AXR3 and *SHY2* interact to regulate root hair development

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Accepted 10 June 2003

Development 130, 5769-5777

doi:10.1242/dev.00659

Summary

Signal transduction of the plant hormone auxin centres on the regulation of the abundance of members of the Aux/IAA family of transcriptional regulators, of which there are 29 in *Arabidopsis*. Auxin can influence Aux/IAA abundance by promoting the transcription of *Aux/IAA* genes and by reducing the half-life of Aux/IAA proteins. Stabilising mutations, which render Aux/IAA proteins resistant to auxin-mediated degradation, confer a wide range of phenotypes consistent with disruptions in auxin response. Interestingly, similar mutations in different family members can confer opposite phenotypic effects. To understand the molecular basis for this functional specificity in the Aux/IAA family, we have studied a pair of Aux/IAAs, which have contrasting roles in root hair development. We have found that stabilising mutations in *AXR3/IAA17* blocks root hair initiation and elongation, whereas similar mutations in *SHY2/IAA3* result in early initiation of root hair development and prolonged hair elongation, giving longer root hairs. The phenotypes resulting from double mutant combinations, the transient induction of expression of the proteins, and the pattern of transcription of the cognate genes suggest that root hair initiation is controlled by the relative abundance of SHY2 and AXR3 in a cell. These results suggest a general model for auxin signalling in which the modulation of the relative abundance of different Aux/IAA proteins can determine which down-stream responses are induced.

Key words: Auxin, Aux/IAAs, Root hairs, *Arabidopsis thaliana*

Introduction

The plant hormone auxin (indole-acetic acid) is a simple molecule, yet it is involved in regulating a wide range of developmental processes in plants, as diverse as root tip patterning, lateral branch growth, vascular differentiation and root hair elongation (Went and Thimann, 1937; Theologis, 1986; Pitts et al., 1998). The complexity of auxin action is reflected in the diverse responses of plants and plant tissues to exogenous auxin addition. Auxin dose-response profiles can be complex, and different tissues can respond in completely different ways. A good example of this is in the induction of gene transcription. Auxin induces the transcription of several classes of genes as a rapid, primary response (Guilfoyle, 1986; Theologis, 1986). One of the best characterised families is the *Aux/IAA* family. In *Arabidopsis* there are 29 *Aux/IAA* genes, which show different dose-response profiles, induction kinetics and tissue specificities (Abel and Theologis, 1995; Liscum and Reed, 2002). This means that for any particular tissue, the response to a particular auxin dose is the activation of a particular sub-set of *Aux/IAA* genes with a particular timing. There is increasing evidence that *Aux/IAA* genes mediate downstream responses to auxin and that they regulate their own transcription. It is possible therefore, that the complex responses of *Aux/IAA* genes to auxin not only reflects the complexity of auxin action but also goes some way toward explaining it.

Aux/IAA gene function

Aux/IAAs encode low abundance, nuclear proteins with extremely short half-lives, some as short as 5 minutes (Abel et al., 1994; Ouellet et al., 2001). The stability of Aux/IAAs is further reduced by auxin treatment (Ramos et al., 2001; Gray et al., 2001). Aux/IAAs are characterised by a highly conserved four domain structure. Domain II contains the destabilisation signal, a 13 amino acid destruction box, necessary and sufficient for the characteristic auxin-regulated instability of the Aux/IAAs (Ramos et al., 2001). Via this domain, Aux/IAAs interact with the ubiquitin ligase SCF^{TIR1} and this interaction is promoted by auxin and results in 26S proteasome-mediated degradation (Gray et al., 2001).

Aux/IAA domains III and IV are required for the formation of both homo- and heterodimers with other Aux/IAAs and with a family of DNA binding proteins, the auxin response factors (ARFs) (Kim et al., 1997; Ulmasov et al., 1997), of which there are 23 in *Arabidopsis*.

ARFs bind to the auxin response elements (AREs) in the promoters of auxin-regulated genes through an N-terminal DNA binding domain. ARFs are required to mediate auxin-regulated transcription from ARE-containing promoters. At their C termini, most ARFs have domains homologous to Aux/IAA domains III and IV, through which they can homo- and heterodimerise within the ARF family, and heterodimerise with Aux/IAAs (Kim et al., 1997; Ulmasov et al., 1997). A sub-set of ARFs act as dimers to promote transcription of auxin responsive genes (Ulmasov et al., 1997; Ulmasov et al., 1999a). However, dimerisation of Aux/IAAs with ARFs appears to block this transcriptional activation (Ulmasov et al., 1999b). Auxin promotes the degradation of Aux/IAAs, and therefore

presumably favours the formation of ARF/ARF dimers, activating transcription of auxin responsive genes. Since the promoters of *Aux/IAA* genes themselves contain AREs, it is predicted that an increase in auxin levels initially reduces Aux/IAA levels by promoting their degradation, but subsequently replenishes Aux/IAA pools by promoting transcription from *Aux/IAA* genes (Kepinski and Leyser, 2002).

This model for auxin action places Aux/IAAs at the centre of an auxin signalling network. In *Arabidopsis*, there are 29 Aux/IAAs, with diverse tissue specificities and auxin response characteristics, with the potential to interact with 23 ARFs, and also with diverse tissue specificities and promoter binding affinities. Therefore, the wide range of auxin responses shown by plants could be encoded in the relative abundance of the different members of this complex network.

AXR3 and SHY2

Support for the central role of Aux/IAAs in mediating diverse auxin responses comes from analysis of the phenotypes conferred by mutations in *Aux/IAA* genes (Liscum and Reed, 2002). Loss-of-function mutations cause relatively mild phenotypes, presumably as a result of redundancy. Phenotypes that are more dramatic result from dominant or semi-dominant mutations, and this has led to the isolation of many such *Aux/IAA* mutants. So far, these mutations have all mapped to the domain II destruction box and result in reduced interactions with SCF^{TIR1}, and hence increased, and auxin resistant, protein stability (Gray et al., 2001). These stabilising mutations in specific *Aux/IAA* family members confer specific phenotypes. For example, the *AXR3/IAA17* gene was originally defined by two semi-dominant stabilising point mutations in domain II (Leyser et al., 1996; Rouse et al., 1998). The two alleles, *axr3-1* and *axr3-3* confer severe phenotypes, largely consistent with an over-response to auxin (Leyser et al., 1996), including short, highly agravitropic roots, with an increased number of adventitious roots and a greatly reduced number of root hairs. In contrast, similar domain II mutations in *SHY2/IAA3*, originally identified as suppressors of the long hypocotyl phenotype of the phytochrome deficient *phyB* mutants (Kim et al., 1996; Reed et al., 1998), result in long straight roots with reduced adventitious rooting (Tian and Reed, 1999) and increased root hair density; opposite to the root phenotype conferred by *axr3*.

These opposite phenotypes conferred by similar stabilising mutations in *SHY2* and *AXR3* provide an opportunity to understand better how Aux/IAAs might mediate particular auxin responses. We have chosen to focus on the root hair phenotypes of these mutants because of the wealth of information available on *Arabidopsis* root hair development.

Root hairs are long tubular outgrowths from the surface of specialised epidermal cells. By greatly increasing the surface area, they are important for nutrients and water uptake and for anchorage (Peterson and Farquhar, 1996). In *Arabidopsis*, the root epidermis is made up of longitudinal cell files, which develop in a distinct pattern (Dolan et al., 1994; Galway et al., 1994). The development of the cell files begins with transverse divisions of initial cells in the root meristem (Schneider et al., 1997). Divisions continue immediately behind the initials in the division zone. Following the cessation of cell division, the cells continue to elongate, in the elongation zone, after which they differentiate into either trichoblasts (root hair cells) or atrichoblasts (hair-less cells) (Grierson et al., 1997). Trichoblasts are always located over the junction between two underlying cortical cells, resulting in a pattern of alternating files of trichoblasts and actrichoblasts around the root. Trichoblasts can be distinguished from atrichoblasts as early as the latter stages of embryogenesis, because of their increased cytoplasmic density (Dolan et al., 1994; Galway et al., 1994) an increased rate of cell division (Berger et al., 1998) and cell surface deposits (Dolan et al., 1994).

Root hair outgrowth itself can be split into three developmental stages: bulge formation, initiation and tip-growth (Grierson et al., 1997). Tip growth is a rapid form of directional elongation, which involves precise targeting of vesicles carrying cell wall precursors to the growing tip (Benfey and Schiefelbein, 1994; Grierson et al., 1997).

In this work, we describe the root hair phenotypes of both gain- and loss-of-function mutants of *AXR3* and *SHY2* and provide evidence for a dose-dependent interaction between AXR3 and SHY2 in regulating the timing of root hair differentiation.

Materials and methods

Plant materials

axr3-1, HS::axr3-1 and *HS::shy2-6* are all in the Columbia ecotype. *shy2-2, axr3-10* and *shy2-31* are all in the Landsberg *erecta* (L*er*) ecotype. *axr3-10* is a *Dissociation* insertion line, originally designated GT 3958 (Parinov et al., 1999). The transposable element is inserted 130 bp downstream of the start codon, between domains I and II (Blilou et al., 2002). *shy2-31* has a point mutation which introduces a stop codon early in exon 1 (Jason Reed, personal communication). *axr3-10* and *shy2-31* were kind gifts from Jason Reed (University of North Carolina at Chapel Hill, USA).

Plant growth conditions

Seeds were surface sterilised with 10% bleach and 0.1% Triton X-100 for 15 minutes, then rinsed once in 70% ethanol and four times in sterile distilled water. Sterile seeds imbibed at 4°C for 2 days prior to planting in Petri dishes containing 20 ml of *Arabidopsis thaliana* salts (ATS) growth medium, as previously described (Wilson et al., 1990). The ATS was solidified with 0.8% Phytagel (Sigma). Plates were orientated vertically in controlled condition growth rooms at 23°C with 16 hours light or in a growth cabinet at 20°C for the heat shock (HS) experiments.

Phenotypic analysis

All images for measurements were taken at 5 days post-germination with a JVC TK-1070E video camera attached to a Nikon SMZ 10A stereo dissecting microscope, apart from those of epidermal cells, which were captured with the camera using a Nikon Optiphot-2 microscope. Images were measured using LUCIA G software (version 3.52a, 1991). At least 50 measurements were taken from at least 10 plants for each parameter. For root hair number, only hairs visible within a 1 mm segment, viewed from above, were counted. For epidermal cell lengths a combination of atrichoblasts and trichoblasts were measured. For root hair lengths, only hairs from mature sections of roots were measured.

For time-lapse analysis, pictures were taken automatically every 10 minutes of a 5-day old root growing on an ATS/Phytagel plate under the dissecting microscope. The images were measured on completion and were taken from three different plants for each genotype.

In situ pictures were taken using a Nikon FX 35DX camera fixed to the Nikon Optiphot-2 microscope using dark-field optics.

Transgenic plants

The transgenic plant line *HS:axr3-1* was created using the cDNA from EST H36782, obtained from the *Arabidopsis* Biological Resource Centre, Ohio. The *axr3-1* point mutation (Rouse et al., 1998) was introduced into the 941 base pair (bp) sequence by site-directed mutagenesis using the Stratagene QuikChange™ kit according to the manufacturer's instructions. Similarly, the *HS:shy2-6* line was created using the cDNA from EST TO4296, obtained from the *Arabidopsis* Biological Resource Centre, Ohio, and the corresponding point mutation to that of *axr3-1* was introduced to the 978 bp sequence in the same way. Each cDNA was cloned into a pJR1Ri vector, in the sense orientation, using the *Xba*I/*Sma*I site, downstream of a 0.4 kb soybean heat shock promoter (Schoffl et al., 1989). The vectors were then transformed into *Agrobacterium tumefaciens* strain *GV3101* (Koncz and Schell, 1986) by freeze-thaw (Höfgen and Willmitzer, 1988) and then into wild-type *Arabidopsis* plants of the Columbia ecotype using the floral dip method (Clough and Bent, 1998). Transformants were selected by kanamycin resistance and were then planted into soil and allowed to self-fertilise. In the T_2 generation, lines showing a 3:1 ratio of kanamycin resistant to sensitive plants, indicative of a single site of transgene integration, were selected for further study. Homozygous lines were selected from the T_3 generation. Preliminary experiments indicated that multiple independent lines for each transgene behaved in a similar way in response to heat shock, so for each construct a single representative line was selected for further work.

Transient activation of gene expression by heat shock

The positions of root tips were marked on the back of Petri dishes and these were placed in a 37°C incubator for 2 hours. The root tip positions were marked again at 4, 8, 12 and 24 hours following heat induction. The length of root hairs was measured at each of these marks, in each of the genotypes.

Whole-mount in situ hybridisation

Probes

For *AXR3* probes, a 133 bp region was amplified from cDNA, in a region between domains I and II using the forward primer 5′-CGGAAGAACGTGATGGTTTCA-3′ and reverse primer 5′-CGT-AGCTTTTATACATCCTC-3′. For *SHY2/IAA3*, a 240 bp region was amplified from the 3′UTR by PCR, using the forward primer 5′-CTCTGTCTGTGCTTGGGTTG-3′ and the reverse primer 5′-CTC-TTCAATCTTCATAACAC-3′. Both products were then cloned into PCR4-TOPO vector (Invitrogen) by TA-cloning. M13 forward and reverse primers were then used to amplify across the probe region including promoter sites for T3 and T7 polymerase, and the product was purified. Both sense and antisense RNA probes were made from the same PCR product, in separate reactions, using the digoxigenin (DIG) RNA labelling kit (Roche) according to the manufacturer's instructions, except the reaction was scaled up fivefold.

Fixing and hybridisation

Throughout the fixing and antibody stages, the seedlings were contained in cell strainers (Falcon), which minimised tissue damage when transferring from one solution to the next (de Almeida Engler et al., 1994).

Four-day-old seedlings were fixed in 4% formaldehyde/0.1% Triton X-100/0.1% Tween 20 by vacuum infiltration for 15 minutes and then overnight at 4°C. The seedlings were then dehydrated through an ethanol series from 50% to 100% over 3 days, at 4°C. They were then taken back down the ethanol series to 30%, prior to being treated with acetone and then acetic anhydride solution (0.1 M triethanolamine/0.5% (v/v) acetic anhydride). Between the treatments, the seedlings were rinsed with phosphate-buffered saline (PBS) for 30 minutes.

The seedlings were then washed in PBS before being placed in a probe and hybridisation buffer mix in Eppendorf tubes. They were then allowed to hybridise overnight at 50°C. The seedlings were transferred back to cell strainers in six-well plates and three post-hybridisation washes in 2× SSC/50% formamide were carried out, at 50°C, for 1.5 hours. The seedlings were then washed twice in NTE (0.5 M NaCl/10 mM Tris pH 7.5/1 mM EDTA) at 37°C for 15 minutes each time. This was followed by seedling incubation in NTE with 20 μg/ml RNaseA, also at 37°C, for 45 minutes. The seedlings were then washed twice with NTE, for 15 minutes each time and then incubated in SSC/50% formamide for 2 hours at 50°C. Then one wash with SSC at 23°C, was carried out for 1 hour, followed by two rinses with PBS (for 15 minutes each) at room temperature. The seedlings were then stored overnight at 4°C. They were prepared for the antibody and detection stages by washing in a salt buffer (0.1 M Tris/0.15 M NaCl) solution for 10 minutes. They were then incubated in a solution of 0.5% Blocking Reagent (Roche) in salt buffer for 1 hour, followed by washing with salt buffer, containing 1% BSA and 0.3% Triton X-100 for 1 hour. The seedlings were then incubated with a 1:2000 dilution of the anti-digoxigenin antibody (Roche) for 1 hour before being washed six times with salt buffer/BSA/Triton X-100 for 15 minutes each wash. A final wash in plain salt buffer was carried out for 30 minutes before the sieves were removed and the seedlings were incubated in the six-well plates with the developer, Western Blue (Promega). The development reaction was stopped by placing the seedlings in PBS, as soon as background signal could be seen in the sense controls.

Results

AXR3 and SHY2 have opposite root hair phenotypes

In order to determine the effects of mutation in *AXR3* and *SHY2* on root hair formation, both gain-of-function and loss-of-function mutants of *AXR3* and *SHY2* were examined with

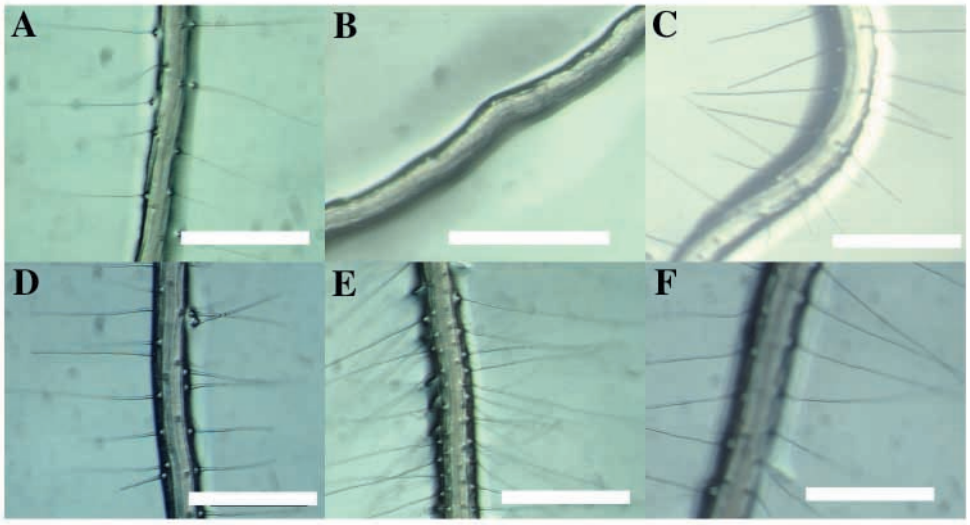

Fig. 1. Root hair phenotypes of 5-day old seedlings: (A) Columbia ecotype, (B) *axr3-1*, (C) *axr3-10*, (D) Landsberg erecta (L*er*) ecotype, (E) *shy2-2* and (F) *shy2-31*. Scale bar: 0.5 mm.

regard to their root hair phenotype. Plants homozygous for the strong gain-of-function alleles *axr3-1* (Col background) and *shy2-2* (L*er* background) and plants homozygous for the likely null alleles *shy2-31* (L*er* background) and *axr3-10* (L*er* background; a transposon insertional mutant), were used for this work. Crude inspection showed that *axr3-1* plants have essentially no root hairs, *shy2-2* roots appear more hairy than wild-type and the loss-of-function mutants have no striking root hair phenotypes (Fig. 1). To quantify these differences we measured root hair number per mm root, epidermal cell length and root hair length in the mutants and their wild-type counterparts (Fig. 2). Excluding a few initiation bumps (approximately seven per 5-day-old root), *axr3-1* roots were found to have only 0.04±0.009 root hairs per unit cell length (Fig. 2C). From its general appearance, *shy2-2* has a hairier root (Fig. 1). Certainly, when the number of root hairs per mm was measured, *shy2-2* was found to have one third more root hairs than the wild type (L*er*) (Fig. 2A). However, *shy2-2* has shorter epidermal cells than L*er* (Fig. 2B), so that when the root hair density was corrected for epidermal cell length, *shy2-2* has a similar number of hairs (3.44±0.14 per cell length) to L*er,* (3.69±0.12 per cell length) (Fig. 2C). Homozygous *axr3-10* plants also had a wild-type number of root hairs (3.81±0.13 per cell length). In contrast, *shy2-31* plants had fewer root hairs per cell length, 2.51±0.1 than wild type, 3.69±0.12 (Fig. 2C).

A comparison of root hair length revealed further differences between the mutants. There were insufficient root hairs on *axr3-1* roots for meaningful measurements, but the root hairs of *shy2-2* plants were found to be one-third longer than wild-type hairs, contributing to the hairy appearance of *shy2-2* roots (Fig. 2D). Both loss-of-function mutants, *shy2-31* and *axr3-10,* had slightly shorter root hairs than wild type with *shy2-31* hairs being the shortest (Fig. 2D). This phenotype is also less reproducible in *axr3-10* plants than in *shy2-31* plants (Fig. 2E).

Since root hair length is known to be regulated by environmental conditions, we tested the ability of the mutants to respond to the root hair growth promoting effects of low phosphate. As previously reported (Bates and Lynch, 1996) removal of phosphate from the medium stimulates elongation of wild-type root hairs, resulting in an 125% increase over hairs growing on 2.5 mM phosphate (Fig. 2E). This effect was even more pronounced in the *axr3-10* root hairs, with hairs achieving significantly longer final lengths than in the wild type, representing an 155% increase. In contrast the root hairs of *shy2-31* plants responded less strongly than those of wild type, increasing by only 84%. Interestingly both gain-of-function mutants were impaired in their ability to respond. The roots of *axr3-1* plants remained completely bald even on medium with no added phosphate (data not shown), while the root hairs of *shy2-2* plants were able to increase their length only 26% over their already long-hair base line.

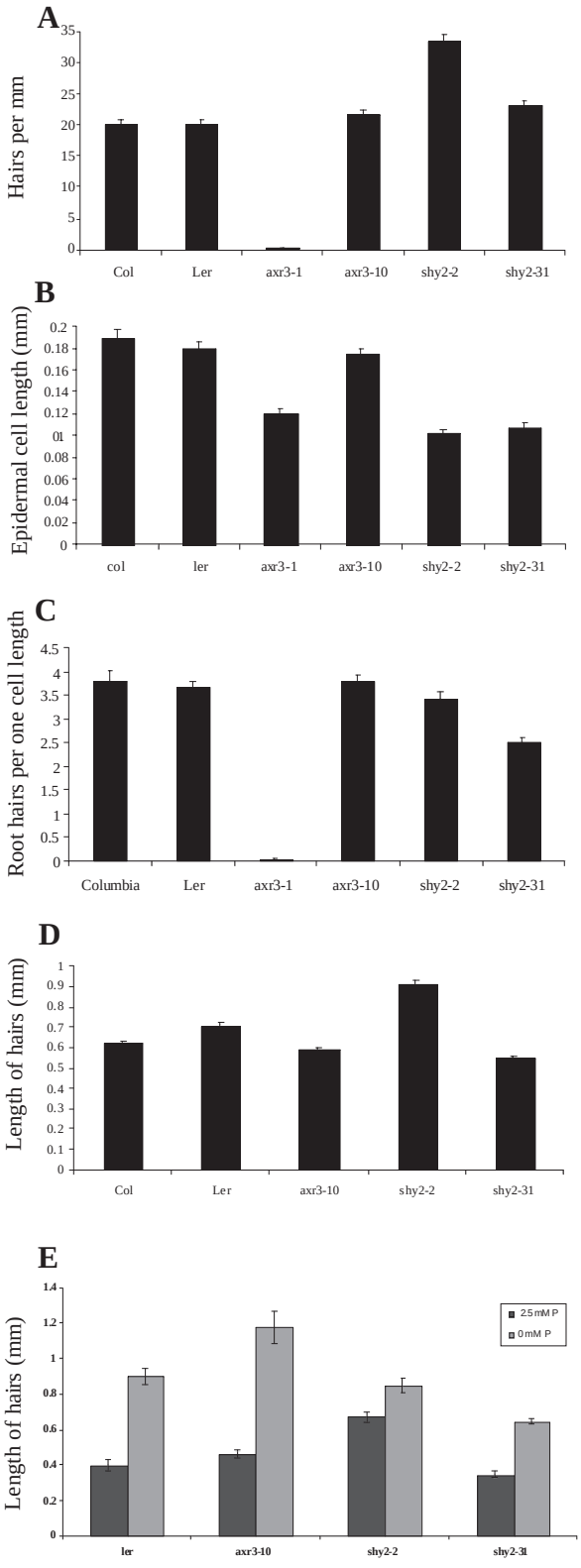

Fig. 2. Quantitative analysis of root hair phenotypes. (A) Mean number of root hairs per mm root. At least 50 measurements were taken for each genotype, visible hairs were counted in a 1mm section of mature root, and only those observed from above were counted. (B) Mean epidermal cell length (mm). One hundred cells, both atrichoblasts and trichoblasts, were measured for each genotype, from at least 10 different plants. (C) Number of root hairs per one cell length. The measurements for number of hairs per mm were multiplied by the corresponding epidermal cell length, to give the mean number of hairs per unit cell length. (D) Mean length of root hairs. Fifty hairs were measured, from at least 10 different plants, for each genotype. Only hairs in mature sections of the root were measured. (E) Mean length of root hairs grown on medium with no phosphate. Fifteen hairs were measured, from at least 3 plants. All measurements were made on 5-day-old plants. Bars represent the standard errors of the means.

The *shy2-2* mutation affects the timing of root hair initiation

To examine the timing and position of root hair differentiation in the mutants, we measured the distance from the root tip to the first root hair.

In *shy2-31* and *axr3-10* plants, the hairs initiate at the same distance from the tip as in wild type (L*er*) (Fig. 3). However, in *shy2-2* roots the hairs were found to initiate much closer to the root tip than in wild type (0.79±0.02 vs 1.45±0.05). This correlates with the observation that the number of cells in the elongation zone below the first hair-bearing cell was 7±0.35 in *shy2-2* seedlings compared with 10±0.61 in wild type.

To investigate the dynamics of s*hy2* mutant root hair initiation and growth more closely, time-lapse videos were taken to record the growth of the root hairs from initiation to full length. Tip growth rates were determined for each genotype from length measurements taken every 10 minutes over a 150 minute period. Tip-growth occurs once the bulge in the cell wall formed during the initiation stages of hair growth reaches approximately 0.04 mm. The results show that *shy2-2* hairs grow at the same rate as wild type (Fig. 4A), 0.23 μm-0.25μm per minute. Similarly, *shy2-31* root hairs have a wild-type mean growth rate, but with a greater variance, caused by the fact that *shy2-31* individual hairs do not grow at a constant rate (Fig. 4A, data not shown).

Interestingly, *shy2-2* hairs were found to start tip growth before the supporting epidermal cell had left the elongation zone, consistent with the observation that they initiate nearer the primary root tip than in the wild type. Wild-type and *shy2-31* trichoblasts increased in length by only 0.03 mm once the root hair had begun tip growth (Fig. 4B). In contrast, *shy2-2* trichoblasts continued to elongate by at least 1 mm after root hair initiation (Fig. 4B), although they never attained full wild-type length (Fig. 2B).

The early initiation of *shy2-2* root hair elongation is not matched by early cessation, so that *shy2-2* hairs grow for a longer time period, than wild type (Fig. 4C). Wild-type hairs complete tip-growth in an average of 4 hours, *shy2-2* hairs grow for 8 hours before reaching full length (Fig. 4C). Hence, the longer length of *shy2-2* root hairs and the reduced distance between the *shy2-2* root tip and first root hair can both be attributed to the ectopic initiation of root hair growth in the elongation zone.

Transient expression of *axr3-1* and *shy2-6*

To determine which stages of root hair growth are affected by the *axr3* and *shy2* gain-of-function mutants, the effect of transient expression of *shy2-6* and *axr3-1* was examined by inducing their transcription from the soybean heat shock (HS) promoter (Schoffl et al., 1989). Heat shock was carried out for 2 hours at 37°C. Following heat shock, the position of the growing tip of the root was marked at 4, 8, 12 and 24 hours. For each of these time points root hair length was measured. Transient expression of *HS:axr3-1* led to an immediate block in root hair formation, which persisted for up to 12 hours (Fig. 5, Fig. 6B). Hairs that were elongating at the time of heat shock stopped. In contrast, heat shock had no effect on wild-type root hair elongation (Fig. 6A), and induction of *shy2-6* expression gradually increased the length of the root hairs over the 24 hour period of the experiment (Fig. 5). An additional striking phenotype resulting from transient expression of *axr3-1* was transient agravitropism (Fig. 6B). Root hair length and

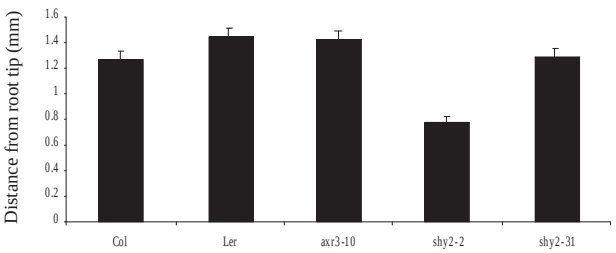

Fig. 3. Distance from the root tip to the first initiating root hair (mm). The values shown are the means for 50 5-day old plants of each genotype. Bars represent standard errors of the means.

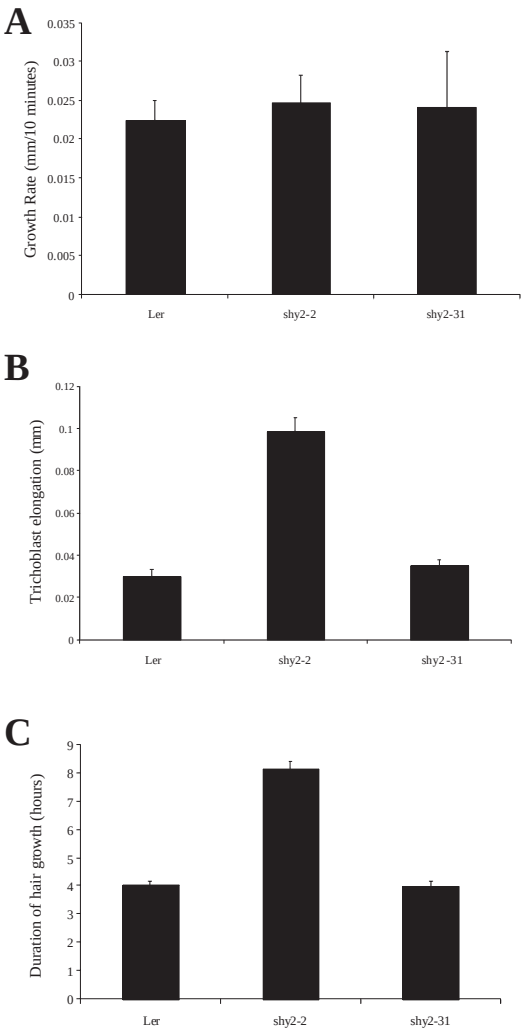

Fig. 4. *shy2-2* and *shy2-31* root hair outgrowth determined by time-lapse analysis. (A) Mean root hair growth rate calculated from images taken at 10-minute intervals after the transition to tip growth. Growth rate is therefore presented as mean increase in length per 10-minute interval. (B) Mean increase in trichoblast cell length following root hair initiation. (C) Time taken for a hair to reach final length from the initiation bump stage. The values shown are the means of at least 5 hairs for A and C and 10 trichoblasts for B. Bars represent standard errors of the means.

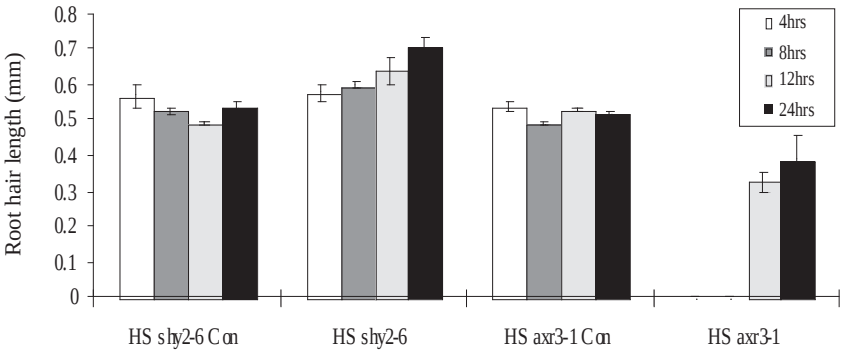

Fig. 5. Mean root hair length at four time points: 4, 8, 12 and 24 hours after the *shy2-6* or *axr3-1* transgene induction from the heat shock promoter (HS). The same transgenic lines without heat shock were used as controls (Con). The mean length of 10 root hairs from at least three plants, at each time point, is shown and bars represent the standard errors of the means.

morphology in the non-heat-shocked controls remained constant through the experiment (Fig. 5).

shy2-2 and *axr3-1* interact in a dose-dependent manner

SHY2 and *AXR3* are both located on the upper arm of chromosome 1, 5 kb apart. Therefore, making a double mutant between *axr3-1* and *shy2-2* would be extremely difficult.

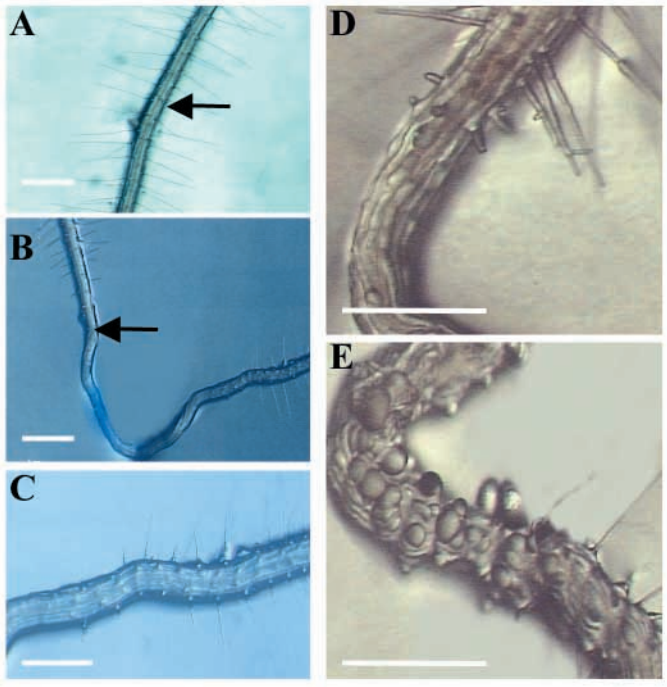

Fig. 6. Root phenotypes induced by transient expression of *axr3-1* or *shy2-6* transgenes from the heat shock promoter. (A) Heat shocked wild-type Columbia root, showing no effect of the heat shock (administered at the time when the part of the root indicated by the arrow was entering the root hair initiation zone). (B) *HS:axr3-1* root following a 2-hour HS (arrow), showing blocked hair growth and root agravitropism. (C) Close up of *HS:axr3-1* root treated as in B showing the transition back to hair growth: a few shorter hairs appear before normal hair growth resumes. (D) Transient induction of *axr3-1* transcription from the heat shock promoter in the *shy2-2* genetic background. Following induction, all hairs arrest at their current stage of development, and initiation is blocked. (E) Close up of *HS:axr3-1, shy2-2* root treated as in D showing the transition back to hair growth: an area of the root becomes gnarled and produces depolarised root hairs. Scale bar: 0.5 mm.

Transheterozygous plants were constructed and found to be indistinguishable from *axr3-1* plants with respect to their root hair phenotypes (data not shown), but in order to analyse further the interactions between *shy2*-2 and *axr3-1*, *HS:shy2-6* was crossed into the *axr3-1* background and *HS:axr3-1* was crossed into a *shy2-2* background. Doubly homozygous F_3 lines were selected and seedlings from these were heat shocked to induce expression of the transgene. Heat shocked *HS:shy2-6, axr3-1* plants were indistinguishable from *axr3-1* plants. In contrast, following induction of *HS:axr3-1*, hair formation was blocked in the *shy2-2* background, in the same manner as in a wild-type background (Fig. 6D). However, in a wild-type background the return to normal hair formation is sharply defined (Fig. 6C), but in *HS:axr3-1, shy2-2*, before the return of hair growth, a dramatic phenotype was variably observed. The roots became very twisted and gnarled, root hair outgrowth became depolarised and the cells appeared as large bubble-like structures (Fig. 6E). This phenotype was variable in severity and is most reliably induced by carrying out repeated heat shocks interspersed by several hours of recovery in normal growth conditions. This may result in a specific ratio of levels of shy2-6 and axr3-1, and at a critical dose where axr3-1 levels are dropping against endogenous shy2-2, the aberrant root hair phenotype is seen. To test the idea that this novel phenotype depends on a low axr3-1 level against a high shy2-2 level, we generated plants heterozygous for both *HS:shy2-6* and *HS::axr3-1*. Heat shock of these plants was predicted to generate high axr3-1 and shy2-6 levels that drop together, so that low axr3-1 levels should only occur in a low shy2-6 background. When this experiment was carried out, heat shock resulted in an *axr3-1*-like bald root phenotype with a sharp boundary in the return to root hair growth. The apolar root hair phenotype was not observed (data not shown). This is consistent with the hypothesis that this phenotype results from a low axr3-1 level relative to shy2-2.

AXR3 and *SHY2* expression in the root tip

To discover where *AXR3* could act during root hair development, we carried out whole-mount in situ hybridisation to detect the location of the *AXR3* transcript. *AXR3* transcript was observed in a region extending from the root tip toward the differentiation zone, where expression dropped away sharply (Fig. 7A). In the sense control, no signal was seen in the root tip (Fig. 7B).

The pattern of expression of *SHY2* has previously been examined using a promoter-reporter fusion (Tian et al., 2002), which showed no expression in the root. However, this is

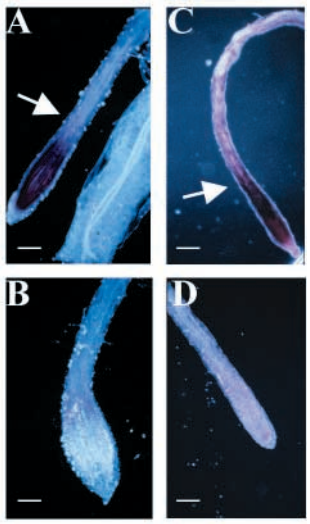

Fig. 7. Whole-mount in situ analysis of AXR3 and SHY2 expression. (A) Antisense AXR3 after 4 hours' development of the signal. Signal is strong throughout the elongation zone and fading into the zone of differentiation (indicated by arrow). (B) Sense AXR3 probe after 4 hours' development of the signal. (C) Antisense SHY2 after 4.5 hours' development of the signal. Dark staining throughout the tip extends into the zone of differentiation (indicated by arrow). (D) Sense SHY2, after 4.5 hours' development of the signal. Slight background signal is visible in the epidermis. Scale bar: 0.1 mm.

difficult to reconcile with the phenotypic effects of *shy2-2* and *shy2-31* in the root, and other work showing expression of *SHY2* in roots by northern blot (Abel et al., 1995) and expression in late-embryonic roots using a different promoter-reporter fusion (Hamann et al., 2002). The results of our whole-mount in situ hybridisation experiments support a root tip expression pattern, with strong hybridisation of a *SHY2*-specific anti-sense probe to the root tip extending back into the differentiation zone (Fig. 7C). A very faint signal was detected using the sense control probe (Fig. 7D).

These data demonstrate that both *AXR3* and *SHY2* transcripts accumulate in root tips, with the zone of *SHY2* expression extending beyond that of *AXR3*, into the root hair differentiation zone.

Discussion

The roots of *axr3-1* plants have no root hairs, whilst *shy2-2* roots have longer root hairs than wild-type roots. These two mutations confer opposite effects on root hair length, yet they are caused by similar semi-dominant point mutations in highly homologous *Aux/IAA* genes, both of which increase the stability of the cognate proteins and result in their accumulation to high levels (Colon-Carmona et al., 2000; Ouellet et al., 2001; Blilou et al., 2002). The effects of the mutations on root hair length are reproduced when the mutant proteins are transiently expressed from the same heat-shock-inducible promoter. This finding suggests that the opposing effect of the mutant alleles is a property of the proteins themselves rather than their expression patterns. Furthermore, these results indicate that the phenotypes are likely to be a direct consequence of expression of the mutant proteins rather than a very indirect consequence as a result of a long-term accumulation of effects.

The mode of action of the two mutant proteins in regulating root hair length is very different. The axr3-1 protein can block root hair elongation at any stage, since in the *HS:axr3-1* plants, heat shock induction resulted in immediate inhibition of root hair initiation and elongation. Growth was blocked even in hairs that were elongating at the time of the heat shock (Fig. 6B). In contrast, the shy2-2 protein appears to affect the timing of the initiation of hair development, rather than the rate of hair growth following initiation. The *shy2-2* root hair phenotype is caused by early initiation of root hair growth, when the trichoblasts are still actively expanding in the longitudinal axis. The hairs then elongate at a wild-type rate but for a longer period of time, resulting in longer hairs. Consistent with this idea, the effects of transient induction of *shy2-6* are only apparent in hairs that initiated (presumably ectopically in the elongation zone) after the heat shock.

When *shy2-2* and *axr3-1* are co-expressed, a novel phenotype is observed in which apolar aberrant root hairs initiate, but fail to undergo tip growth. This phenotype is not observed in the *axr3-1* mutant background, when *shy2-6* is transiently expressed, but only in the *shy2-2* mutant background when *axr3-1* is transiently expressed. Furthermore, it only occurs after a period when root hair formation is completely blocked, as axr3-1 levels are dropping back to zero. The aberrant roots hairs presumably develop at a point when the axr3-1 protein falls below a critical level. However, the phenotype is not simply related to the level of axr3-1 because it is dependent on the presence of shy2-2 and is not observed when axr3-1 is transiently expressed in a wild-type background. Taken together these data suggest that it is not the absolute level of axr3-1 that is important, but rather the relative amounts of shy2-2 and axr3-1. This hypothesis is supported by the observation that the apolar root hair phenotype is not observed when expression of both *shy2-6* and *axr3-1* are transiently induced together, and so, presumably, levels of both proteins fall off together. This suggests an interaction between shy2 and axr3 in regulating root hair development, although not necessarily direct or physical.

These results are consistent with the model outlined above in which the specificity of auxin responses is mediated by the dimerisation network of Aux/IAAs (and ARFs), and hence the transcriptional

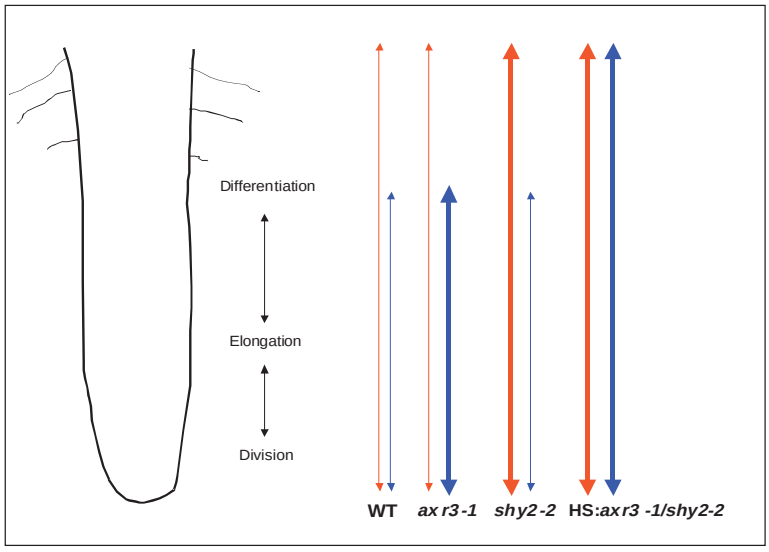

Fig. 8. Model to explain the root hair phenotypes of the genotypes studied in this work. Red and blue arrows indicate sites of SHY2 and AXR3 protein accumulation, respectively.

regulation of downstream genes. However, it is important to note that all these data are derived from the study of dominant mutant proteins. It is unclear whether these alleles are operating through hypermorphic, hypomorphic or neomorphic mechanisms. Therefore, it is difficult to interpret the data to understand the wild-type function of the *AXR3* and *SHY2* genes. For this reason we also examined loss-of-function alleles and gene expression patterns of *AXR3* and *SHY2*.

The in situ hybridisation data show that the *AXR3* gene is expressed in the elongation zone of roots, with expression dwindling into the differentiation zone and the more mature parts of the root. This is consistent with the *axr3-1* allele being hypermorphic and the wild-type role for AXR3 being to repress root hair initiation and growth in the elongation zone. In the *axr3-1* mutant, the stable axr3-1 protein may persist into the differentiation zone blocking root hair development. The phenotype of *axr3-10* root hairs is weak, probably reflecting functional redundancy in the Aux/IAA family. None-the-less the phenotype does support the proposed hypermorphic nature of the gain-of-function alleles, because when grown on medium with no added phosphate, *axr3-10* plants show a hyper-induction of root hair elongation compared to wild type, consistent with a wild-type role for AXR3 in suppressing root hair elongation.

A similar case can be made for the *SHY2* gene. The phenotype of the *shy2-31* mutant is in general the opposite of that conferred by the *shy2-2* dominant allele. The roots of *shy2-31* plants have fewer root hairs per cell, indicating reduced root hair initiation. Furthermore, the loss-of-function phenotype reveals a minor role for *SHY2* in tip growth since root hairs are slightly shorter in the mutant, elongate at erratic rates and show a reduced growth response to phosphate. *SHY2* transcript was found to accumulate in the differentiation zone, but transcripts were also detected more apically in the root tip. These data suggest that the dominant *shy2* alleles are hypermorphic, and that SHY2 functions in the root tip to promote the initiation of root hair growth and elongation. In the *shy2-2* mutant, shy2 protein may accumulate in the elongation zone above a threshold level sufficient to trigger root hair initiation. In this model, the relative amounts of AXR3 and SHY2 would control the timing of root hair initiation on trichoblast cells as they pass through the elongation zone. Initially AXR3 is high relative to SHY2, but as the trichoblasts stop elongating, AXR3 expression is reduced and SHY2 expression increased, resulting in high SHY2 relative to AXR3, and triggering root hair initiation (Fig. 8). Because SHY2 and AXR3 can dimerise with themselves, with each other and with ARFs, it is tempting to speculate that the AXR3:SHY2 ratio is measured directly in the relative abundance of different dimers and hence the relative activity of different ARF-regulated genes. Certainly the data presented here are consistent with this idea.

We would like to thank Dean Rouse and Pamela McKay for help with the HS-fusion constructs, and the University of York horticulture technicians for expert plant care. This work was funded by the Biotechnology and Biological Sciences Research Council of the UK.

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