the plant journal

SOCIETY FOR EXPERIMENTAL BIOLOGY

The Plant Journal (2009) **59**, 63–76 doi: 10.1111/j.1365-313X.2009.03849.x

Identification of the Arabidopsis *dry2/sqe1-5* mutant reveals a central role for sterols in drought tolerance and regulation of reactive oxygen species

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Received 11 December 2008; revised 9 February 2009; accepted 17 February 2009; published online 1 April 2009.

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SUMMARY

Squalene epoxidase enzymes catalyse the conversion of squalene into 2,3-oxidosqualene, the precursor of cyclic triterpenoids. Here we report that the Arabidopsis *drought hypersensitive/squalene epoxidase 1-5* (*dry2/sqe1-5*) mutant, identified by its extreme hypersensitivity to drought stress, has altered stomatal responses and root defects because of a point mutation in the *SQUALENE EPOXIDASE 1* (*SQE1*) gene. GC-MS analysis indicated that the *dry2/sqe1-5* mutant has altered sterol composition in roots but wild-type sterol composition in shoots, indicating an essential role for SQE1 in root sterol biosynthesis. Importantly, the stomatal and root defects of the *dry2/sqe1-5* mutant are associated with altered production of reactive oxygen species. As RHD2 NADPH oxidase is de-localized in *dry2/sqe1-5* root hairs, we propose that sterols play an essential role in the localization of NADPH oxidases required for regulation of reactive oxygen species, stomatal responses and drought tolerance.

Keywords: squalene epoxidase, sterol biosynthesis, drought tolerance, NADPH oxidase, reactive oxygen species.

INTRODUCTION

Sterols are isoprenoid-derived lipids that play essential roles in plant growth and development (Benveniste, 2004; Phillips *et al.*, 2006). Bulk sterols are integral components of the membrane lipid bilayer, and are important for its permeability and fluidity. The plasma membrane sterol content has also been shown to modulate the activity of membrane-bound proteins (Hartmann, 1998). Sterols are synthesized from isopentenyl diphosphate (IPP) produced through the mevalonic acid pathway located in the cytosol/endoplasmic reticulum. IPP is further converted to the linear 30-carbon intermediate squalene (SQ), the first committed precursor of all known angiosperm cyclic triterpenoids (Phillips *et al.*, 2006; Rasbery *et al.*, 2007), which is subsequently oxidized to 2,3-oxidosqualene by the enzyme squalene epoxidase (SQE) (Phillips *et al.*, 2006; Rasbery *et al.*, 2007). Of the 3 genes that show squalene epoxidase activity, mutations in *SQE1* cause reduced root and hypocotyl elongation, diminished stature and unviable seeds, indicating an essential role for this gene in plant development (Phillips *et al.*, 2006; Rasbery *et al.*, 2007). From 2,3-oxidosqualene, plant cells use a sterol biosynthetic pathway that is different to that of other eukaryotes (Figure 1) (Schaller, 2003, 2004; Benveniste, 2004). Following conversion of 2,3-oxidosqualene to cycloartenol, the

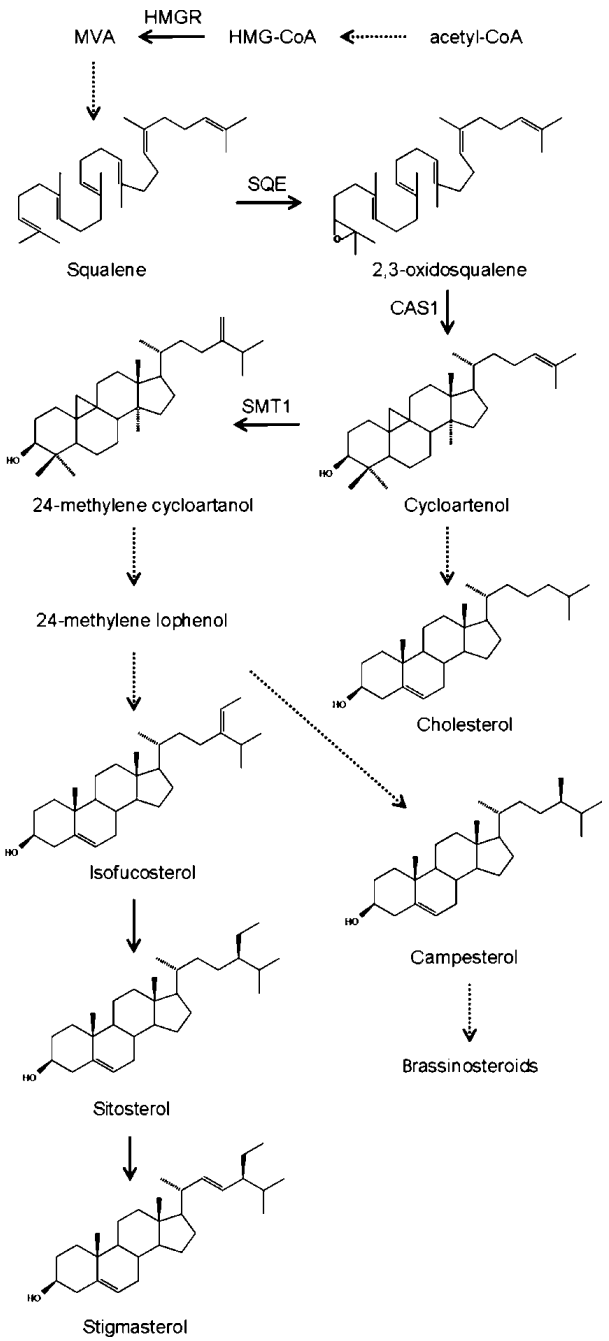

Figure 1. Sterol biosynthesis in Arabidopsis.
Squalene is oxidized by squalene epoxidase (SQE) to produce 2,3-oxidosqualene, which is further metabolized to produce steroids, including membrane sterols and brassinosteroids. Only those intermediates whose levels have been determined in this study are shown. Dashed arrows indicate multiple reactions. HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; MVA, mevalonic acid; CAS, cycloartenol synthase; SMT1, sterol methyltransferase 1.

first cyclic intermediate of plant sterol biosynthesis, the pathway is essentially linear until reaching 24-methylene lophenol. After formation of this compound, there is a bifurcation leading to either 24-methyl sterols, which include campesterol and its derivatives, the brassinosteroids, or 24-ethyl sterols, which include the structural sterols sitosterol and stigmasterol (Clouse, 2002).

Critical for elucidation of the biological functions of sterols and their role as brassinosteroid precursors has been the identification of *Arabidopsis thaliana* mutants with altered sterol profiles due to disruption of specific enzymatic steps of the post-squalene segment of the sterol pathway (Clouse, 2002; Schaller, 2004). Some of these mutants are lethal due to multiple developmental defects (Diener *et al.*, 2000; Jang *et al.*, 2000; Schrick *et al.*, 2000, 2002, 2004a; Carland *et al.*, 2002; Souter *et al.*, 2002; Willemsen *et al.*, 2003; Kim *et al.*, 2005; Babiychuk *et al.*, 2008; Men *et al.*, 2008). Some of these developmental defects may be partly explained by an enhanced and unregulated ethylene signalling activity (Souter *et al.*, 2002, 2004) and the inability to establish proper cell polarity, exemplified by the aberrant localization of the auxin efflux carriers PIN1 and PIN3 in mutants of the sterol methyl transferase (*SMT1*) gene (Willemsen *et al.*, 2003). Details of the mechanism of how sterols influence cell polarity have been analysed using the *cyclopropylsterol isomerase1-1* (*cpi1-1*) mutant in Arabidopsis. This study indicated that sterol composition affects post-cytokinetic acquisition of PIN2 polarity by endocytosis, suggesting a mechanism for sterol action on establishment of asymmetric protein localization (Men *et al.*, 2008).

Reactive oxygen species (ROS) are emerging as essential regulators of plant development. The location at which ROS production takes place is an important factor controlling plant form (Gapper and Dolan, 2006; Kwak *et al.*, 2006). ROS that have been shown to play a role in development are produced by NADPH oxidases that generate the superoxide radical (O_2^-) using NADPH as an electron donor (Torres and Dangl, 2005; Gapper and Dolan, 2006). In Arabidopsis, this class of genes is referred to as Arabidopsis respiratory burst oxidase homologues (*Atrboh*) (Keller *et al.*, 1998; Torres *et al.*, 1998). The activity of three members of this family has been shown to be involved in various aspects of root growth (Foreman *et al.*, 2003) and stomatal regulation (Kwak *et al.*, 2003).

In a search for genes that are essential for drought tolerance, we have identified an Arabidopsis mutant (*dry2/sqe1-5*) that is affected in the *squalene epoxidase 1* gene, resulting in reduced squalene epoxidase activity. This mutant shows pleiotropic developmental defects with various changes in shoot and root sterol composition. Genetic and biochemical analyses suggest that *dry2/sqe1-5* phenotypes are caused by reduced activity of SQE1. This reduced activity causes an altered sterol composition, which in turn produces defective functioning of NADPH oxidases and ROS production, leading to the developmental defects observed in the mutant.

RESULTS

Identification of an Arabidopsis mutant with drought hypersensitivity and root developmental defects

To identify genes required for drought tolerance, we performed a mutant screen for drought hypersensitivity on a population of approximately 50 000 EMS-mutagenized M_2 Landsberg *erecta* (L*er*) seedlings. A recessive mutant named *drought hypersensitive2* (*dry2*) was identified as extremely sensitive to dehydration (Figure 2a). The phenotype was verified in selfed M_3 progeny and in the F_2 of a back cross to wild-type L*er*. In addition to its drought hypersensitivity, the *dry2* mutant also had other developmental defects. Under our growth conditions (16 h light/8 h dark, 35% relative humidity), *dry2* adult plants were smaller than wild-type and their leaves were pale green (Figure 2b). The reduced size of *dry2* plants was obvious early in development (Figure 2c), and seedlings also had both reduced chlorophyll content (Figure 2e) and increased accumulation of proline (Figure 2f), an osmolyte that accumulates under conditions of water deficit.

The extreme sensitivity of *dry2* mutants to dehydration suggested that shoot developmental defects could be caused by a defective water balance. Therefore, we charac-

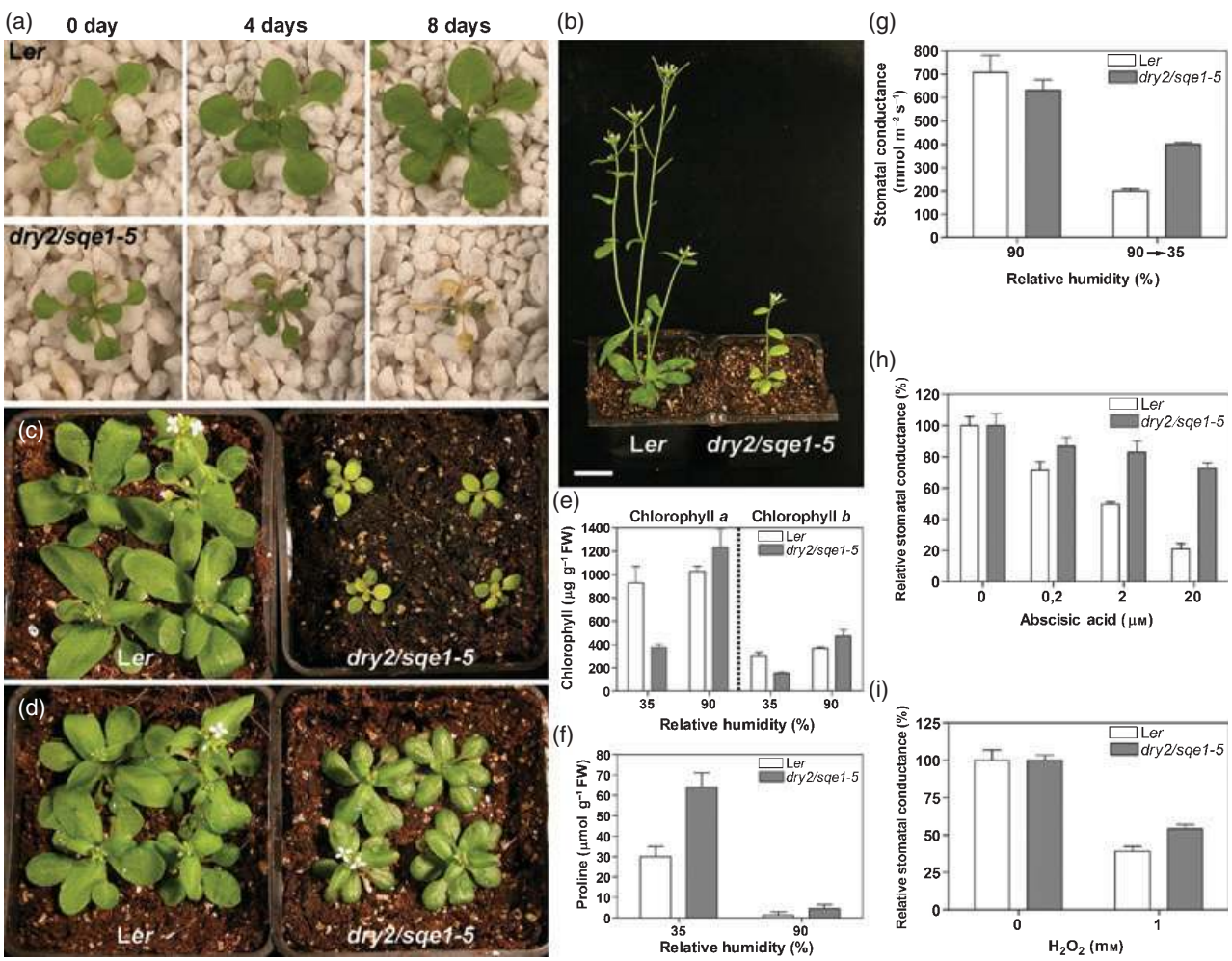

Figure 2. The *dry2/sqe1-5* mutant is hypersensitive to dehydration and has developmental defects and impaired stomatal responses.
(a) Drought tolerance assay. Twelve-day-old wild-type L*er* and *dry2/sqe1-5* seedlings grown on MS medium were transferred to perlite and grown for an additional 7 days with abundant nutrient solution. Thereafter, watering was withheld, and photographs were taken at the times indicated.
(b) Thirty four-day-old plants of L*er* and the *dry2/sqe1-5* mutant grown at 35% relative humidity (RH). *dry2/sqe1-5* plants show reduced size and pale green leaves compared to wild-type plants. Scale bar = 2 cm.
(c,d) *dry2/sqe1-5* leaves show reduced size at 35% RH (c), but this phenotype is partially suppressed when grown at 90% RH (d).
(e,f) The chlorophyll *a* and *b* content (e) and proline content (f) of L*er* and *dry2/sqe1-5* plants grown under the same conditions as in (c) and (d). Data represent mean values ± SD (n = 3).
(g) The *dry2/sqe1-5* mutant shows impaired stomatal response to changes in the RH. Data represent mean values ± SD (n = 3).
(h) The *dry2/sqe1-5* mutant shows reduced stomatal responses to exogenous ABA compared to L*er*. Data represent mean values ± SD (n = 3).
(i) L*er* and *dry2/sqe1-5* leaves show similar stomatal responses to H_2O_2 application. Data represent mean values ± SD (n = 3).

terized *dry2* growth in a high-humidity environment. When the relative humidity (RH) was increased to approximately 90%, the defective growth of *dry2* plants was attenuated (Figure 2d), and both chlorophyll and proline content were similar to that of wild-type (Figure 2e,f). This indicates that the growth defects, low chlorophyll content and increased proline content are severe at lower humidity and ameliorated at high humidity.

As the control of transpirational movement of water through stomata is a major factor in drought tolerance and water balance, the drought sensitivity and presence of defective phenotypes at low humidity suggested that stomatal function may be defective in the *dry2* mutant (Hetherington and Woodward, 2003). We therefore investigated whether the *dry2* mutant was impaired in stomatal responses. When the *dry2* mutant was grown at high RH, it had similar stomatal conductance to the wild-type (Figure 2g). In the wild-type, there is a rapid decrease in stomatal conductance when plants are subjected to a decrease in RH. However, this rapid decrease in stomatal conductance was abolished in the *dry2* mutant (Figure 2g), causing an increased sensitivity to dehydration.

The opening and closing of stomata is regulated by hormones (Li *et al.*, 2006). The concentration of the plant stress hormone ABA increases under drought and induces stomatal closure through second messengers such as ROS (Pei *et al.*, 2000; Borsani *et al.*, 2002). When we analysed the stomatal responses of the *dry2* mutant, we found that they were much more insensitive to ABA than wild-type plants were (Figure 2h), indicating that *DRY2* is required for full ABA-induced stomatal closure. We then determined the stomatal responses to ROS by exogenous application of H_2O_2 in both wild-type and the *dry2* mutant. Our analysis showed that stomatal responses were similar (Figure 2i), indicating that signalling downstream of ROS is functional in the *dry2* mutant.

In addition to defects in shoot development (Figure 3a), the root architecture was defective in *dry2* mutants (Figure 3b). The length of the *dry2* primary root was 60% shorter than that of the wild-type 10 days after germination (Figure 3c). In addition, *dry2* seedlings also developed approximately six times more lateral roots than wild-type plants (Figure 3d).

DRY2 encodes a squalene epoxidase protein involved in sterol biosynthesis

To determine the molecular basis of the *dry2* phenotypes, we identified the mutant gene using map-based cloning (Figure S1). *dry2* phenotypes were caused by a point mutation in the 4th exon of the At1g58440 gene, which is annotated as squalene epoxidase 1 (*SQE1*), resulting in substitution of a conserved glycine by an arginine (Figures S1 and S2). The activity of the protein encoded by *SQE1* was recently demonstrated by complementation of a yeast mutant lacking this activity (Rasbery *et al.*, 2007).

To verify that the *dry2* mutation was caused by a mutation in the SQE1 gene, we transformed the *dry2* mutant with a 4.1 kb genomic fragment of *SQE1*, including 0.6 kb of the promoter region, or the cDNA driven by the 35S promoter. All defective *dry2* phenotypes, including reduced shoot growth under low RH and root defects, were complemented by transformation with these gene constructs (Figures 3a–c

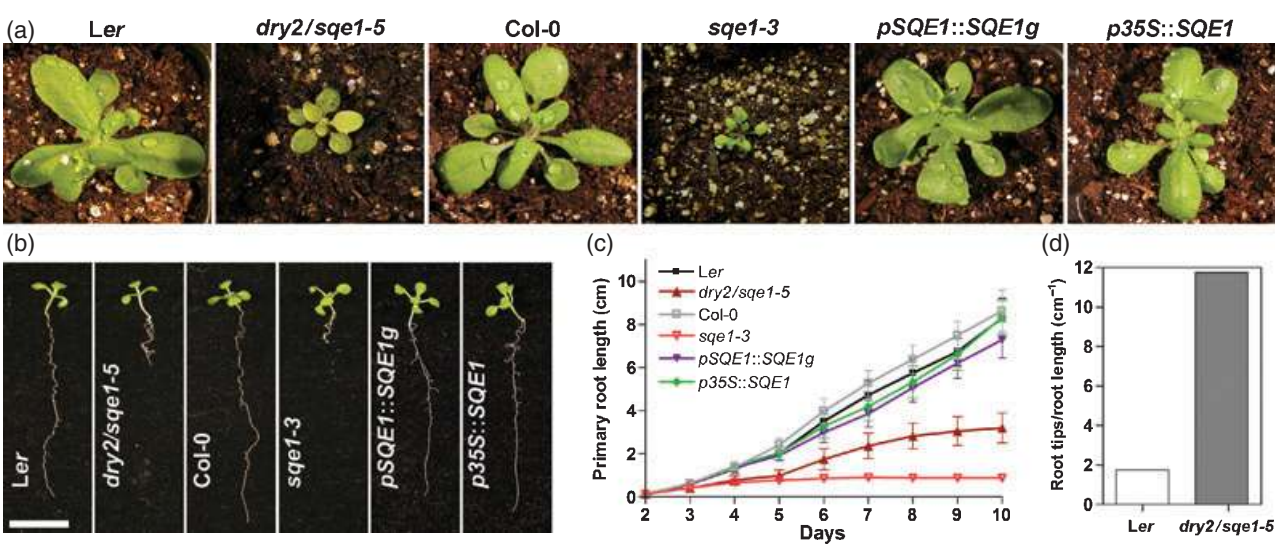

Figure 3. *dry2/sqe1-5* phenotypes are caused by a mutation in the *DRY2/SQE1* gene.
(a, b) Shoot (a) and root (b) phenotypes of L*er*, *dry2/sqe1-5*, wild-type Col-0, *sqe1-3* and one line of *dry2/sqe1-5* complemented with the either a genomic fragment of *DRY2/SQE1* (*pSQE1*::*SQE1g*) or *DRY2/SQE1* cDNA driven by the 35S promoter (*p35S*::*SQE1*). Scale bar = 1 cm.
(c) Primary root growth over 10 days for L*er*, *dry2/sqe1-5*, Col-0, *sqe1-3* and complemented *dry2/sqe1–5* lines.
(d) Root branching of L*er* and the *dry2/sqe1-5* mutant was determined by counting the number of root tips per length of primary root.

and S3). We designated our *dry2* allele of squalene epoxidase as *sqe1-5,* as four other *sqe1* alleles have been reported (Rasbery *et al.*, 2007), and refer to it below as *dry2/sqe1-5*.

The increased sensitivity of the *dry2/sqe1-5* mutant to terbinafine, a specific inhibitor of squalene epoxidase activity, is consistent with a decreased squalene epoxidase activity in the mutant (Figure S4). We then analysed the phenotypes of the loss-of-function *sqe1-3* mutant allele (Rasbery *et al.*, 2007), and compared them to the phenotypes of *dry2/sqe1-5*. The *sqe1-3* (SALK_022763) mutation is caused by a T-DNA insertion in the 7th exon (Figure S1). We found that *sqe1-3* phenotypes are similar but more severe than those exhibited by the *dry2/sqe1-5* mutant. The *sqe1-3* mutant shows both smaller shoot size (Figure 3a) and more reduced root growth compared with the *dry2/sqe1-5* mutant (Figure 3b,c), and, in contrast to *dry2/sqe1-5*, is sterile, suggesting that *dry2/sqe1-5* is a hypomorphic allele of *DRY2/SQE1*.

Auxin and ethylene signalling are not impaired in the *dry2/sqe1-5* mutant

Defective auxin and ethylene signalling are phenotypes that are often associated with mutations in sterol biosynthetic genes, which in turn cause defective root development (Souter *et al.*, 2002, 2004). To investigate whether *dry2/sqe1-5* root phenotypes could be caused by defective auxin signalling as observed in *hyd1* and *fk* mutants, we analysed expression of the GUS reporter gene under the control of the auxin-inducible synthetic promoter *DR5* in a *dry2/sqe1-5* background (Friml *et al.*, 2003). We also analysed the localization of the PIN2–GFP fusion protein in the *dry2/sqe1-5* mutant because polar localization of PIN2 has been shown to be defective in the sterol biosynthesis mutant *cyclopropylsterol isomerase1-1 (cpi1-1)* (Men *et al.*, 2008). Analysis of the *DR5*::*GUS* lines and localization of the PIN2–GFP protein in the *dry2/sqe1-5* mutant indicated a normal auxin distribution and polar localization of PIN2 in roots (Figure 4a,b). Partial phenotypic rescue of root development in the *hyd1* and *fk* mutants occurred when ethylene signalling was inhibited (Souter *et al.*, 2002, 2004). However, *dry2/sqe1-5* seedlings in media containing 10 μM $AgNO_3$, which blocks ethylene perception, were morphologically identical to untreated controls (Figure 4c). In addition, the *dry2/sqe1-5* mutant does not exhibit altered ethylene responses when its triple response is analysed (Figure 4d). These results suggest that auxin or ethylene signalling are not defective in the *dry2/sqe1-5* mutant.

Mutations in *dry2/sqe1-5* alter ROS production and RHD2 localization in roots

Analysis of stomatal responses in the *dry2/sqe1-5* mutant suggests that the production and/or regulation of ROS production might be compromised in the mutant due to altered sterol composition. We therefore analysed whether H_2O_2 accumulation was altered in the *dry2/sqe1-5* mutant using 3,3′-diaminobenzidine (DAB) staining (Davletova *et al.*, 2005). As shown in Figure 5(a), under our growth conditions, the leaves of wild-type plants accumulated high levels of H_2O_2, while *dry2/sqe1-5* mutant leaves did not accumulate detectable levels of H_2O_2. This result, together with the finding that guard cells of the *dry2/sqe1-5* mutant are responsive to exogenous H_2O_2, suggests that the impaired stomatal function in the *dry2/sqe1-5* mutant is due to defective ROS production in the mutant.

A well-characterized role for ROS as second messenger is their involvement in polarized root hair growth, which requires the localized production of ROS by RHD2/AtrbohC NADPH oxidase (Foreman *et al.*, 2003; Carol *et al.*, 2005; Takeda *et al.*, 2008). Therefore, we predicted that, as the control of ROS production is defective in the *dry2/sqe1-5* mutant, root hair growth might also be expected to be defective in this mutant. Almost 80% of root hairs of wild-type plants were over 300 μm in length, but 55% of root hairs of the *dry2/sqe1-5* mutant were shorter than 200 μm (Figure 5b,c). In addition, branched root hairs developed on *dry2/sqe1-5* mutants (Figure 5d, arrow), and more than one hair per trichoblast was often induced (Figure 5d, arrowhead), implicating *DRY2/SQE1* in both root hair initiation and the subsequent maintenance of tip growth. This suggests that the spatial regulation of ROS production is defective in *dry2/sqe1-5* root hairs. To investigate this possibility, we stained apoplastic ROS in wild-type and *dry2/sqe1-5* hairs with nitroblue tetrazolium (NBT) (Carol *et al.*, 2005). ROS accumulates on the surface of the bulge during root hair initiation in wild-type plants and at the tip during growth of the hair (Figure 5e, arrow). In *dry2/sqe1-5* mutants, ROS was not localized to the tip of the root hair, with further sites of production of ROS being observed in the same hair cell (Figure 5e, arrowheads). Therefore, in contrast to *dry2/sqe1-5* leaves, where the main defect was a reduction in the production of ROS, the main defects we observed in root hairs were in ROS localization.

We next hypothesized that the ectopic ROS accumulation observed in the root hairs of the *dry2/sqe1-5* mutant could result from de-localization of the RHD2 NADPH oxidase protein. To investigate this possibility, we introduced a GFP–*RHD2* construct (with *RHD2* promoter and terminator regulatory sequences) into the *dry2/sqe1-5* background. This construct has been shown to complement the defective phenotypes of *rhd2* mutants (Takeda *et al.*, 2008). RHD2 localizes to the tip of wild-type root hairs at early stages of root hair development (Figure 5f,g), and this polar localization is maintained during subsequent root hair growth (Figure 5h,i, arrow). The localization of RHD2 at the site of root hair emergence is identical in *dry2/sqe1-5* mutants and wild-type (Figure 5j,k, arrow). However, RHD2 does not maintain its polar localization during root hair growth in the mutant (Figure 5l–q, arrowheads). Thus it is likely that

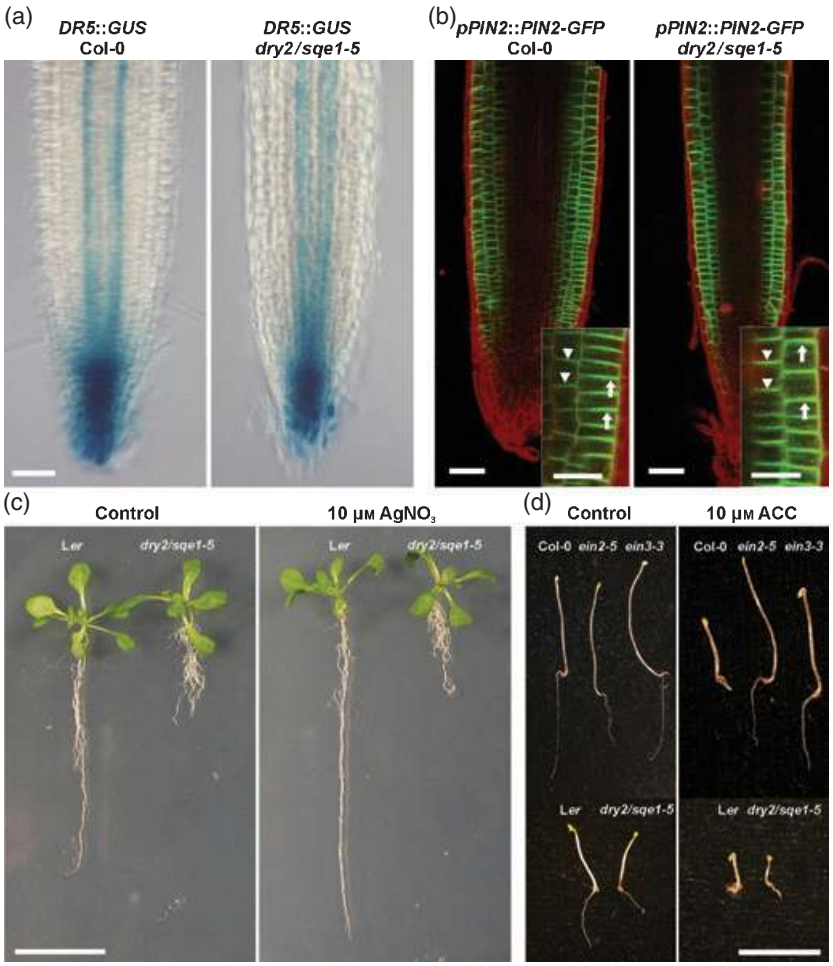

Figure 4. The *dry2/sqe1-5* mutant does not have altered auxin distribution or ethylene signalling.
(a) Expression of the auxin-responsive *DR5::GUS* reporter in roots of a wild-type and a *dry2/sqe1-5* seedling. Scale bar = 50 μm.
(b) Expression of *pPIN2::PIN2-GFP* in Col-0 and *dry2/sqe1-5* roots. The polar PIN2–GFP localization is not altered in the *dry2/sqe1-5* mutant, with the fusion being localized in the basal membranes of cortical cells (arrowheads) and apical membranes of epidermal cells (arrows) in the elongation zone. Scale bar = 50 μm (that of inset = 25 μm).
(c) Treatment with 10 μM of silver nitrate does not rescue *dry2/sqe1-5* root defects. The higher growth of a wild-type root in medium containing 10 μM of silver nitrate indicates that the treatment was effective. Scale bar = 1 cm.
(d) Analysis of ethylene sensitivity using the triple response. The *dry2/sqe1-5* mutant shows a similar sensitivity to ethylene as wild-type plants. The ethylene-insensitive mutants *ein2-5* and *ein3-3* were used as controls. Scale bar = 1 cm.

the ectopic localization of RHD2 in *dry2/sqe1-5* root hairs causes defective ROS production, which in turn can explain the observed root hair defects.

To determine whether defective ROS accumulation was specific to the *dry2/sqe1-5* allele, we investigated ROS production in shoots and during root hair development in *sqe1-3* plants. The *sqe1-3* mutant did not accumulate H_2O_2 in leaves, as shown above for the *dry2/sqe1-5* mutant (Figure 5a). In addition, root hairs of the *sqe1-3* mutant were shorter than those of wild-type (Figure 5b,c), and were defective in both morphology (Figure 5d, arrow) and ROS accumulation (Figure 5e, arrowhead). This shows that this phenotype is caused by the lack of *SQE1* function, and reveals a previously unrecognized role for sterols in the regulation of NADPH oxidases and ROS production.

DRY2/SQE1 is expressed in the shoot and in the elongation zone of the root

To obtain further insight into *dry2/sqe1-5* phenotypes, we investigated the *in planta* expression patterns of *DRY2/SQE1*. To this end, we generated transgenic lines containing fused *GUS–GFP* genes under the control of the *DRY2/SQE1 cis*-regulatory sequences located upstream of the open reading frame. The promoter region selected was identical to that used to complement the *dry2/sqe1-5* mutant. Therefore, we expected that this fragment would indicate wild-type expression of the wild-type *DRY2/SQE1* gene. The GUS expression patterns for eight of the ten transgenic lines analysed were very similar, and Figure 6 shows the GUS expression pattern of a representative transgenic line.

DRY2/SQE1 was expressed in expanded cotyledons and in the tip of primary and secondary roots of 6-day-old seedlings (Figure 6a). This expression in roots was specific and confined to the cortical cells of the elongation zone (Figure 6b–d). These patterns of expression are supported by the publicly available microarray profiling data on tissue-specific expression in Arabidopsis roots (Brady *et al.*, 2007). Interestingly, no expression was observed in root hair cells despite the defects in hair development in *dry2/sqe1-5* mutants (Figure 6e). It is possible that an intermediate downstream of the reaction catalysed by DRY2/SQE1, probably a sterol, travels from

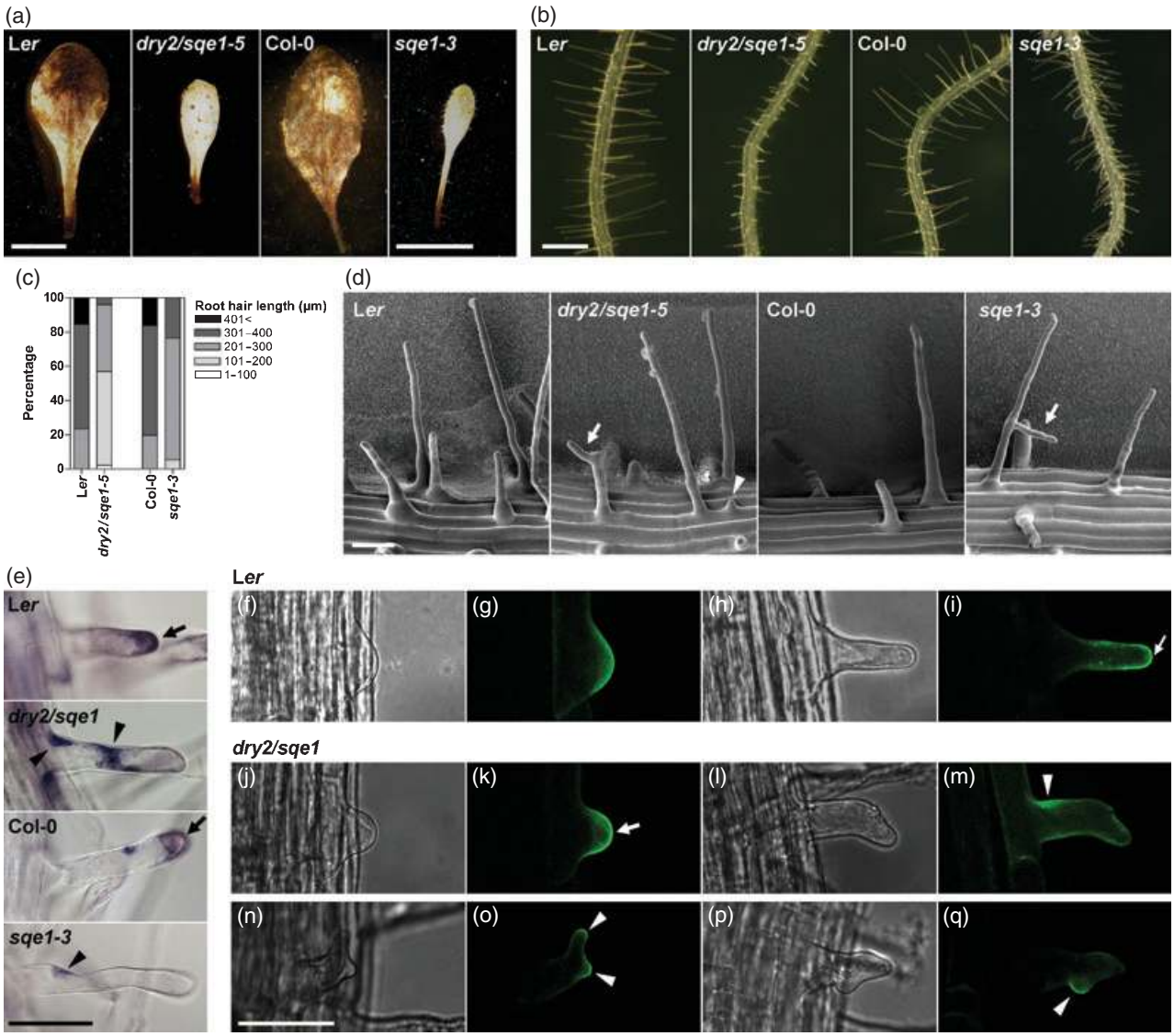

Figure 5. ROS accumulation in both leaves and root hairs is defective in *dry2/sqe1-5* and *sqe1-3* mutants.
The altered morphology of root hairs in *dry2/sqe1-5* mutants is caused by de-localization of RHD2 NADPH oxidase.
(a) Accumulation of H_2O_2 in L*er*, *dry2/sqe1-5*, Col-0 and *sqe1-3*. Compared with wild-type, *dry2/sqe1-5* and *sqe1-3* accumulate almost undetectable levels of H_2O_2. Scale bar = 0.5 cm.
(b–d) Defective root hairs develop on *dry2/sqe1-5* and *sqe1-3* roots.
(b) Roots of L*er*, *dry2/sqe1-5*, Col-0 and *sqe1-3*. Scale bar = 500 μm.
(c) Percentage root hair length of L*er*, *dry2/sqe1-5*, Col-0 and *sqe1-3*. (d) Scanning electron micrograph showing root hair phenotypes. In *dry2/sqe1-5* and *sqe1-3* mutants, approximately 40% of the root hairs were branched (arrows) and showed more than a single site of growth per cell (arrowhead). Scale bar = 50 μm.
(e) ROS localization by staining roots with NBT. O_2^- is localized at the tips of wild-type L*er* and Col-0 root hairs (arrows). In *dry2/sqe1-5* and *sqe1-3* mutants, O_2^- production is localized either at the base or the middle of root hairs (arrowheads), and eventually more than one focus are stained in the same cell (arrowheads). Scale bar = 50 μm.
(f–i) Bright-field (f, h) and confocal images (g, i) of L*er* root hairs showing GFP–RHD2 located at the tip of emerging (f, g) and growing (h, i, arrow) root hairs. Scale bar = 50 μm.
(j–q) Bright-field (j, l, n, p) and confocal images (k, m, o, q) of *dry2/sqe1-5* root hairs showing GFP–RHD2 located at the tip of an emerging root hair (j, k, arrow) but with ectopic accumulation later in development (l, m, arrowhead). This ectopic location eventually results in the development of lateral bulges (n–q, arrowheads). Scale bar = 50 μm.

the cells in which *DRY2/SQE1* is expressed to the root hairs to regulate ROS production and growth. It may also be that the levels of expression of *DRY2/SQE1* are below the detection limit for our reporter construct. In adult leaves, *DRY2/SQE1* was expressed in most cells (Figure 6f), with very strong expression in stomata (Figure 6g), consistent with the altered stomatal responses and defective ROS production found in the *dry2/sqe1-5*

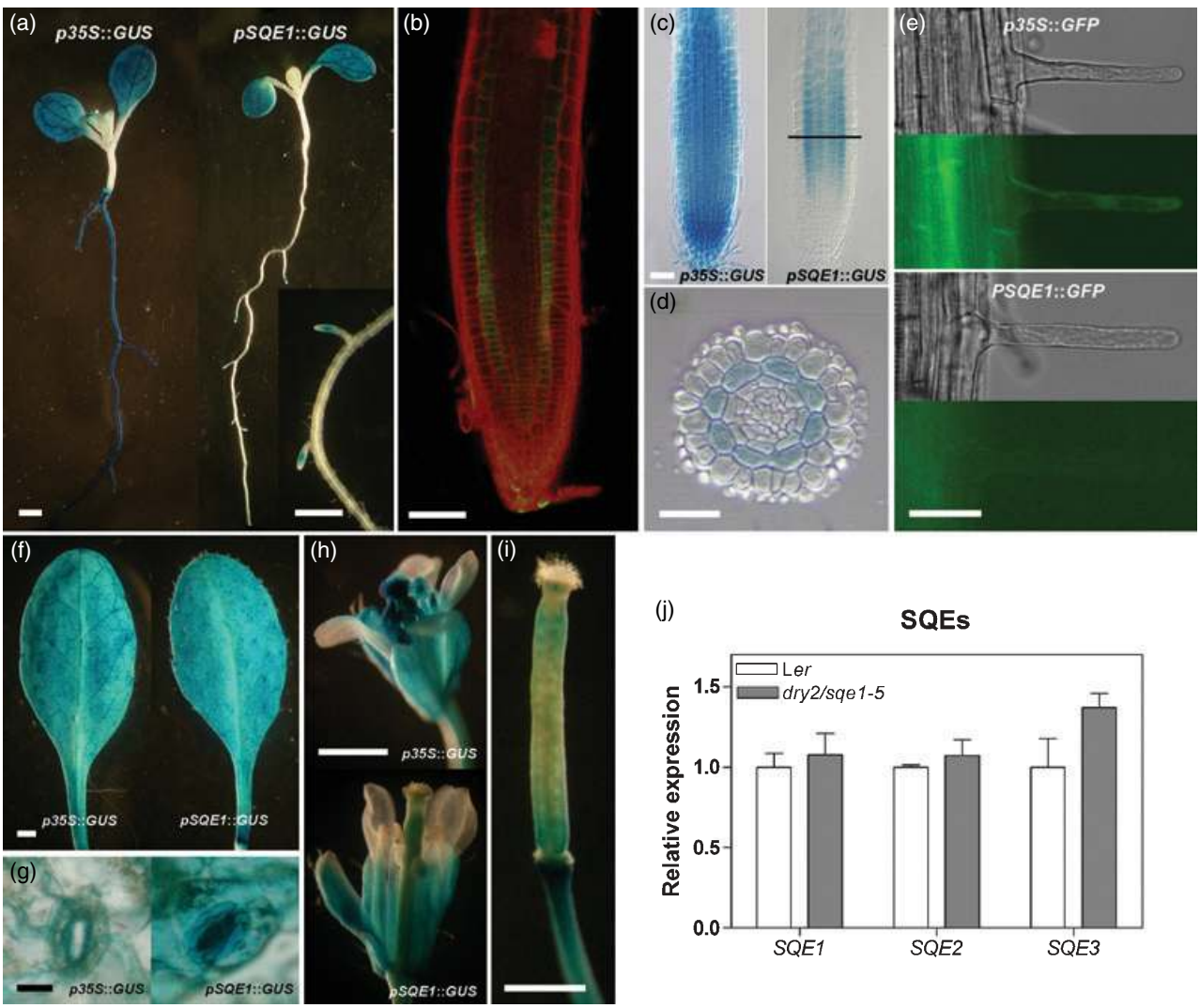

Figure 6. Expression profile of *pSQE1::GUS-GFP* and analysis of squalene epoxidase genes expression using quantitative RT-PCR.
(a) Expression patterns of *p35S::GUS-GFP* and *pSQE1::GUS-GFP* in 6-day-old seedlings. Scale bar = 1 mm. A detail of the root is shown in the inset (scale bar = 0.5 mm).
(b–d) The *SQE1* promoter is expressed in the cortex cells of the elongation zone in the roots. Expression was visualized using a GFP reporter (b). GUS expression is shown in a transverse section (d) at the level indicated by a black line in (c). Scale bar = 50 μm.
(e–i) Expression of *p35S::GUS-GFP* and *pSQE1::GUS-GFP* in a root hair (e), adult leaf (f), stomata cells (g), flower (h) and young silique (i). Scale bars = 50 μm (e), 1 mm (f, h, i) and 10 μm (g)
(j) Quantitative RT-PCR analysis of *SQE1*, *SQE2* and *SQE3* transcripts in wild-type L*er* and the *dry2/sqe1-5* mutant. RNA was extracted from leaves of 20-day-old plants. The signal was normalized relative to the constitutively expressed β-tubulin gene. Data represent mean values ± SD (n = 3).

mutant (Figures 2g–i and 5a). GUS staining was observed in most tissues of the inflorescent stems, sepals, style and developing siliques (Figure 6h,i).

Six genes that are very similar to SQEs have been identified in Arabidopsis. However, only the products of three, *DRY2/SQE1*, *SQE2* and *SQE3*, have been shown to posses SQE activity by functional complementation of yeast. Therefore, the function of *SQE2* and *SQE3* could potentially overlap with that of *DRY2/SQE1* (Rasbery *et al.*, 2007). Data from the Arabidopsis expression database for the *DRY2/SQE1*, *SQE2* and *SQE3* genes were compiled. *DRY2/SQE1* is the SQE that is most highly expressed in roots, *SQE2* is expressed at low levels in most tissues and *SQE3* is highly expressed in leaves and expressed at low levels in roots, which may explain why *dry2/sqe1-5* mutants have defects in root architecture early in development.

Expression analysis of the *SQE* genes in wild-type and the *dry2/sqe1-5* mutant using quantitative RT-PCR revealed a slight increase of *SQE3* transcripts in the mutant (Figure 6j) that could explain the similar sterol profile in shoots of L*er* and the *dry2/sqe1-5* mutant (see below).

Microarray analysis shows that genes involved in stress responses are induced in *dry2/sqe1-5* mutants

Global changes of gene expression in the *dry2/sqe1-5* mutant relative to wild-type shoots were investigated using microarray analysis. The complete data set for the microarray analysis is provided in Table S1. Genes with altered expression in the *dry2/sqe1-5* mutant were classified using gene ontology (GO) categories (Al-Shahrour *et al.*, 2007) (Table S2). 'Response to abiotic stimulus' and 'response to stress' are two of the most over-represented biological processes in the group of up-regulated genes, whereas 'thylakoid'-like cellular components and 'photosynthesis'-like biological processes are over-represented GO categories in the group of down-regulated genes (Table S2). We found that genes related to abiotic stress responses, genes involved in ROS production and detoxification, and genes involved in sterol biosynthesis were de-regulated in the *dry2/sqe1-5* mutant, consistent with the biochemical and molecular analyses (Table S3).

Chemical analysis of the *dry2/sqe1-5* mutant indicates that DRY2/SQE1 is the main squalene epoxidase in the root

We next investigated metabolic changes caused by the *dry2/sqe1-5* mutation by determining the sterol composition in leaves and roots of 15-day-old wild-type and *dry2/sqe1-5* Arabidopsis seedlings (Table 1). Interestingly, and despite the altered expression of some sterol biosynthetic genes in shoots (Table S3), no differences in major sterols were found. On the other hand, in roots of the *dry2/sqe1-5* mutant, there is a 42% reduction of sitosterol and a 60% reduction of stigmasterol, the two major bulk sterols, and a significant accumulation of the 4,4-dimethylsterols cycloartenol and 24-methylene cycloartanol. Interestingly, the effect of the *dry2/sqe1-5* mutation on the sterol profile in shoots and roots is fully consistent with its effect on the levels of squalene, the substrate of SQE enzymes. Slightly increased levels of squalene are detected in the shoots, and a dramatic accumulation of the SQE substrate was observed in the roots (Figure 7a). Moreover, the activity of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR), the major rate-limiting enzyme in the mevalonic acid pathway (Schaller *et al.*, 1995), remains unaltered in shoots but is enhanced approximately 2.8-fold in roots (Figure 7b). This suggests that HMGR is up-regulated in roots in an attempt to compensate for the reduced DRY2/SQE1 activity, which in turn may lead to the dramatic accumulation of squalene observed in this organ. Campesterol levels were similar in wild-type and the *dry2/sqe1-5* mutant, suggesting that defective brassinosteroid biosynthesis does not cause the defective mutant phenotype. This was supported by the inability of epibrassinolide to complement the defective growth of the *dry2/sqe1-5* mutant (Figure S5) or the complete loss-of-function *sqe1-3* allele (Rasbery *et al.*, 2007). The differences in sterol

Table 1 Mass spectral analyses of sterols from L*er* and the *dry2/sqe1-5* mutant

	Root		Shoot	
Sterol	WT (L*er*)	*dry2/sqe1-5*	WT (L*er*)	*dry2/sqe1-5*
Cycloartenol	32[a] (0.76)[b]	125 (4.48)	25 (0.91)	31 (1.14)
24-methylene cycloartanol	46 (1.09)	89 (3.19)	46 (1.67)	47 (1.72)
Isofucosterol	67 (1.59)	50 (1.79)	60 (2.18)	65 (2.38)
Sitosterol	2723 (64.68)	1576 (56.49)	2133 (77.34)	2086 (76.52)
Stigmasterol	894 (21.23)	358 (12.83)	57 (2.07)	52 (1.91)
Campesterol	410 (9.74)	574 (20.57)	395 (14.32)	401 (14.71)
Cholesterol	38 (0.90)	18 (0.65)	42 (1.52)	44 (1.61)

[a]Values are given in micro gram per gram dry weight.
[b]The percentage of the total sterol content is shown in parentheses.

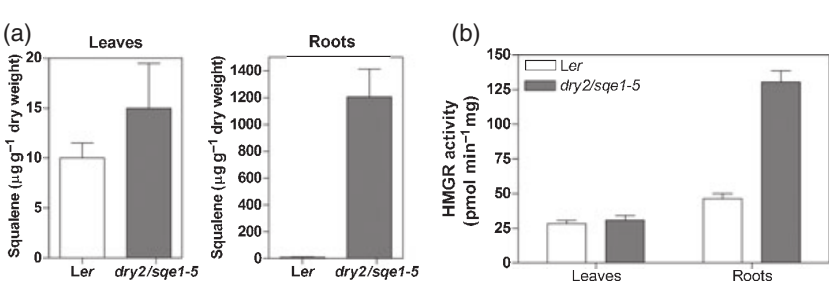

Figure 7. The *dry2/sqe1-5* mutant shows increased accumulation of squalene and higher HMGR activity than wild-type in roots but not in shoots.
(a) Squalene content in leaves and roots of 15-day-old wild-type L*er* and *dry2/sqe1-5* seedlings. Data represent mean values ± SD (n = 3).
(b) HMGR activity in leaves and roots of 15-day-old *dry2/sqe1-5* seedlings. Data represent mean values ± SD (n = 3).

composition in roots suggest that SQE1 provides the main squalene epoxidase activity in this organ, consistent with the low expression of *SQE2* and *SQE3* in roots.

DISCUSSION

The *dry2/sqe1-5* mutant, which has a defective *squalene epoxidase1* gene, was identified because of its extreme sensitivity to dehydration. This sensitivity is caused by the inability of *dry2/sqe1-5* to regulate stomatal closure, resulting in accumulation of proline, chlorophyll degradation and the induction of stress-related genes. Consistent with the stomatal defects, growing *dry2/sqe1-5* plants at high humidity can ameliorate but does not fully restore defective phenotypes. In addition, the morphology of the *dry2/sqe1-5* root system is abnormal, which could also contribute to its impaired growth. These developmental defects correlate with an abnormal ROS production and de-localization of the NADPH oxidase RHD2/AtrbohC, thus suggesting a role for sterols in plant development through the regulation of ROS production.

DRY2/SQE1 has an essential role in sterol composition and plant development

The phenotypes of the *dry2/sqe1-5* mutant are caused by the replacement of a conserved glycine by an arginine in the DRY2/SQE1 protein, resulting in reduced activity of the enzyme, as revealed by the terbinafine hypersensitivity of the mutant. In contrast to other sterol biosynthetic genes in Arabidopsis, the presence of three functional *SQE* genes further complicates elucidation of the role of *DRY2/SQE1* in sterol biosynthesis and plant development. Phenotypic analyses of *DRY2/SQE1* mutant alleles indicate an important role of this DRY2/SQE1 isozyme in root sterol biosynthesis and morphogenesis. Roots of both the *dry2/sqe1-5* mutant (this work) and *sqe1-3* (Rasbery *et al.*, 2007) show an accumulation of the SQE substrate squalene and an increase in HMGR activity, the key regulatory enzyme in sterol biosynthesis (Babiychuk *et al.*, 2008). A similar up-regulation of HMGR activity has been observed in response to pharmacological (Wentzinger *et al.*, 2002; Nieto *et al.*, 2009) and genetic (Babiychuk *et al.*, 2008) blockage of the sterol biosynthetic pathway. These metabolic effects are consistent with the observation that *DRY2/SQE1* expression in roots is higher than that of the remaining two *SQE* genes, and also with the fact that the levels of several downstream sterols are different between *dry2/sqe1-5* and wild-type roots. The levels of the two main bulk sterols sitosterol and stigmasterol are reduced in the mutant, which is expected because of the reduced production of intermediates of sterol biosynthesis. We also found a significant accumulation of cycloartenol and 24-methylene cycloartanol. This result is also to be expected, as sterol-depleted tobacco BY-2 cells with inhibited SQE activity also showed increased levels of these sterols, probably due to secondary regulatory effects (Wentzinger *et al.*, 2002). Thus it is reasonable to assume that changes in membrane sterol composition in root cells may lead to structural defects in cellular membrane networks, and, ultimately, to altered root architecture. However, signalling roles for sterols have previously been proposed in plants (Schrick *et al.*, 2000; Clouse, 2002). This is supported by the identification of lipid/sterol-binding StAR-related lipid transfer (START) protein domains in plants (Schrick *et al.*, 2004b). In fact, START domains are more common in plants than in animals, and are primarily found within homeodomain (HD) transcription factors, suggesting a mechanism by which lipid/sterol ligands can directly modulate transcription in plants (Clouse, 2002). Therefore, we cannot discount that the *dry2/sqe1-5* mutation could cause defective sterol signalling.

Shoots of wild-type and *dry2/sqe1-5* seedlings show similar squalene content, sterol composition and HMGR activity, suggesting that *SQE2* and/or *SQE3* play major roles in sterol biosynthesis in shoots. This is consistent with *SQE3* having the highest expression levels in shoots of all *SQE* genes, and with the increase in *SQE3* expression in *dry2/sqe1-5* shoots. However, the phenotypic differences in the shoots of *sqe1* mutants indicate that the *SQE* genes are not entirely functionally redundant. Therefore, wild-type DRY2/SQE1 activity is needed in shoots or alternatively, root-derived sterols produced by DRY2/SQE1 function during shoot development. In addition, the stomatal defects exhibited by the *dry2/sqe1-5* mutant rule out the possibility that *SQE3* and *DRY2/SQE1* are functionally redundant, as *SQE3* expression is around ten times higher that of *DRY2/SQE1* in stomata (Winter *et al.*, 2007).

dry2/sqe1-5 analysis suggests sterol movement in plants

Promoter–GUS fusion analyses indicate that *DRY2/SQE1* expression is cell type-specific. In roots, the expression of *DRY2/SQE1* is restricted to the cortical cells of the elongation region. This is somewhat unexpected because it is accepted that sterols are essential structural components of the plasma membrane in most cell types. Interestingly, the expression of other sterol biosynthetic genes such *FACKEL*, *HYDRA* and *CPI1* (Jang *et al.*, 2000; Souter *et al.*, 2002; Men *et al.*, 2008) also showed non-overlapping cell type-specific expression in roots, supporting the idea that sterol intermediates move between cells.

The lack of detectable GUS expression in root hairs with an altered morphology supports the idea that sterol intermediates move between cells. Consistent with the high levels of *DRY2/SQE1* expression during embryogenesis, null *sqe1-3* mutant plants do not produce viable seeds. Nevertheless, homozygous *sqe1-3* seeds undergo normal development in heterozygous plants, suggesting that an essential mobile component produced by DRY2/SQE1 (possibly a

sterol) must move from the maternal tissue to allow embryo development. Sterol movement to distal parts in *Solanum chacoense* plants has been demonstrated using ^{14}C-labelled obtusifoliol (O'Brien *et al.*, 2005).

The *dry2/sqe1-5* mutation causes ectopic localization of RHD2 NADPH oxidase and ROS production

Mutations in sterol biosynthetic genes result in structural defects in cellular membranes caused by a depletion of essential sterols. Alterations in hormone signalling (Souter *et al.*, 2002), de-localization of polar proteins (Willemsen *et al.*, 2003; Men *et al.*, 2008) and defective plastid biogenesis (Babiychuk *et al.*, 2008) have been reported in these mutants. According to our results, the *dry2/sqe1-5* mutant does not appear to be defective in auxin or ethylene signalling. We did find, however, that under high-light and low-humidity conditions, the *dry2/sqe1-5* mutant had pale green leaves caused by a reduced chlorophyll content similar to that previously reported for mutants in the *CAS1* gene (Babiychuk *et al.*, 2008), supporting a role for sterols in plastid biogenesis.

Interestingly, many of the developmental defects exhibited by the *dry2/sqe1-5* mutant can be explained by an alteration in the production of ROS. Because at least some of the ROS involved in development are produced by NADPH oxidases, the correct spatial localization of these enzymes is essential. The defective root hairs that develop in the *dry2/sqe1-5* mutant produced ectopic ROS, caused by de-localization of the RHD2/AtrbohC NADPH oxidase. Because RHD2/AtrbohC polar localization depends on endocytic turnover (Takeda *et al.*, 2008), we speculate that this process is defective in the *dry2/sqe1-5* mutant because of the altered sterol composition. Defective endocytosis leading to post-cytokinetic acquisition of PIN2 has also been reported in the sterol *cpi1-1* mutant (Men *et al.*, 2008).

In stomata, H_2O_2 produced by AtrbohD and AtrbohE NADPH oxidases induces ABA activation of Ca^{2+}channels, which in turn increase the cytosolic concentration of Ca^{2+} and stomatal closure (Kwak *et al.*, 2003; Torres *et al.*, 2005). The defective ROS production in *dry2/sqe1-5* leaves may cause insensitivity of guard cells to ABA. There is no information available as to whether the localization of AtrbohD and/or AtrbohE NADPH oxidases is dependent on vesicle trafficking, although *dry2/sqe1-5* analysis supports the model that sterol composition is important for the localization and/or activity of these proteins.

In summary, sterols are essential components in maintaining protein–lipid interactions, and for vesicle trafficking, docking and recycling in plant cells. In this study, we report a previously unrecognized role for sterols in the regulation of ROS through localization of RHD2 NADPH oxidase. Defective localization of NADPH oxidases is expected to have a profound impact in many aspects of plant development, and may account for many of the developmental abnormalities observed in sterol-deficient mutants.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

The *Arabidopsis thaliana* ecotypes Landsberg *erecta* (L*er*) and Columbia-0 (Col-0) were used as wild-type controls. The Col-0 T-DNA insertion mutants were identified in the SIGnAL website (http://signal.salk.edu) and obtained from the Arabidopsis Biological Resource Stock Center (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/index.html). Other mutant and transgenic lines used in this study have been described previously: *DR5*::*GUS* (Ulmasov *et al.*, 1997), *pPIN2*::*PIN2-GFP* (Xu and Scheres, 2005), *ein2-5* (Alonso *et al.*, 1999) and *ein3-3* (Chao *et al.*, 1997). The plants were grown using a mixture of organic substrate and vermiculite (4:1 v/v) under controlled conditions: 21–22°C, 16 h light/8 h dark cycle with a photon flux density (PFD) of approximately 150 μE m^{-2} sec^{-1}, 35% RH or 90% RH using Conviron growth chambers (http://www.conviron.com). For experiments in plates, seeds were surface-sterilized and sown on Phytagel (Sigma, http://www.sigmaaldrich.com) or agar-solidified MS medium including 1.5% sucrose, 0.5 g/l MES, pH 5.7, in culture rooms with a 16 h light/8 h dark cycle at 23°C.

Mutant screening and dehydration experiment

For identification of *dry2/sqe1*-5, a population of approximately 50 000 EMS-mutagenized M_2 L*er* seeds (Lehle Seeds, http://www.arabidopsis.com) was used for the screening. Plants were grown for 2 weeks with abundant nutrient solution. Watering was withheld until mild shoot wilting had occurred. Mutants with decreased drought tolerance relative to wild-type plants were visually identified and used for further analysis. For the dehydration experiments, L*er* and *dry2/sqe1-5* seeds were grown for 12 days in MS medium, transferred to perlite and grown for 7 days with abundant half-strength Hoagland nutrient solution. After that, watering was withheld and the phenotype was monitored for 8 days.

Determination of proline and chlorophyll contents

Proline was extracted and quantified as described previously (Borsani *et al.*, 2001). Chlorophyll extraction and quantification were performed in 20-day-old plants as previously described (Canfield *et al.*, 1995).

Imaging and microscopy

A Leica Wild M10 stereomicroscope (http://www.leica.com) coupled to a Nikon Coolpix 4500 digital camera (http://www.nikon.com) was used for the observation and documentation of seedlings, roots and root hairs, DAB staining of leaves, and GUS analysis of seedlings, leaves, flowers and siliques. The GUS analysis in roots and stomata cells and NBT staining were documented using a Nikon Eclipse E800 microscope with DIC optics. Nikon Eclipse E600 and confocal Leica TCS SP microscopes were used for GFP visualization. *pPIN2*::*PIN2GFP* seedlings were stained using 10 μg/ml propidium iodide (Sigma) for 5 min. For double labelling using propidium iodide and GFP, propidium iodide was visualized at wavelengths 605–665 nm and GFP at wavelengths 521–561 nm. Scanning electron micrographs were obtained using an FEI/Philips XL30-FEG (FEI Philips, http://www.fei.com) microscope as previously described (Carol *et al.*, 2005). For confocal and SEM analysis, the seedlings were grown on Phytagel medium and analysed at 2–5 days old. Images were processed using ImageJ (http://rsb.info.nih.gov/ij/index.html).

Brassinosteroids, silver nitrate and triple response experiments

dry2/sqe1-5 seeds were germinated and grown on MS medium supplemented with the indicated concentrations of 24-epibrassinolide and silver nitrate (Sigma-Aldrich, http://www.sigmaaldrich.com/). The triple response assay was performed as previously described (Guzmán and Ecker, 1990).

Mapping

The F_2 population from a cross between *dry2/sqe1-5* (L*er*) and ecotype Col-0 was used for mapping of the *DRY2/SQE1* gene. Genomic DNA was extracted from 556 F_2 plants showing the phenotype conferred by *dry2/sqe1-5*. All information regarding the genetic markers used in the map-based cloning was obtained from TAIR (http://www.arabidopsis.org). The primers used in the diagnostic PCRs of SALK_022763 plants were as follows: T-DNA-specific primer SALK LBa1, 5′-TGGTTCACGTAGTGGGCCATCG-3′, *SQE1* DPCR-F, 5′-TGAACATTTGGTTTCTCCAACT-3′, and *SQE1*DPCR-R, 5′-AGCATGCCAGCTTCTCCTTA-3′.

dry2/sqe1-5 complementation

For complementation of the *dry2/sqe1-5* mutation, two constructs were used. For the first, a 4.1 kb fragment containing the wild-type *DRY2/SQE1* sequence was cloned into the pBINPLUS binary vector (van Engelen *et al.*, 1995). For the second, the U24589 clone, provided by ABRC, which contains *SQE1* cDNA, was cloned into the pENTR™/D-TOPO vector (Gateway® system, Invitrogen, http://www.invitrogen.com). The cDNA was sub-cloned into the binary pMDC32 vector (Curtis and Grossniklaus, 2003) using LR clonase mix (Invitrogen) to put the cDNA under the control of the 35S promoter. The resulting plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101, and then introduced into the *dry2/sqe1-5* mutant by the floral dipping method (Clough and Bent, 1998).

Whole-plant stomatal conductance

Leaf stomatal conductance to water vapour was measured using a Model SC-1 leaf porometer (Decagon Devices, http://www.decagon.com) in 20–30-day-old leaves. Three leaves each from three different plants were analysed for each genotype. Measurements were performed in leaves of plants grown at 90% RH, 4 h after being transferred to 35% RH conditions, after being sprayed with 0, 0.2, 2 or 20 μM of ABA with 0.1% Tween-20, or after being sprayed with 0 or 1 mM of H_2O_2 with 0.1% Tween-20.

Histochemical analyses

Hydrogen peroxide was localized in leaves using 3,3′-diaminobenzidine (DAB) (Sigma) according to a procedure described by Orozco-Cardenas and Ryan (1999). The leaves were cleared in 96% boiling ethanol.

Nitroblue tetrazolium (NBT Colour Development Substrate, Promega, http://www.promega.com) was used to stain for the site of superoxide production (Fryer *et al.*, 2002). Five-day-old seedlings grown on Phytagel-solidified MS medium were stained as previously described (Carol *et al.*, 2005).

T_2 *pSQE1::GUS* homozygous lines were used for the histochemical localization of GUS activity. The GUS activity in plant samples (except for root analysis) or whole seedlings was detected as previously described (Rosado *et al.*, 2006b).

Roots were stained for GUS activity as previously described (Ortega-Martínez *et al.*, 2007). Stained and fixed roots were embedded in Technovit 7100® resin (Kulzer GmbH, http://www.heraeus-kulzer.com) according to the manufacturer's instructions, and 10–20 μm transverse sections were obtained.

Sterol analysis and measurement of HMGR activity

Sterol levels were measured as previously described (Masferrer *et al.*, 2002). HMGR activity was assayed in the 200 ***g*** supernatant fraction of extracts from shoots and roots of plants as previously described (Masferrer *et al.*, 2002).

Terbinafine sensitivity analysis

L*er* and *dry2/sqe1-5* seeds were sown on MS medium supplemented with the indicated concentrations of terbinafine (Tb) (supplied by Novartis Farmacéutica, http://www.novartis.es). Plates were incubated in a growth chamber for 20 days with an 8 h light/16 h dark cycle at 23°C.

Quantitative RT-PCR analysis

The protocols used for RT-PCR were essentially as previously described (Rosado *et al.*, 2006a). Total RNA was isolated from 20-day-old leaves using TRIZOL® reagent (Invitrogen) according to the manufacturer's instructions. One microgram of total RNA was used for the reverse transcription using an iScript™ cDNA synthesis kit (Bio-Rad, http://www.bio-rad.com) according to the manufacturer's protocol. PCR was performed using iQ™ SYBR® Green Supermix (Bio-Rad). PCR conditions were one cycle of 94°C for 3 min, 30 cycles of 94°C for 35 sec, annealing temperature (see below) for 40 sec and 72°C for the extension time (see below). The primers used were as follows: *SQE1* (annealing temperature 61°C; extension time 18 sec), forward, 5′-ATCTTTGCTTTCGGGTTTGA-3′ and reverse 5′-GCCTAACTCCTTCCGCTTTT-3′; *SQE2* (annealing temperature 61°C; extension time 30 sec), forward, 5′-CTCTACATTTTGCGTCGGAGT-3′ and reverse, 5′-CAACGATTCTGTCTTGCTCAG-3′; *SQE3* (annealing temperature 61°C; extension time 18 sec), forward, 5′-CGAATGGAGCAAGGAACAGT-3′ and reverse, 5′-CCAATACCAGACCAACGAAA-3′; *TUBULIN* β-5 chain (annealing temperature 55°C; extension time 50 sec), forward, 5′-CCTGATAACTTCGTCTTTGG-3′ and reverse, 5′-GTGAACTCCATCTCGTCCAT-3′.

Microarray analysis

Genome-wide expression studies were performed with three pools of 15 different plants each using the Complete Arabidopsis Transcriptome Microarray (CATMA) (Appendix S1). The RNA extraction was performed in 20-day-old L*er* and *dry2/sqe1-5* leaves using the TRIZOL® method. The RNA was purified using a RNeasy Plant Mini Kit (Qiagen, http://www.qiagen.com).

Over-represented GO categories were obtained from microarray data for de-regulated genes with a P value < 0.01 using the web-based tool FatiGO+ (Al-Shahrour *et al.*, 2007) and CYTOSCAPE software (Shannon *et al.*, 2003) with the BinGO plugin (Maere *et al.*, 2005), using the hypergeometric statistical test with the Benjamini and Hochberg false discovery rate correction and a P value < 0.01.

ACKNOWLEDGEMENTS

This work was supported by the Ministerio de Educación y Ciencia (grant BIO2005-4733 and grant BIO2008-1709 to M.A.B. and grant BFU2006-0544 to A.F.) and by grant FCE2007_498 to O.B. D.P. and I.C. were supported by FPU (Formación del Profesorado Universitario) and FPI (Formación del Personal Investigador) fellowships, respectively, from the Ministerio de Educación y Ciencia. We thank Roberto Solano (CNB-CSIC, Madrid, Spain) for providing us

with *ein2-5* and *ein3-3* mutant seeds. We thank J. Botella (The University of Queensland, Brisbane, Australia) for critical reading of the manuscript, and M. Pernas, C. Kim, M. Izaguirre, S. Takeda (John Innes Centre, Norwich, UK) and A. Esteban for technical assistance.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. The *DRY2/SQE1* gene encodes a squalene epoxidase involved in sterol biosynthesis.

Figure S2. Sequence alignment of SQE enzymes.

Figure S3. *SQE1* complements the defective root hair phenotypes of the *dry2/sqe1-5* mutant.

Figure S4. *dry2/sqe1-5* seedlings are more sensitive to the squalene epoxidase inhibitor terbinafine than wild-type is.

Figure S5. Brassinosteroids do not rescue *dry2/sqe1-5* root defects.

Table S1. Complete data set for the microarray analysis.

Table S2. Gene ontology (GO) categories that are statistically over-represented, and functional classification of genes deregulated in the *dry2/sqe1-5* mutant.

Table S3. De-regulated genes in *dry2/sqe1-5* plants.

Appendix S1. Experimental procedures for CATMA arrays.

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REFERENCES

Alonso, J.M., Hirayama, T., Roman, G., Nourizadeh, S. and Ecker, J.R. (1999) EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. *Science*, **284**, 2148–2152.

Al-Shahrour, F., Minguez, P., Tárraga, J., Medina, I., Alloza, E., Montaner, D. and Dopazo, J. (2007) FatiGO+: a functional profiling tool for genomic data. Integration of functional annotation, regulatory motifs and interaction data with microarray experiments. *Nucleic Acids Res.* **35**, W91–W96.

Babiychuk, E., Bouvier-Navé, P., Compagnon, V., Suzuki, M., Muranaka, T., Van Montagu, M., Kushnir, S. and Schaller, H. (2008) Allelic mutant series reveal distinct functions for Arabidopsis cycloartenol synthase 1 in cell viability and plastid biogenesis. *Proc. Natl Acad. Sci. USA*, **105**, 3163–3168.

Benveniste, P. (2004) Biosynthesis and accumulation of sterols. *Annu. Rev. Plant Biol.* **55**, 429–457.

Borsani, O., Cuartero, J., Fernandez, J.A., Valpuesta, V. and Botella, M.A. (2001) Identification of two loci in tomato reveals distinct mechanisms for salt tolerance. *Plant Cell*, **13**, 873–887.

Borsani, O., Cuartero, J., Valpuesta, V. and Botella, M.A. (2002) Tomato tos1 mutation identifies a gene essential for osmotic tolerance and abscisic acid sensitivity. *Plant J.* **32**, 905–914.

Brady, S.M., Orlando, D.A., Lee, J.Y., Wang, J.Y., Koch, J., Dinneny, J.R., Mace, D., Ohler, U. and Benfey, P.N. (2007) A high-resolution root spatiotemporal map reveals dominant expression patterns. *Science*, **318**, 801–806.

Canfield, M.R., Guiamet, J.J. and Nooden, L.D. (1995) Alteration of soybean seedling development in darkness and light by the stay-green mutation *cytG* and Gd_1d_2. *Ann. Bot.* **75**, 143–150.

Carland, F., Fujioka, S., Takatsuto, S., Yoshida, S. and Nelson, T. (2002) The identification of CVP1 reveals a role for sterols in vascular patterning. *Plant Cell*, **14**, 2045–2058.

Carol, R., Takeda, S., Linstead, P., Durrant, M., Kakesova, H., Derbyshire, P., Drea, S., Zarsky, V. and Dolan, L. (2005) A RhoGDP dissociation inhibitor spatially regulates growth in root hair cells. *Nature*, **438**, 1013–1016.

Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W. and Ecker, J. (1997) Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. *Cell*, **89**, 1133–1144.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.

Clouse, S. (2002) Arabidopsis mutants reveal multiple roles for sterols in plant development. *Plant Cell*, **14**, 1995–2000.

Curtis, M.D. and Grossniklaus, U. (2003) A gateway cloning vector set for high-throughput functional analysis of genes *in planta*. *Plant Physiol.* **133**, 462–469.

Davletova, S., Rizhsky, L., Liang, H., Shengqiang, Z., Oliver, D., Coutu, J., Shulaev, V., Schlauch, K. and Mittler, R. (2005) Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of Arabidopsis. *Plant Cell*, **17**, 268–281.

Diener, A., Li, H., Zhou, W., Whoriskey, W., Nes, W. and Fink, G. (2000) Sterol methyltransferase 1 controls the level of cholesterol in plants. *Plant Cell*, **12**, 853–870.

van Englen, F.A., Molthoff, J.W., Connor, A.J., Nap, J.P., Pereira, A. and Stiekema, W.J. (1995) pBINPLUS: an improved plant transformation vector based on pBIN19. *Transgenic Res.* **4**, 288–290.

Foreman, J., Demidchik, V., Bothwell, J. *et al.* (2003) Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature*, **422**, 442–446.

Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R. and Jürgens, G. (2003) Efflux-dependent auxin gradients establish the apical–basal axis of Arabidopsis. *Nature*, **426**, 147–153.

Fryer, M.J., Oxborough, K., Mullineaux, P.M. and Baker, N.R. (2002) Imaging of photo-oxidative stress responses in leaves. *J. Exp. Bot.* **53**, 1249–1254.

Gapper, C. and Dolan, L. (2006) Control of plant development by reactive oxygen species. *Plant Physiol.* **141**, 341–345.

Guzmán, P. and Ecker, J. (1990) Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. *Plant Cell*, **2**, 513–523.

Hartmann, A.M. (1998) Plant sterols and the membrane environment. *Trends Plant Sci.* **3**, 170–175.

Hetherington, A.M. and Woodward, F.I. (2003) The role of stomata in sensing and driving environmental change. *Nature*, **424**, 901–908.

Jang, J., Fujioka, S., Tasaka, M., Seto, H., Takatsuto, S., Ishii, A., Aida, M., Yoshida, S. and Sheen, J. (2000) A critical role of sterols in embryonic patterning and meristem programming revealed by the *fackel* mutants of *Arabidopsis thaliana*. *Genes Dev.* **14**, 1485–1497.

Keller, T., Damude, H.G., Werner, D., Doerner, P., Dixon, R.A. and Lamb, C. (1998) A plant homolog of the neutrophil NADPH oxidase *gp91phox* subunit gene encodes a plasma membrane protein with Ca^{2+} binding motifs. *Plant Cell*, **10**, 255–266.

Kim, H., Schaller, H., Goh, C., Kwon, M., Choe, S., An, C., Durst, F., Feldmann, K. and Feyereisen, R. (2005) Arabidopsis *cyp51* mutant shows postembryonic seedling lethality associated with lack of membrane integrity. *Plant Physiol.* **138**, 2033–2047.

Kwak, J.M., Mori, I.C., Pei, Z.M., Leonhardt, N., Torres, M.A., Dangl, J.L., Bloom, R.E., Bodde, S., Jones, J.D. and Schroeder, J.I. (2003) NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in Arabidopsis. *EMBO J.* **22**, 2623–2633.

Kwak, J.M., Nguyen, V. and Schroeder, J.I. (2006) The role of reactive oxygen species in hormonal responses. *Plant Physiol.* **141**, 323–329.

Li, S., Assmann, S. and Albert, R. (2006) Predicting essential components of signal transduction networks: a dynamic model of guard cell abscisic acid signaling. *PLoS Biol.* **4**, e312.

Maere, S., Heymans, K. and Kuiper, M. (2005) BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. *Bioinformatics*, **21**, 3448–3449.

Masferrer, A., Arro, M., Manzano, D., Schaller, H., Fernandez-Busquets, X., Moncalean, P., Fernandez, B., Cunillera, N., Boronat, A. and Ferrer, A. (2002) Overexpression of *Arabidopsis thaliana* farnesyl diphosphate synthase (FPS1S) in transgenic Arabidopsis induces a cell death/senescence-like response and reduced cytokinin levels. *Plant J.* **30**, 123–132.

Men, S., Boutté, Y., Ikeda, Y., Li, X., Palme, K., Stierhof, Y.D., Hartmann, M.A., Moritz, T. and Grebe, M. (2008) Sterol-dependent endocytosis mediates post-cytokinetic acquisition of PIN2 auxin efflux carrier polarity. *Nature Cell Biol.* **10**, 237–244.

Nieto, B., Forés, O., Arró, M. and Ferrer, A. (2009) Arabidopsis 3-hydroxy-3-methylglutaryl-CoA reductase is regulated at the post-translational level in response to alterations of the sphingolipid and the sterol biosynthetic pathways. *Phytochemistry*, **70**, 58–64.

O'Brien, M., Chantha, S.C., Rahier, A. and Matton, D.P. (2005) Lipid signaling in plants. Cloning and expression analysis of the obtusifoliol 14α-demethylase from Solanum chacoense Bitt., a pollination- and fertiliza-

tion-induced gene with both obtusifoliol and lanosterol demethylase activity. *Plant Physiol.* **139**, 734–749.

Orozco-Cardenas, M. and Ryan, C.A. (1999) Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. *Proc. Natl Acad. Sci. USA*, **96**, 6553–6557.

Ortega-Martínez, O., Pernas, M., Carol, R. and Dolan, L. (2007) Ethylene modulates stem cell division in the *Arabidopsis thaliana* root. *Science*, **317**, 507–510.

Pei, Z.M., Murata, Y., Benning, G., Thomine, S., Klusener, B., Allen, G.J., Grill, E. and Schroeder, J.I. (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature*, **406**, 731–734.

Phillips, D., Rasbery, J., Bartel, B. and Matsuda, S. (2006) Biosynthetic diversity in plant triterpene cyclization. *Curr. Opin. Plant Biol.* **9**, 305–314.

Rasbery, J., Shan, H., Leclair, R., Norman, M., Matsuda, S. and Bartel, B. (2007) *Arabidopsis thaliana* SQUALENE EPOXIDASE 1 is essential for root and seed development. *J. Biol. Chem.* **282**, 17002–17013.

Rosado, A., Amaya, I., Valpuesta, V., Cuartero, J., Botella, M. and Borsani, O. (2006a) ABA- and ethylene-mediated responses in osmotically stressed tomato are regulated by the TSS2 and TOS1 loci. *J. Exp. Bot.* **57**, 3327–3335.

Rosado, A., Schapire, A., Bressan, R., Harfouche, A., Hasegawa, P., Valpuesta, V. and Botella, M. (2006b) The Arabidopsis tetratricopeptide repeat-containing protein TTL1 is required for osmotic stress responses and abscisic acid sensitivity. *Plant Physiol.* **142**, 1113–1126.

Schaller, H., Grausem, B., Benveniste, P., Chye, M.L., Tan, C.T., Song, Y.H. and Chua, N.H. (1995) Expression of the Hevea brasiliensis (H.B.K.) Mull 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 in tobacco results in sterol overproduction. *Plant Physiol.* **109**, 761–770.

Schaller, H. (2003) The role of sterols in plant growth and development. *Prog. Lipid Res.* **42**, 163–175.

Schaller, H. (2004) New aspects of sterol biosynthesis in growth and development of higher plants. *Plant Physiol. Biochem.* **42**, 465–476.

Schrick, K., Mayer, U., Horrichs, A., Kuhnt, C., Bellini, C., Dangl, J., Schmidt, J. and Jürgens, G. (2000) FACKEL is a sterol C-14 reductase required for organized cell division and expansion in Arabidopsis embryogenesis. *Genes Dev.* **14**, 1471–1484.

Schrick, K., Mayer, U., Martin, G., Bellini, C., Kuhnt, C., Schmidt, J. and Jürgens, G. (2002) Interactions between sterol biosynthesis genes in embryonic development of Arabidopsis. *Plant J.* **31**, 61–73.

Schrick, K., Fujioka, S., Takatsuto, S., Stierhof, Y.D., Stransky, H., Yoshida, S. and Jürgens, G. (2004a) A link between sterol biosynthesis, the cell wall, and cellulose in Arabidopsis. *Plant J.* **38**, 227–243.

Schrick, K., Nguyen, D., Karlowski, W. and Mayer, K. (2004b) START lipid/sterol-binding domains are amplified in plants and are predominantly associated with homeodomain transcription factors. *Genome Biol.* **5**, R41.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B. and Ideker, T. (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* **13**, 2498–2504.

Souter, M., Topping, J., Pullen, M., Friml, J., Palme, K., Hackett, R., Grierson, D. and Lindsey, K. (2002) *hydra* mutants of Arabidopsis are defective in sterol profiles and auxin and ethylene signaling. *Plant Cell*, **14**, 1017–1031.

Souter, M.A., Pullen, M.L., Topping, J.F., Zhang, X. and Lindsey, K. (2004) Rescue of defective auxin-mediated gene expression and root meristem function by inhibition of ethylene signalling in sterol biosynthesis mutants of Arabidopsis. *Planta*, **219**, 773–783.

Takeda, S., Gapper, C., Kaya, H., Bell, E., Kuchitsu, K. and Dolan, L. (2008) Local positive feedback regulation determines cell shape in root hair cells. *Science*, **319**, 1241–1244.

Torres, M. and Dangl, J. (2005) Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. *Curr. Opin. Plant Biol.* **8**, 397–403.

Torres, M., Jones, J. and Dangl, J. (2005) Pathogen-induced, NADPH oxidase-derived reactive oxygen intermediates suppress spread of cell death in *Arabidopsis thaliana*. *Nat Genet.* **37**, 1130–1134.

Torres, M.A., Onouchi, H., Hamada, S., Machida, C., Hammond-Kosack, K.E. and Jones, J.D. (1998) Six *Arabidopsis thaliana* homologues of the human respiratory burst oxidase (gp91phox). *Plant J.* **14**, 365–370.

Ulmasov, T., Murfett, J., Hagen, G. and Guilfoyle, T. (1997) Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell*, **9**, 1963–1971.

Wentzinger, L., Bach, T. and Hartmann, M. (2002) Inhibition of squalene synthase and squalene epoxidase in tobacco cells triggers an up-regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Plant Physiol.* **130**, 334–346.

Willemsen, V., Friml, J., Grebe, M., van den Toorn, A., Palme, K. and Scheres, B. (2003) Cell polarity and PIN protein positioning in Arabidopsis require STEROL METHYLTRANSFERASE1 function. *Plant Cell*, **15**, 612–625.

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G.V. and Provart, N.J. (2007) An 'electronic fluorescent pictograph' browser for exploring and analyzing large-scale biological data sets. *PLoS ONE*, **2**, e718.

Xu, J. and Scheres, B. (2005) Dissection of Arabidopsis ADP-RIBOSYLATION FACTOR 1 function in epidermal cell polarity. *Plant Cell*, **17**, 525–536.

