutant roots also expressed ProDc3:GUS in the root vasculature in addition to meristems (Fig. 2d). As previously reported (Rock 2000; Subramanian et al. 2002), wild-type Ler roots showed moderate induction of ProDc3:GUS activity in the root trichoblasts (root hair cells), in the cortex of the proximal (relative to the root primary meristem) zone of differentiation, in the mature vasculature, and in a diffuse band in the zone of elongation in response to 100- μM ABA treatment (Fig. 2e). In the zone of elongation, distinct files of epidermal cells that had strong GUS staining could be discerned under higher magnification (Fig. 2e inset). As previously described (Subramanian et al. 2002), root growth was affected by ABA treatment, with root hairs showing a short and bulbous phenotype (Fig. 2e; data not shown). Swelling was observed in the zones of elongation and differentiation due to inhibition of root growth (Himmelbach et al. 1998; Schnall and Quatrano 1992). Interestingly, in *aux1* mutant roots treated with 100 μM ABA, ProDc3:GUS activity was imperceptible in the trichoblasts and cortex of zones of elongation and differentiation, with induction only observed in the mature vasculature (Fig. 2f). Similarly, the *axr4* mutant showed a hypomorphic effect of ABA treatment on ProDc3:GUS expression compared to wild type, although the hypomorphic effect on ProDc3:GUS was not as strong as observed with *aux1*, with some ProDc3:GUS activity in cortex cells of *axr4* roots and stronger vascular expression (compare Fig. 2e-g). The *rtv* mutants had a stunted root-growth phenotype and an abundance of lateral roots and trichoblasts and showed strong ProDc3:GUS induction by ABA throughout the roots (Fig. 2h). Our results with the *aux1*, *axr4* and *rtv* mutants are consistent with those of Oono et al. (1998), who studied the effects of these mutations on the

auxin-inducible expression of the 183 bp auxin-responsive region of the *PS-IAA4/5* promoter. Heterozygous *rtγ*⁺ seedlings showed wild-type ProDc2:GUS and root-hair phenotypes in response to ABA (data not shown), which can be taken as evidence that the observed decreases in ProDc3:GUS activity in the various mutants was not because of mixed genetic backgrounds due to segregation of the two ProDc3:GUS insertions in the wild-type Ler control stock crossed into the homozygous mutant lines.

The tissue-specific patterns of ProDc3:GUS expression in wild-type Ler were distinctive for each auxin

tested, with the relative intensities of ProDc3:GUS staining in response to equimolar (1 or 10 μM) amounts of auxins being 2, 4-D ~ NAA > IAA (compare Fig. 2i, q, u; data not shown). There was a positive dose-response of ProDc3:GUS expression for each auxin tested. A root-growth phenotype of swelling in the zone of elongation was observed in all auxin treatments (e.g. compare Fig. 2a with Fig. 2i, m; data not shown). In response to 24 h 1 μM IAA treatment, ProDc3:GUS expression in wild-type Ler was observed in the cortex of the proximal zone of differentiation and as two bands of expression in the middle and distal regions of the zone

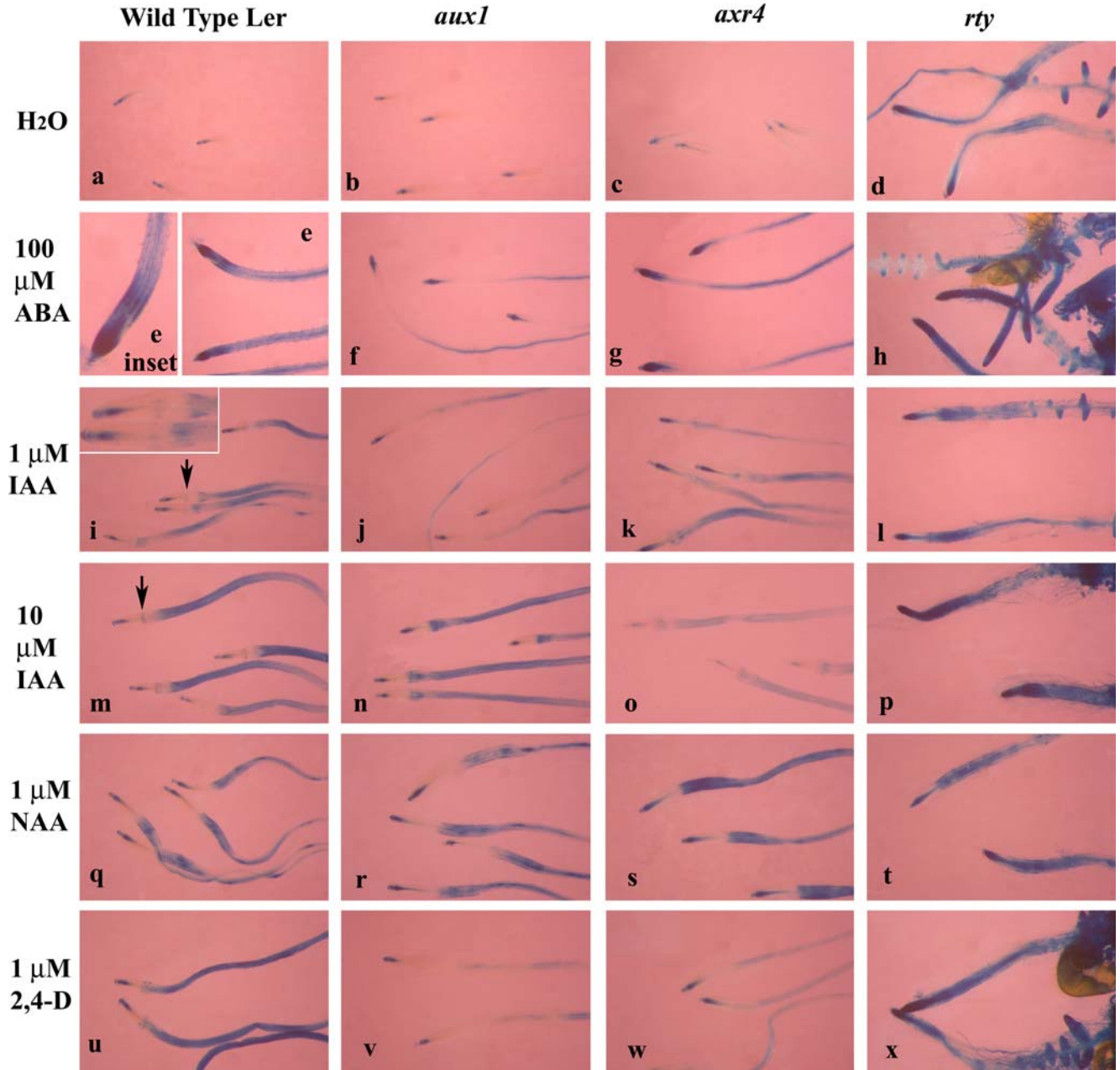


Fig. 2 Histochemical staining with X-Gluc of ProDc3:GUS activity in 6-day-old wild-type Ler and homozygous auxin mutants *aux1*, *axr4*, and *rtγ* treated with ABA or natural and synthetic auxins. All images are magnified 12×, except panels for *rooty* mutant magnified 24×. The arrows in panels **i** and **m** correspond to the middle band of GUS in the zone of elongation

of elongation (Fig. 2i arrow; see inset); the distal band was somewhat obscured in the 10 μ M IAA treatment by increased ProDc3:GUS expression in the overlapping cortex of the proximal zone of differentiation (Fig. 2m). In response to 1 μ M NAA, wild-type Ler roots expressed ProDc3:GUS in the proximal zone of differentiation, which also had a swelling phenotype (Fig. 2q). The pattern of ProDc3:GUS staining was distinctive, with diffuse and variable staining punctuated with dark patches of GUS activity associated with the protovascularature and correlated with increased frequencies of lateral root primordia (Fig. 2q; data not shown); this patterning was not observed in 10 μ M NAA treatments, which showed uniform staining throughout the cortex and a strong band of staining in the distal zone of elongation (data not shown). In response to 2, 4-D treatments, wild-type Ler showed a similar pattern to the IAA treatments (Fig. 2u) except there was stronger staining throughout the cortex of the zone of differentiation and at 10 μ M the stronger staining in the zone of elongation appeared to obscure the bands (data not shown). No apparent differences in tissue-specific patterns of ProDc3:GUS expression in response to auxins were observed between wild-type Ler and *abi2-1* mutant roots (data not shown).

As expected, we observed ProDc3:GUS expression and growth phenotypes in roots of the auxin insensitivity mutants *aux1* and *axr4* and the auxin homeostasis mutant *rtv* in response to exogenous treatment with the natural IAA and synthetic auxin and 2, 4-D (Fig. 2i–p, u–x), which require auxin uptake carriers for activity (Delbarre et al. 1996), but not in response to treatments with the diffusible auxin NAA (Fig. 2q–t). Specifically, *aux1* and *axr4* mutant roots showed reduced swelling in the zone of elongation and pronounced reductions in inducible ProDc3:GUS expression in response to 1 μ M IAA and 2, 4-D (compare Fig. 2i and j; u and v; i and k; u and w), and a slight reduction of cortex-specific expression and interestingly, an increase in ProDc3:GUS expression in a diffuse band in the distal zone of elongation in response to 10 μ M IAA and 2, 4-D (compare Fig. 2m and n; data not shown). The hypomorphic effects on ProDc3:GUS expression were more pronounced in the *aux1* mutant compared to *axr4* (e.g. compare Fig. 2j and k; v and w). *Rtv* mutant responses to IAA and 2, 4-D appeared normal in that ProDc3:GUS expression was additive compared to the water-alone control treatments (compare Fig. 2d, i, l; d, u and x), as expected if *rtv* mutants contain higher (but non-saturating) concentrations of endogenous auxin.

Discussion

Genetic and genomic analyses in the model plant *Arabidopsis* have uncovered genetic redundancy in ABA and auxin signaling. An auxin mutant, *axr2*, is insensitive to ABA and encodes IAA-7 (Nagpal et al. 2000), a one of \sim 30 homologous transcription factors (including

AXR3) that undergo rapid turnover by ubiquitin-mediated degradation and act by binding with auxin-responsive factors (ARFs), members of the family of 34 B3 domain transcription factors whose cognate is ABA-INSENSITIVE-3/VIVIPAROUS1 (Hagen et al. 2004; Tiwari et al. 2003). A dominant-gain-of-function allele of IAA28 suppresses lateral root development (Rogg et al. 2001), whereas overexpression of the ABI5-like basic leucine zipper transcription factor ABF2 promotes lateral root development under salt stress (Kim et al. 2004), supporting the link between auxins and ABA signaling in root development. Although AXR2 is a good candidate for an ABA-IAA crosstalk factor, the use of upstream-acting mutants *abi2* and *aux1/axr4* in this study rather than downstream transcription factor mutants like *abi3* and *axr2* may have revealed crosstalk between the ABA and IAA pathways leading to ProDc3:GUS expression because specific transcription factors that are members of large gene families may not interact with the *Dc3* promoter nor act upstream in a hierarchy that includes *Dc3* as a target.

It has recently been shown that ABI3/VIVIPAROUS1 modulates lateral root formation and some auxin-inducible gene-expression phenotypes in *Arabidopsis* (Brady et al. 2003; Suzuki et al. 2001). The *hyl* mutant is ABA hypersensitive and auxin insensitive, and HYL1 encodes a double-stranded RNA-binding nuclear protein that mediates metabolism of microRNAs known to target ARFs (Han et al. 2004; Park et al. 2002; Rhoades et al. 2002; Vazquez et al. 2004). Taken together with the recent findings that some microRNA levels are modulated by ABA and stress (Sunkar and Zhu 2004), it is speculated that microRNAs, which are recently discovered regulators of development in plants and animals (Bartel and Bartel 2003; Carrington and Ambrose 2003), may be a mechanistic bridge between hormone signaling pathways. We are currently testing this hypothesis by physiological studies of ABA and auxin sensitivities of microRNA effector mutants.

We have previously characterized some tissue-specific phenotypes of ProDc3:GUS expression in *Arabidopsis* as genetic markers for characterization and dissection of the interaction and crosstalk between ABA-, auxin, and drought-stress signaling pathways in vegetative tissues (Chak et al. 2000; Rock 2000; Subramanian et al. 2002). Our quantitative ProDc3:GUS expression results in the *abi1-1* and *abi2-1* mutants shown in Fig. 1, and our observation (unpublished) that *abi1* and *abi2* mutants germinate and grow better than wild type on auxin-containing media, provide additional evidence for the crosstalk between ABA and auxin signaling. The lack of qualitative tissue-specific ProDc3:GUS expression phenotypes in *abi2-1* mutants treated with auxins suggests that the interaction between ABA and auxin signaling pathways in roots reported here is limited. Consistent with this conclusion is our observation that *abi1* and *abi2-1* mutants exhibit normal gravitropism, unlike the *aux1* and *axr4* mutants.

Our quantitative ProDc3:GUS results (Fig. 1) do not take into account the possibility of “separate but overlapping” tissue-specific responses to ABA versus auxins, a phenomenon seen with ABA- and osmoticum (simulating drought)-inducible ProDc3:GUS expression in roots (Rock 2000). Tissue-specific differences of ProDc3:GUS expression (lateral root primordia, variable bands of expression in root zone of elongation, intensities of responses to equimolar amounts of auxins; ABA but not auxin induction in vasculature) and similarities (all hormone treatments induce expression in cortical cells) suggests some limited crosstalk between ABA and auxin pathways. It is known that AXR1 functions in a signaling pathway that is independent of either AUX1 or AXR4 (Hobbie and Estelle 1995; Timpte et al. 1995). The basis for the differences in phenotypes between types and concentrations of auxins is not known, but these observations point out the importance of performing experiments with physiological concentrations of exogenous hormone.

In the case of natural (IAA) versus synthetic auxins which enter the cell by diffusion (NAA) or carrier-mediated uptake (2, 4-D), the observation that all three auxin treatments gave similar (but distinct) tissue-specific phenotypes is evidence for at least some ProDc3:GUS phenotypes being mediated by the same auxin pathway(s). A similar conclusion can be drawn from the additive effects of exogenous auxins to the ProDc3:GUS expression levels in the auxin-overproducing mutant *rtv* compared to wild type (Fig. 2) and from the observation that auxin insensitivity phenotypes were not seen in the *aux1* and *axr4* mutants treated with NAA, a diffusible auxin (Fig. 2). This result is consistent with previous results showing that the gravitropic phenotype of *aux1* and *axr4* mutant roots could be rescued by adding to the medium low concentrations of NAA, but not by adding 2, 4-dichlorophenoxyacetic acid (2, 4-D) or IAA (Marchant et al. 1999; Simmons et al. 1995; Yamamoto and Yamamoto 1999). Other naturally occurring auxins such as indole-3-butyric acid (IBA) and phenyl acetic acid were not tested for their effects on ProDc3:GUS. There is genetic evidence that IBA functions in a different signaling pathway from IAA (Poupart and Waddell 2000) and therefore the results presented here are indicative of other potential tissue-specific or hormone-specific interactions between ABA and auxins.

Mutant analysis is a powerful means to test for crosstalk between signaling pathways. Our quantitative ProDc3:GUS results with the *abi1-1* and *abi2-1* mutants in response to auxins (Fig. 1a) and unexpected qualitative and physiological results of the *aux1* and *axr4* mutants in response to ABA and the carrier uptake-mediated auxins IAA and 2, 4-D (Fig. 2) provide strong evidence for crosstalk between some tissue-specific ABA and auxin pathways leading to ProDc3:GUS expression in roots. Specifically, the ProDc3:GUS and physiological phenotypes of *aux1* and *axr4* compared to wild type in response to ABA look remarkably similar to the *abi2-1* phenotype, but not the *abi1-1* ABA phenotype

(Subramanian et al. 2002), with a hypomorphic effect seen on ProDc3:GUS expression in the mutant root zones elongation and differentiation and vasculature. The morphological and growth effects of 24-h ABA treatments, namely root hair bulging and root tip swelling (Fig. 2a vs. e) could not be readily seen in *aux1* and *axr4* mutants treated with ABA. Taken together with the observation that ABI1 affects auxin response, but not the ABA response of ProDc3:GUS in roots (Fig. 1a) (Subramanian et al. 2002), these data suggest that the crosstalk between auxin and ABA signaling in roots is limited to a subset of responses and effectors, with *AUX1* and *AXR4* not affecting ABA growth physiology and *ABI1* not affecting ProDc3:GUS expression. Consistent with our conclusion of ABA and IAA interactions in roots that differentially affect physiological processes are the results of Takahashi et al. (2002), who showed that *abi2-1* mutants have reduced root hydrotropism, whereas agravitropic *axr1* and *axr2* roots paradoxically have enhanced hydrotropism.

It was reported that *aux1* is resistant to ethylene effects on root elongation inhibition (Pickett et al. 1990), while *axr4* is resistant to IAA and 2, 4-D but has a similar response to wild type to ABA inhibition of root elongation (Hobbie and Estelle 1995). The apparent discrepancy between our results and those of Hobbie and Estelle (1995) with regard to ABA effects on root growth of *axr4* may be due to differences in the assays—the ProDc3:GUS induction and swelling phenomena occur within hours, whereas the growth inhibition by ABA occurs over a period of days. The *axr4* mutant was isolated based on its resistance to 2, 4-D and is more insensitive to 2, 4-D than IAA (our unpublished observations); this phenotype is seen in our results with ProDc3:GUS expression in the *axr4* mutant being relatively more induced by IAA than 2, 4-D (compare Fig. 2u and w vs. i and k). The hypomorphic *aux1* and *axr4* ProDc3:GUS expression phenotypes in response to IAA and 2, 4-D are consistent with the results of Oono et al. (1998), who observed hypomorphic effects of the *aux1*, *axr1*, and *axr4* mutants on IAA induction of the *PS-IAA4/5* promoter.

The observation of elevated ProDc3:GUS expression in the *rtv1/sur1/hls3* mutant, which accumulates high levels of auxin (Boerjan et al. 1995), is evidence that ProDc3:GUS expression can mark locally elevated concentrations of endogenous auxin, as has been previously demonstrated for the GH3 promoter of tobacco (Li et al. 1999). *RTY1/SUR1/HLS3* encodes an aminotransferase-like protein, but its mechanism of action in auxin homeostasis is still not clear (Boerjan et al. 1995; Gopalraj et al. 1996; King et al. 1995). It is possible that the punctuate ProDc3:GUS expression observed in lateral root primordia and primary meristems (Fig. 2) is due to elevated auxin levels. However, the ABI3 gene is also expressed in lateral root primordia (Brady et al. 2003) and therefore may regulate GUS expression in that tissue via ABA-responsive elements found in the *Dc3* promoter (Thomas et al. 1997). Recently, it has

been shown that ABA production sites may be determined by the cellular localization of an ABA biosynthetic gene promoter ProAAO3:GFP fusion, which is expressed in the root tips, vascular bundles of roots, hypocotyls and inflorescence stems, and along the leaf veins (Koiwai et al. 2004). These are many of the same tissues to which ProDc3:GUS expression has been localized in response to osmotic stress (Chak et al. 2000; Rock 2000), therefore ProDc3:GUS may mark locally elevated levels of ABA. Characterization of double and triple homozygous ABA/auxin/ethylene mutants for patterns of ProDc3:GUS expression or effector gene cross-regulation could be informative in mapping the crosstalk between these pathways and elucidating mechanisms of hormone homeostasis and action in tissue-specific gene expression, growth, and development.

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