RESEARCH ARTICLE

Auxin represses stomatal development in dark-grown seedlings via Aux/IAA proteins

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ABSTRACT

Stomatal development is tightly regulated through internal and external factors that are integrated by a complex signalling network. Light represents an external factor that strongly promotes stomata formation. Here, we show that auxin-resistant aux/iaa mutants, e.g. axr3-1, exhibit a de-repression of stomata differentiation in dark-grown seedlings. The higher stomatal index in dark-grown axr3-1 mutants when compared with the wild type is due to increased cell division in the stomatal lineage. Excessive stomata in dark-grown seedlings were also observed in mutants defective in auxin biosynthesis or auxin perception and in seedlings treated with the polar auxin transport inhibitor NPA. Consistent with these findings, exogenous auxin repressed stomata formation in light-grown seedlings. Taken together, these results indicate that auxin is a negative regulator of stomatal development in dark-grown seedlings. Epistasis analysis revealed that axr3-1 acts genetically upstream of the bHLH transcription factors SPCH, MUTE and FAMA, as well as the YDA MAP kinase cascade, but in parallel with the repressor of photomorphogenesis COP1 and the receptor-like protein TMM. The effect of exogenous auxin required the ER family of leucine-rich repeat receptor-like kinases of the ERECTA (ER) family. The ERf, but in hypocotyls and stems, it promotes stomatal development and thereby antagonises ERf function (Bhave et al., 2007). Additional negative regulators act upstream of this cascade: leucine-rich repeat (LRR) receptor-like kinases of the EPIDERMAL PATTERNING FACTOR-1 (EPF1) family, acting in concert with the aforementioned bHLH proteins, and the repressor of light signalling, consisting of CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1) and SUPPRESSOR OF PHOTOMORPHOGENESIS1 (SUPP1). The meristemoid can divide asymmetrically twice more before it differentiates into a guard mother cell (GMC). The GMC undergoes one symmetric division, producing two guard cells that form a stoma. The surrounding SLGCs can either differentiate into pavement cells or can undergo additional asymmetric divisions, thereby giving rise to satellite meristemoids. These divisions are referred to as spacing divisions, as the new meristemoid is always formed away from the existing stoma. As a consequence, two stomata are normally separated by at least one pavement cell ('one-cell-spacing rule') (Pillitteri and Torii, 2012; Wengier and Bergmann, 2012).

Three bHLH transcription factors, SPEECHLESS (SPCH), MUTE and FAMA, control consecutive steps of stomatal development. SPCH promotes entry and amplifying divisions in the stomatal lineage (MacAlister et al., 2007; Pillitteri et al., 2007). MUTE is required for the termination of amplifying divisions and the cell-state transition from the meristemoid to the GMC (Pillitteri et al., 2007). FAMA restricts symmetric division of the GMC to one round and promotes the transition to guard cells (Ohashi-Ito and Bergmann, 2006). Two additional bHLH proteins, SCREAM (SCRM)/INDUCER OF CBF1 (ICE1) and SCRM2, with largely redundant functions, act in concert with the aforementioned bHLH proteins to promote all steps of stomatal development (Kanaoka et al., 2008). Upstream of these bHLH transcription factors, a MAP kinase cascade, comprising the MAP kinase kinase YDA, the MAP kinase kinases MKK4 and MKK5 and the MAP kinases MPK4, MPK5, MPK7 and MPK9, suppresses stomatal development (Bergmann et al., 2004; Lampard et al., 2008, 2009; Wang et al., 2007). Additional negative regulators act upstream of this cascade: leucine-rich repeat (LRR) receptor-like kinases of the ERECTA (ER) family, consisting of ER, ERECTA-LIKE1 (ERL1) and ERL2, control stomatal patterning and differentiation (Shpak et al., 2005). Activity of the ERF is modulated by the LRR receptor-like protein TOO MANY MOUTHS (TMM). In cotyledons and leaves, it restricts stomata differentiation and guides spacing divisions in concert with the ERF, but in hypocotyls and stems, it promotes stomatal development and thereby antagonises ERF function (Bhave et al., 2009; Nadeau and Sack, 2002; Shpak et al., 2005; Yang and Sack, 1995). Activity of the TMM-ERF module is controlled by cysteine-rich peptide ligands of the EPIDERMAL PATTERNING FACTOR-LIKE (EPFL) family (Lee et al., 2012; Rychel et al., 2010).

Light strongly promotes stomatal development, as stomata are found at very low numbers in the cotyledon epidermis of dark-grown seedlings. The photoreceptors cryptochromes, phytochrome A (phyA) and phyB promote the differentiation of stomata in the light. phyB has been recently shown to act systemically in stomata cell fate determination, indicating that phyB action in mature leaves modulates stomatal differentiation in young leaves (Casson and Hetherington, 2014). Downstream of these photoreceptors, a repressor of light signalling, consisting of CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1) and SUPPRESSOR OF PHOTOMORPHOGENESIS1 (SOP1)
PHOTOTROPISM A-105 (SPA) proteins, acts to inhibit stomatal development in darkness (Kang et al., 2009). In addition, phyB and PHYTOCHROME INTERACTING FACTOR4 (PIF4) regulate stomata formation in true leaves in response to high light intensities (Boccalandro et al., 2009; Casson et al., 2009).

Phytohormones also control stomatal development. Brassinosteroids (BRs) regulate the phosphorylation state of SPCH and components of the YDA MAPK cascade and thereby affect stomatal development (Gudesblat et al., 2012; Khan et al., 2013; Kim et al., 2012). It has been recently shown that polar auxin transport is crucial for maintaining the one-cell-spacing rule in stomatal patterning. High auxin activity has been observed during unequal cell divisions, whereas a decrease in auxin activity promotes GMC fate and its subsequent equal division into two guard cells (Le et al., 2014).

Auxin is perceived by two sets of receptors: the plasma membrane-localised AUXIN BINDING PROTEIN1 (ABP1) and the nuclear TRANSPORT INHIBITOR RESPONSE1 (TIR1)/AUXIN-BINDING F-BOX (AFB) proteins (Hayashi, 2012). TIR1/AFB proteins control the abundance of AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) repressors. At low auxin concentrations, AUX/IAA proteins inhibit the transcriptional regulatory function of AUXIN RESPONSE FACTORS (ARFs) (Tiwari et al., 2001; Ulmasov et al., 1999). Auxin promotes the formation of a TIR1/AFB-AUX/IAA co-receptor complex, leading to ubiquitylation and subsequent degradation of AUX/IAA proteins, which results in de-repression of ARFs (Dharmasiri et al., 2005a,b; Gray et al., 2001; Kepinski and Leyser, 2005; Tan et al., 2007).

Mutant AUX/IAA proteins that fail to interact with the auxin receptor are not degraded in the presence of auxin and, therefore, lead to auxin insensitivity (Reed, 2001). Such gain-of-function mutations in the AUX/IAA genes SHORT HYPOCOTYL2 (SHY2)/IAA5, AUXIN RESISTANT2 (AXR2)/IAA7 and AXR3/IAA17 result in a constitutive morphogenesis phenotype characterised by a short hypocotyl and open cotyledons in dark-grown seedlings (Leyser et al., 1996; Reed et al., 1998; Timpte et al., 1994). Here, we report that these mutants are defective in the suppression of stomatal development in darkness and that AXR3 acts genetically upstream of the YDA MAP kinase cascade to regulate stomata formation in response to light and auxin signals.

RESULTS
Gain-of-function aux/iaa mutants fail to suppress stomata differentiation in dark-grown seedlings
The gain-of-function aux/iaa mutants axr-1, axr-3-1 and shy-2-2 display a constitutive morphogenesis phenotype similar to, although less pronounced than, the cop1-4 mutant, with short hypocotyls and partially opened cotyledons when grown in darkness (Leyser et al., 1996; Reed et al., 1998; Timpte et al., 1994) (Fig. 1A). We investigated whether the aux/iaa mutants also exhibit a defect in the suppression of stomatal development in dark-grown seedlings. Indeed, the stomatal index (SI), i.e. the proportion of stomata among all epidermal cells, was increased in dark-grown axr-2-1, axr-3-1 and shy-2-2 mutant seedlings when compared with the wild type (Fig. 1B,C; supplementary material Fig. S1). Again, this aspect of the aux/iaa mutant phenotype was similar to, but less dramatic, than that of the cop1-4 mutant (Fig. 1B,C). The proportion of stomatal precursors, i.e. meristemoids and GMCs, was not changed or only mildly reduced in the three aux/iaa mutants. In hypocotyls of dark-grown aux/iaa mutant seedlings, stomata differentiation was also enhanced when compared with the wild type (Fig. 1F). The axr-2-1 axr-3-1 double mutant displayed no additional increase in stomata formation when compared with the single mutants (supplementary material Fig. S2). Clustered stomata were not observed in any of the axr single or double mutants.

The axr-3-1 mutation causes a post-embryonic increase in the division of stomatal lineage cells
Cotyledons of dark-grown seedlings were examined over a time course from 2 to 10 days post germination (dpg). Virtually no mature stomata were observed in the cotyledon epidermis of 2-day-old wild-type, axr-2-1 and axr-3-1 mutant seedlings; the first stomata appeared between 3 and 4 dpg and excessive stomata developed in axr-2-1 and axr-3-1 in the following days (Fig. 2A). Hence, stomata in aux/iaa mutants do not form prematurely but post-embryonically, similar to the wild type.

We subsequently investigated how the increased SI in dark-grown axr-3-1 reflects the total, absolute number of stomata and pavement cells per cotyledon. Fig. 2B shows that at 2 dpg the total number of epidermal cells was similar in wild type and axr-3-1. At 10 dpg, the total number of stomata/cotyledon was approximately five times higher in axr-3-1 than in the wild type (Fig. 2B). Notably, not only the number of stomata but also the number of non-stomatal cells increased strongly between 2 and 10 dpg in the axr-3-1 mutant, but not in the wild type (Fig. 2B). Similar results were obtained from the analysis of transgenic seedlings harbouring a TMM::TMM-GFP construct, which is expressed in meristemoids, GMCs and SLGCs (Nadeau and Sack, 2002), and can thus be used as a marker for incompletely differentiated stomatal lineage cells. In the wild-type background, only the number of GFP-positive cells increased significantly from 2 to 10 dpg, whereas the number of both GFP-positive and GFP-negative cells increased in the axr-3-1 background during this period (Fig. 2C).

To investigate the effects of the axr-3-1 mutation in the absence of cell division activity in the stomatal lineage, we analysed the spch-3 mutant, which lacks entry into the stomatal lineage and therefore contains an epidermis solely composed of pavement cells (MacAlister et al., 2007). The total epidermal cell number did not increase between day 2 and day 10 after germination in spch-3 (Fig. 2D). Similarly, in the axr-3-1 spch-3 double mutant, which is also devoid of stomata (see also below), the total epidermal cell number was essentially the same in 2-day- and 10-day-old seedlings, indicating no further cell division. These results demonstrate that the axr-3-1 mutation increases the total epidermal cell number (Fig. 2B) by enhancing cell division exclusively within the stomatal lineage. Hence, the higher number of non-stomatal cells in the axr-3-1 mutant is also solely a result of cell division activity in the stomatal lineage and, therefore, derived from SLGCs.

Auxin inhibits stomata formation
The stomata-overproducing phenotype of the auxin-insensitive mutants axr-2-1, axr-3-1 and shy-2-2 suggests that auxin is involved in the regulation of stomatal development. To further test this hypothesis, we investigated the effect of exogenous auxin on stomata formation. In wild-type seedlings, concentrations of 10 µM or higher of the synthetic auxin 1-Naphthaleneacetic acid (NAA) strongly reduced the SI, an effect that was much weaker in axr-2-1
and axr3-1 (Fig. 3A; supplementary material Fig. S3). These results indicate that auxin inhibits stomatal development in a dosage-dependent manner. Reduction in the SI was accompanied by a reduction in the number of stomata as well as non-stomatal epidermal cells (Fig. 3B), thus further corroborating the hypothesis that auxin controls cell division in the stomatal lineage.

We subsequently analysed stomatal phenotypes of mutants with perturbed auxin biosynthesis or signalling. The wei8 tar2 mutant with a defect in auxin biosynthesis (Stepanova et al., 2008) exhibited excessive stomata in darkness, similar to axr3-1, and occasionally formed clusters of two stomata in continuous white light (Wc) (Fig. 3D; supplementary material Fig. S4). tir1 afb2 afb3 auxin receptor mutants exhibit variation in penetrance: class A seedlings are similar to the wild type, but have an agravitropic root and lack an apical hook in darkness; class B seedlings lack a root, form a rudimentary hypocotyl and often have only one cotyledon; class C seeds do not germinate (Dharmasiri et al., 2005b; Parry et al., 2009). Seedlings of classes A and B also differed in their stomatal phenotypes: dark-grown class A seedlings displayed a higher SI and formed both more stomata and more non-stomatal epidermal cells than the wild type, whereas class B seedlings showed not only more stomata in darkness but also clusters of two to three stomata (Fig. 3D,E; supplementary material Fig. S4). With the exception of this cluster formation, mutations in auxin biosynthesis and receptor genes affected stomata differentiation only in dark-grown and not in light-grown seedlings when compared with the wild type (Fig. 3D).

Many auxin-regulated processes require the establishment of auxin gradients via polar auxin transport (Tanaka et al., 2006). Dark-grown seedlings treated with the polar auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA) showed an increased SI in darkness compared with the mock-treated control (Fig. 3C). This indicates that proper distribution of auxin via polar auxin transport is required for the suppression of stomatal development in darkness.

**Auxin acts independently of brassinosteroids to suppress stomatal differentiation in darkness**

BRs have recently been shown to regulate stomatal development (Gudesblat et al., 2012; Khan et al., 2013; Kim et al., 2012). Auxin and BRs are known to co-regulate light responses such as the shade avoidance response (Keller et al., 2011; Keuskamp et al., 2011).

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![Fig. 1. The constitutively photomorphogenic mutants axr2-1 and axr3-1 form excessive stomata in darkness.](image-url)

(A) Visual phenotype of 4-day-old dark-grown seedlings of the indicated genotypes. (B) Confocal images of the cotyledon epidermis of dark-grown seedlings of the genotypes Col-0, axr2-1, axr3-1 and cop1-4. Cell outlines were visualised by propidium iodide (PI) staining. Scale bar: 50 µm. (C-E) Quantification of stomata and stomatal precursors in the cotyledon epidermis of dark- (C) or white light (Wc)-grown (D) seedlings or in true leaves of 21-day-old adult plants (E). (F) Quantification of stomata in hypocotyls of dark- and Wc-grown seedlings of the indicated genotypes. Error bars represent s.e.m. Asterisks indicate significant differences in the proportion of stomatal precursors or stomata, respectively, when compared with the wild type (***, P<0.001, **, P<0.01, *, P<0.05).
To test whether BRs and auxin interdependently regulate stomatal development, we tested the effect of NAA on stomata formation in combination with bikinin, an inhibitor of GSK3-like kinases in BR signalling (De Rybel et al., 2009). NAA and bikinin reduced the SI in the wild type and additive effects of both chemicals were evident in the tmm-1 mutant. axr3-1 was less responsive to NAA but still showed a wild type-like response to bikinin (supplementary material Fig. S5A-C). Moreover, auxin treatment could reduce the SI of the BR biosynthesis mutant det2-1 (supplementary material Fig. S5D), suggesting that auxin does not act via BR biosynthesis to regulate stomatal development.

The stomata-overproducing phenotype of axr3-1 depends on functional SPCH, MUTE and FAMA

SPCH, MUTE and FAMA are master regulators of consecutive steps in stomatal development. Loss of function of any of these genes results in the arrest of stomatal development at the step controlled by the respective gene: spch mutants display an epidermis completely devoid of stomata, whereas mute and fama mutants have arrested meristemoids and GMC clusters (‘fama tumours’), respectively (MacAlister et al., 2007; Ohashi-Ito and Bergmann, 2006; Pillitteri et al., 2007). To investigate genetic interactions of the Aux/IAA genes with SPCH, MUTE and FAMA, respective double mutants were generated. The cotyledon epidermis of dark-grown axr3-1 spch-3 double mutants formed no stomata and, thus, exhibited the same phenotype as the spch-3 single mutant (Fig. 4A). Hence, SPCH is required for expression of the axr3-1 mutant phenotype. Phenotypes of the axr3-1 mute-1 and axr3-1 fama-1 double mutants also qualitatively resembled those of the respective mute-1 and fama-1 single mutants in that no guard cells were formed. Quantitatively, the proportions of meristemoids and ‘fama tumours’, respectively, were increased by the additional axr3-1 mutation (Fig. 4A-C). This probably reflects an
increased entry of protodermal cells into the stomatal lineage in axr3-1 mutants. Taken together, these observations suggest that axr3-1 acts genetically upstream of the three bHLH genes and requires their function to enhance differentiation of mature stomata.

As the axr3-1 phenotype depended on the three bHLH genes, we tested whether axr3-1 affects their transcript levels. SPCH, MUTE and FAMA mRNA levels were only slightly elevated in dark-grown axr2-1 and axr3-1 mutants when compared with the wild type, whereas NAA treatment slightly reduced their expression in the wild type, but also in axr3-1 (supplementary material Fig. S6). In dark-grown cop1-4 mutants, in contrast, at least SPCH and FAMA transcript levels were much more strongly increased when compared with the wild type (Kang et al., 2009) (supplementary material Fig. S6). Thus, any major effect axr3-1 might have on the three bHLH transcription factors is probably post-translational.

A constitutively active YDA MAPK cascade completely suppresses stomata formation in axr3-1

The YDA MAPK cascade acts upstream of SPCH, and probably also of MUTE and FAMA, to suppress stomatal development (Lampard et al., 2008; Ohashi-Ito and Bergmann, 2006; Pillitteri et al., 2007). It integrates signals from the light and BR signalling pathways (Kang et al., 2009; Khan et al., 2013; Kim et al., 2012), thus raising the possibility that it is also a downstream factor in AXR3 signalling.

Loss-of-function yda and mpk3 mpk6 mutants as well as MKK4-MKK5RNAI plants have an epidermis consisting almost exclusively of stomata (Bergmann et al., 2004; Wang et al., 2007). Therefore, additional promoting effects of the axr3-1 mutation would be difficult to detect in these backgrounds. By contrast, expression of constitutively active versions of YDA, MKK4 and MKK5 results in an epidermis completely devoid of stomata (Bergmann et al., 2004; Wang et al., 2007), but many lines expressing these constructs show strong silencing effects (Wang et al., 2007) and were thus not suitable for crossing and subsequent epistasis analysis. Instead, we crossed axr3-1 into a GVG-Nt-MEK2DD background harbouring a dexamethasone (DEX)-inducible construct of a constitutively active version of tobacco MEK2, which was shown to be functionally interchangeable with Arabidopsis MKK4 and MKK5 (Ren et al., 2002). This transgenic line was reported to reliably express the transgene in Arabidopsis (Wang et al., 2007). Both in the wild-type and the axr3-1 background, induction of Nt-MEK2DD expression resulted in the complete absence of stomata (Fig. 5), indicating that the positive effect of the axr3-1 mutation on stomatal development cannot overcome the effect of a constitutively active YDA MAPK cascade. Therefore, AXR3 appears to act genetically upstream of MPK3, MPK6, MKK4 and MKK5.

Genetic interactions between axr3-1, tmm and er(l) mutations

Upstream of the YDA MAPK cascade, TMM and ERF act cooperatively to restrict stomatal development (Bergmann et al., 2004; Lee et al., 2012; Shpak et al., 2005). To test whether AXR3 acts through these proteins to affect stomatal development, we investigated genetic interactions between axr3-1, tmm-1 and various er(l) mutations.

When grown in the light, the tmm-1 mutant exhibits a strong stomata-overproducing and clustering phenotype in leaves and cotyledons (Yang and Sack, 1995) (supplementary material Fig. S7A,B). In darkness, however, tmm-1 hardly forms any mature stomata at all, but shows an increased proportion of meristemoids compared with the wild type (Kang et al., 2009) (Fig. 6A,B). This phenotype was drastically altered by an
additional axr3-1 mutation: the axr3-1 tmm-1 double mutant not only displayed a higher proportion of stomata than the axr3-1 single mutant, but also developed stomatal clusters in darkness, which neither single mutant did (Fig. 6A, B). In the light, in contrast, the axr3-1 tmm-1 stomatal phenotype was similar to the tmm-1 single mutant (supplementary material Fig. S7A, B). Thus, the two mutations have synergistic effects in darkness, and axr3-1 appears to provide a sensitised background in which lack of TMM causes the formation of stomatal clusters in darkness as well.

Different ERf genes affect different steps of stomatal development: in the light, ER primarily prevents entry divisions into the stomatal lineage, whereas ERL1 predominantly inhibits subsequent development of stomatal precursors; ERL2 has minor functions in both processes. A knock out of all three ERf genes results in a stomata-clustering phenotype that is even stronger than that of a tmm mutant (Shpak et al., 2005).

Functional divergence of ERf members was also seen in dark-grown seedlings: er and er erl2 mutants showed an increased proportion of stomatal precursors when compared with the wild type, whereas erl1 and erl1 erl2 mutants formed more mature stomata than the wild type (Fig. 6C,D). Addition of the axr3-1 mutation increased the proportion of stomata in each of these mutants, but to a lesser extent than in the wild-type background (Fig. 6C,D). Only a slight increase in clustered stomata due to axr3-1 was detected in the er erl1 erl2 triple mutant (Fig. 6C,E), and was only of low statistical significance in two experiments ($P=0.085$ for the experiment shown in Fig. 6E; $P=0.046$). Taken together, these results suggest that axr3-1 acts – at least in part – through ERf proteins to affect stomatal development.

Fig. 4. The axr3-1 mutation requires functional SPCH, MUTE and FAMA to promote stomatal development in darkness. (A) Confocal images of the cotyledon epidermis of 10-day-old dark-grown seedlings of the indicated genotypes. Cell outlines were visualised by PI staining. Square brackets highlight ‘fama tumours’. Scale bar: 50 µm. (B,C) Quantification of stomata, stomata precursors and ‘fama tumours’ in the cotyledon epidermis of dark-grown seedlings of the indicated genotypes. Error bars indicate s.e.m. Letters indicate significance groups for each cell type; samples with the same letters are not significantly different ($P<0.05$).

Fig. 5. A constitutively active MKK suppresses stomata formation in the axr3-1 background. Confocal images of the cotyledon epidermis of dark-grown seedlings of Col-0 and axr3-1 and of transgenic Col-0 and axr3-1 seedlings expressing the DEX-inducible GVG-NI-MEK2DD construct. Seedlings were grown on medium supplemented with 0.02 µM DEX. Cell outlines were visualised by PI staining. Scale bar: 50 µm.
YDA and the ERf are important for the regulation of stomatal development by exogenous auxin and light

We subsequently investigated the auxin responsiveness of stomata patterning mutants. To this end, seedlings were grown on plates containing 10 µM NAA, a concentration that reduces the SI in the wild type (Fig. 3A and Fig. 7A). NAA treatment also reduced the SI in epfl1 epfl2 and tmm-1 mutants, whereas it had no significant effect in er erl1 erl2 triple mutants and in loss-of-function yda-10 mutants (Fig. 7A). Hence, the inhibitory effect of NAA on stomata formation was dependent on YDA and members of the ERf.

The effect of light/darkness on the stomatal phenotypes of the stomata-overproducing mutants was investigated as well. yda-10 and er erl1 erl2 also exhibited excessive stomata and stomata clusters in darkness, whereas the stomata-overproducing phenotypes of light-grown tmm-1 and epfl1 epfl2 were strongly attenuated when grown in darkness (Fig. 7B). As a consequence, stomata differentiation/clustering was more responsive to light in epfl1 epfl2 and, in particular, in tmm-1 mutants than in the wild type, er erl1 erl2 and yda-10 mutants.

As light and auxin seem to require the ERf to properly control stomatal development, we investigated whether the expression of the receptor-like kinases and their ligands of the EPFL family is altered in axr mutants or in NAA-treated seedlings. Transcript levels of TMM, all ERf members and several EPFLs were slightly elevated in dark-grown axr mutants when compared with the wild type (supplementary material Fig. S8A-D and Fig. S9A-D), but were not strongly affected by NAA treatment (supplementary material Figs S8E and S9E). Most of these genes act as negative regulators of stomatal development; it is thus unlikely that their elevated transcript levels are the cause for the stomatal phenotype of the axr mutants, but might rather be a consequence of the increased number of stomatal lineage cells.

COP1 and AXR3 act independently in suppressing stomata differentiation in darkness

Light affects stomatal development via inhibition of the COP1/SPA complex, and COP1 has been shown to act genetically upstream of YDA, but in parallel with TMM (Kang et al., 2009). Thus, axr3-1 and cop1 mutations exhibit similar genetic interactions with mutations in stomata patterning genes. The axr3-1 cop1-4 double mutant produced a larger fraction of stomata than either single mutant (Fig. 8A,B), suggesting that both genes act in independent pathways. This is further corroborated by the fact that neither COP1 protein levels nor its subcellular localisation were changed in dark-grown axr2-1 and axr3-1 mutants when compared with the wild type (supplementary material Fig. S10A,B). Furthermore, the

Fig. 6. The axr3-1 mutation shows additive and synergistic effects with loss-of-function mutations in TMM and genes of the ERf.

(A,C) Confocal images of the cotyledon epidermis of dark-grown seedlings of the indicated genotypes. Cell outlines were visualised by PI staining. Scale bars: 50 µm. (B,D) Quantification of stomata and stomatal precursors of the genotypes shown in A and C, respectively. Error bars represent s.e.m. Letters indicate significance groups for each cell type; samples with the same letters are not significantly different (P<0.05).
Expression of axr3-1-YFP in the epidermis partially mimics the axr3-1 mutant phenotype

The axr3-1 mutation requires the stomatal signalling pathway to promote stomatal differentiation; however, it is unclear in which cell type the axr3-1 protein acts. To address this, the axr3-1 mutant coding sequence fused to YFP was expressed under different cell type-specific promoters in the wild-type background. The set of promoters used comprised the 35S and AXR3 promoters, the MERistem Layer1 (ML1) promoter for epidermal specificity (Sessions et al., 1999), the chlorophyll a/b binding protein3 (CAB3) promoter for mesophyll specificity (Susek et al., 1993) and the SPCH and ICE1 promoters for specificity to the stomatal lineage. SPCH is expressed broadly in the protoderm in leaf primordia, but its expression is restricted to MMCs and young meristemoids later in development (MacAlister et al., 2007), whereas the ICE1 expression domain encompasses meristemoids, GMCs, young guard cells and SLGCs (Kanaoka et al., 2008).

No transgenic plants with visible AXR3-1-YFP accumulation were found among those transformed with the 35S::axr3-1-YFP construct, implying that ubiquitous expression of the AXR3-1 mutant protein is lethal. Furthermore, most T1 plants expressing axr3-1-YFP from the CAB3 or ICE1 promoter did not set seed; therefore, none of the CAB3::axr3-1-YFP and only two of the ICE1::axr3-1-YFP lines could be propagated and analysed.

In dark-grown AXR3::axr3-1-YFP seedlings, a YFP signal was detected in epidermal and cortical cells of the hypocotyl, in the upper part of the root and, in some lines, also in pavement cells close to the margin of the cotyledons, but no YFP signal was detected in stomatal lineage cells of the abaxial cotyledon epidermis in any of the lines analysed (Fig. 9A-C; supplementary material Fig. S12 top panel). Accumulation of AXR3-1-YFP in pavement cells and stomatal lineage cells was found in dark-grown ML1::axr3-1-YFP seedlings (Fig. 9E; supplementary material Fig. S12 middle panel). The SPCH::axr3-1-YFP lines accumulated AXR3-1-YFP weakly in pavement cells and most strongly in small cells that lacked any sign of differentiation and often occurred in pairs (Fig. 9G; supplementary material Fig. S12 bottom panel), a pattern that was also observed for transcriptional and translational SPCH reporters (MacAlister et al., 2007). The YFP signal in the epidermis of ICE1::axr3-1-YFP lines was restricted to meristemoids, GMCs and cells adjacent to these (Fig. 9I; supplementary material Fig. S12 bottom panel). No YFP signal was detected in the mesophyll in any line expressing axr3-1-YFP (Fig. 9D,F,H,J).

All transgenic axr3-1-YFP lines were analysed for whether they mimic the axr3-1 stomatal phenotype in dark-grown seedlings. The AXR3::axr3-1-YFP lines showed a significantly higher SI than the wild type, although it was slightly lower than the SI of the axr3-1 mutant (Fig. 9K). A significant increase in the SI was also observed for all ML1::axr3-1-YFP lines, except for line 3-4. By contrast, seedlings expressing axr3-1-YFP from the SPCH and ICE1 promoters exhibited an SI similar to the wild type. Hence, expression of axr3-1-YFP in the stomatal lineage was insufficient to promote stomata formation, thus implying that AXR3-1 acts non-cell-autonomously.
We also investigated whether other facets of the axr3-1 mutant phenotype were observed in the axr3-1-YFP lines. The short hypocotyl phenotype of a dark-grown axr3-1 mutant was mimicked by all AXR3::axr3-1-YFP lines (supplementary material Fig. S13A,B). Two MLI::axr3-1-YFP and one SPCH::axr3-1-YFP line also showed a reduction in hypocotyl length when compared with the wild type, but to a much lesser extent. All of the ICE1::axr3-1-YFP lines appeared similar to the wild type (supplementary material Fig. S13A,B). The vegetative growth phenotype of the axr3-1 mutant, characterised by dwarfism and leaf hyponasty, was not mimicked entirely by any transgenic line. Four AXR3::axr3-1-YFP lines, however, were dwarfed compared with the wild type and also displayed hyponastic leaves (supplementary material Fig. S14). Expression of axr3-1-YFP from the MLI and SPCH promoters caused leaf epinasty and reduced plant size. Hence, AXR3-1-YFP can have opposite effects on leaf curvature when expressed in different tissues. ICE1::axr3-1-YFP lines appeared similar to the wild type.

**DISCUSSION**

Light strongly promotes the differentiation of stomata in cotyledons and leaves, which is consistent with the role of stomata in photosynthetic gas exchange. It has been shown previously that photoreceptors, the COP1/SPA complex and PIF proteins are involved in light-regulated stomatal development (Boccalandro et al., 2009; Casson et al., 2009; Kang et al., 2009). Here, we have identified auxin as an important regulator that suppresses stomata differentiation in dark-grown seedlings. Moreover, we have placed AXR3 within the genetic signalling network that regulates stomatal development in response to intrinsic and external signals. Taken together with a recent report demonstrating the important role of polar auxin transport and auxin distribution in stomatal patterning of light-grown seedlings (Le et al., 2014), our results support the notion that auxin is a negative regulator of stomatal differentiation.

**Auxin via AUX/IAA proteins inhibits stomata differentiation in dark-grown seedlings**

Several lines of evidence indicate that auxin and nuclear auxin signalling repress stomata formation in darkness: first, we have shown that the aux/iaa gain-of-function mutants shy2-2, axr2-1 and axr3-1, which are resistant to auxin, produce more stomata in hypocotyls and cotyledons of dark-grown seedlings when compared with the wild type. Second, exogenous application of NAA reduces the SI in the wild type, but less in axr3-1 mutant seedlings. Third, mutants that are either defective in auxin biosynthesis or in auxin perception via the

![Fig. 9. Expression of axr3-1-YFP exclusively in the epidermis increases the SI.](image)

Fig. 9. Expression of axr3-1-YFP exclusively in the epidermis increases the SI. (A-J) Confocal images of the hypocotyl (A), the cotyledon epidermis (B,C,E,G,I) and the cotyledon mesophyll (D,F,H,J) showing the accumulation of AXR3-1-YFP protein in transgenic seedlings expressing the fusion genes from the indicated promoters. Scale bars: 50 µm in A, 25 µm in B-J. (K) SI of the cotyledon epidermis of dark-grown seedlings of the indicated promoter::axr3-1-YFP lines. Numbers denote independent transgenic lines. Error bars represent s.e.m. Asterisks indicate significant differences to the wild type (**P<0.01, ***P<0.001, *P<0.05).
TIR1/ABF auxin receptor family show an enhanced production of stomata in darkness similar to the auxin-resistant axr mutants. Hence, genetic perturbation on any level, either auxin biosynthesis, auxin perception or auxin signalling, led to a de-repression of stomata formation in dark-grown seedlings. Genetic lesions in auxin biosynthesis or signalling affected the SI exclusively in dark-grown, but not in light-grown seedlings. Auxin signalling is thus required to suppress stomata differentiation primarily in darkness. However, light-grown wt seedlings show weak stomatal clustering, and strongly penetrant tir1 afb2 afb3 mutant seedlings produce stomata clusters at a similar frequency in the light and in darkness. The formation of excessive clusters has also been recently reported for the tir1 afb1 afb2 afb3 quadruple mutant (Le et al., 2014). Hence, auxin has a dual role in stomata differentiation: a dark-dependent role in suppressing stomata formation without an apparent function in stomata spacing, and a possibly light-independent role in maintaining proper spacing of stomata.

Light can affect auxin levels, distribution and responsiveness. Increased auxin biosynthesis and polar auxin transport are crucial events during the shade avoidance growth response to a low red/far-red ratio (Casal, 2012; Tao et al., 2008), a condition that also reduces stomata differentiation (Boccalandro et al., 2009; Casson and Hetherington, 2014). The molecular mechanisms underlying the effect of auxin on the morphogenesis of a dark-grown seedling are, in contrast, poorly understood. It appears unlikely that light prevents auxin-dependent suppression of stomatal development by reducing overall auxin levels, because these levels are lower in dark-grown than in light-grown seedlings, at least when examining the whole shoot tissue of a seedling (Bhalerao et al., 2002). However, although polar auxin transport is not required for hypocotyl elongation in darkness (Jensen et al., 1998), auxin transport might influence local auxin concentrations in cells of the stomatal lineage, as was recently demonstrated for light-grown seedlings (Le et al., 2014). Consistent with this idea, our results show that NPA treatment also led to excessive stomata in dark-grown seedlings, although without forming stomata clusters. Interestingly, long-distance signalling also controls stomatal differentiation in response to light intensity, as shading of a mature leaf causes a reduction in the SI of an unshaded young leaf (Casson and Hetherington, 2014; Lake et al., 2001).

The axr3-1 mutation promotes cell division in the stomatal lineage of dark-grown seedlings

The observed increase in the SI in dark-grown auxin-resistant seedlings could principally be the result of a reduced number of pavement cells and/or an increased number of stomata. Our analysis of total cell numbers in axr3-1 cotyledons and in axr3-1 spch cotyledons lacking a stomatal lineage clearly indicates that the axr3-1 mutation increases cell division activity exclusively in the stomatal lineage, thereby also producing excess pavement cells derived from SLGCs. Hence, the increase in the SI in dark-grown axr3-1 mutant seedlings is associated with an increase in cell division activity in the stomatal lineage post germination. This suggests that the consistent changes of total cell numbers observed in tir1 afb2 afb3 seedlings and in NAA-treated wild-type seedlings are also a result of changes in cell division activity exclusively in the stomatal lineage. The axr3-1 mutation appears to also cause a very slight, although statistically non-significant, increase in total cell numbers in 2-day-old dark-grown cotyledons, i.e. prior to stomata differentiation. Therefore, we cannot fully exclude the possibility that disruption of auxin signalling also increases cell division activity during embryogenesis or very early after seed imbibition.

Two pieces of evidence suggest that a disruption of auxin signalling by the axr3-1 mutation promotes both the entry divisions into the stomatal lineage and the differentiation of stomatal precursors into mature guard cells. First, dark-grown axr3-1 mutants exhibit a higher number of mature stomata without decreasing the proportion of stomatal precursor cells. Second, the axr3-1 mutation increases the number of meristemoids and ‘fama-tumours’ in the mute-1 and fama-1 backgrounds, respectively. This demonstrates that if progression through the stomatal lineage is arrested by mute or fama mutations, axr3-1 still increases entry divisions into the stomatal lineage. Hence, we suggest that the axr3-1 mutation enhances the cell division-promoting activity of the bHLH transcription factors. However, we also consider it possible that the axr3-1 mutation, moreover, enhances cell division activity in the stomatal lineage by promoting earlier cell divisions that provide cells for the stomatal lineage. Taken together, it appears that proper auxin signalling is required for the inhibition of at least two transitions in stomatal differentiation in darkness. Auxin levels dynamically change during stomatal patterning of light-grown seedlings and thereby direct the switch from unequal to equal cell division (Le et al., 2014). An analysis of auxin activity using reporter lines might resolve auxin action on cell division patterns in dark-grown seedlings.

Although the effect of axr3-1 on stomatal development requires the presence of the stomatal lineage, expression of an axr3-1-YFP fusion gene from stomatal lineage-specific promoters (SPCH, MUTE and FAMA genes to promote stomata formation in dark-grown seedlings, suggesting that auxin and auxin signalling act upstream of these transcription factors. The effect of axr3-1 also requires a functional YDA MAPK cascade, thus placing AXR3 upstream of this MAPK cascade. This is supported by the failure of exogenous NAA to reduce the SI in an yda loss-of-function mutant.

axr3-1 exhibits synergistic effects with tmm-1, suggesting that AXR3 and TMM do not act in a common linear signalling pathway, but that mutations in AXR3 or TMM mutually sensitise components of the signalling pathways for the respective loss of the other. The axr3-1 mutation shows additive interactions with mutations in ERf genes. However, the effect of axr3-1 was reduced in an er el1 el2 triple mutant background, implying that axr3-1 might act at least in part via the ERf to affect stomatal development in darkness. In agreement with this idea, auxin-induced reduction in stomata formation was abolished in er el1 el2 mutants. Hence, the YDA MAPK cascade, possibly via ERf proteins, is a likely target of auxin and auxin signalling in the regulation of stomatal development. Interestingly, auxin treatment was shown to activate a MAPK cascade in Arabidopsis roots, and it has been suggested that this induction is independent of stress associated with this treatment (Mockaitis and Howell, 2000). Apart from the EPFL-ERf pathway (Jewaria et al., 2013; Lee et al., 2012), the YDA MAPK cascade also integrates signals from the BR signalling cascade (Khan et al., 2013; Kim et al., 2012) and the light signalling pathway (Kang et al., 2009). Thus, the YDA MAPK cascade is a central switch...
responding to intrinsic and environmental signals in the decision of whether or not to form a stoma.

**MATERIALS AND METHODS**

**Plant material**

axr3-1 (Timppe et al., 1994), axr3-1 (Leyser et al., 1996), cop1-4 (Deng and Quail, 1992), det2-1 (Chory et al., 1991), epfl-1 epfl-2-3 (Hara et al., 2009), er-105 (Torii et al., 1996), er-105 erl1-2 erl2-1, er-105 erl1-2, erl1-2 erl2-1 (all Shpak et al., 2004), fama-1 (Ohashi-Ito and Bergmann, 2006), mute-1 (Pillitteri et al., 2007), shy-2-2 (Reed et al., 1998), spch-3 (MacAlister et al., 2007), tir1-1 afb2-3 afb3-4 (Parry et al., 2009), trim-1 (Yang and Sack, 1995), wei8-1 tar2-1 (Stepanova et al., 2008) and yda-10 (Kang et al., 2009) as well as the transgenic lines GYG-NtMEK2DD (Ren et al., 2002) and TMM::TMM-GFP (Nadeau and Sack, 2002) have been described previously. All axr3-1 genotypes used for epistasis analysis were generated by crossing and were confirmed using the primers listed in supplementary material Table S1.

**Plant growth**

Light sources and general growth conditions have been described previously (Laubinger et al., 2004). Seedlings were grown in darkness or Wc (25 µM m⁻² s⁻¹) on MS medium containing 1% (w/v) sucrose. For NAA treatment, medium was supplemented with various concentrations of NAA. For treatment with NPA, DEX and bikinin, medium was supplemented with 10 µM NPA, 0.02 µM DEX and 30 µM bikinin, respectively, or the equivalent amounts of DMSO for mock treatment.

**Microscopy**

For observation by bright-field microscopy, seedlings were cleared in chloral hydrate solution as described by Kang et al. (2009) and analysed using an Eclipse E800 compound light microscope (Nikon Instruments). For confocal microscopy, samples were stained in 10 µg/ml PI for several minutes and then mounted in water. They were visualised by a DM5500Q confocal laser-scanning microscope (Leica Microsystems).

**Quantification of epidermal cells**

For quantification of epidermal cells, calculation of the percentage of stomata/stomatal precursors and subsequent statistical analysis see supplementary materials and methods.

**Plasmid construction and selection of transgenic plants**

For generation of promoter::axr3-1-YFP constructs, see supplementary materials and methods. Constructs were transformed into Col-0 wild type, and 30-40 independent transgenic lines were analysed for each construct. Four to five representative lines showing a 3:1 segregation of the resistance marker and correct cell type-specific accumulation of the AXR3-1-YFP fusion protein were propagated further to obtain homozygous transgenic lines.

**Real-time PCR, total protein preparation and nuclear fractionation**

Purification of total RNA, qPCR, total protein isolation and nuclear fractionation of proteins have been described previously (Balcerowicz et al., 2011). Primers used for qPCR are listed in supplementary material Table S2.

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**Competing interests**

The authors declare no competing financial interests.

**Author contributions**

The project was designed by U.H. and M.B. Most experiments were conducted by M.B. A.R. performed the initial characterisation of the axr3-1 mutant stomata phenotype. L.R. genotyped and characterised mutants used for epistasis analysis. G.F. generated transgenic axr3-1-YFP plants. M.B. and U.H. wrote the manuscript, with all other authors commenting on it.

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**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.109181/-/DC1

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