The SUD1 Gene Encodes a Putative E3 Ubiquitin Ligase and Is a Positive Regulator of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Activity in Arabidopsis

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The 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) enzyme catalyzes the major rate-limiting step of the mevalonic acid (MVA) pathway from which sterols and other isoprenoids are synthesized. In contrast with our extensive knowledge of the regulation of HMGR in yeast and animals, little is known about this process in plants. To identify regulatory components of the MVA pathway in plants, we performed a genetic screen for second-site suppressor mutations of the Arabidopsis thaliana highly drought-sensitive drought hypersensitive2 (dry2) mutant that shows decreased squalene epoxidase activity. We show that mutations in SUPPRESSOR OF DRY2 DEFECTS1 (SUD1) gene recover most developmental defects in dry2 through changes in HMGR activity. SUD1 encodes a putative E3 ubiquitin ligase that shows sequence and structural similarity to yeast Degradation of α factor (Doa10) and human TEB4, components of the endoplasmic reticulum–associated degradation C (ERAD-C) pathway. While in yeast and animals, the alternative ERAD-L/ERAD-M pathway regulates HMGR activity by controlling protein stability, SUD1 regulates HMGR activity without apparent changes in protein content. These results highlight similarities, as well as important mechanistic differences, among the components involved in HMGR regulation in plants, yeast, and animals.

INTRODUCTION

For sessile organisms such as plants, metabolic plasticity is essential to survive in their changing environments (Nicotra et al., 2010). A good example of this plasticity is the thousands of isoprenoid compounds and derivatives that higher plants synthesize from the five-carbon building units isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (Bouvier et al., 2005). Plants synthesize IPP and dimethylallyl diphosphate by two independent pathways: the mevalonic acid (MVA) pathway, which produces cytosolic IPP (McGarvey and Croteau, 1995; Newman and Chappell, 1999); and the methylerythritol phosphate pathway, which is localized in the plastids (Eisenreich et al., 2001; Rodríguez-Concepción and Boronat, 2002). In higher plants, isoprenoids carry out numerous essential roles in developmental processes, including respiration, photosynthesis, growth, and reproduction, as well as adaptation to environmental challenges and involvement in plant defense mechanisms against different types of organisms (Tholl and Lee, 2011; Hemmerlin et al., 2012). The main MVA-derived isoprenoid end products in plants are sterols, which are integral components of the membrane and are essential for plant growth and developmental processes. Other important MVA products are the steroid hormones brassinosteroids, dolichols, which are involved in protein glycosylation, and the prenyl groups used for protein prenylation and cytokinin biosynthesis (Benveniste, 2004; Phillips et al., 2006; Schaller, 2010). A number of studies over the years have shown the importance of correct sterol composition in plants because of their roles in embryonic pattern formation (Jang et al., 2000), cell division, elongation and polarity (Schrick et al., 2000; Willemsen et al., 2003; Men et al., 2008), vascular patterning (Carland et al.,
chemical analysis indicated that the been recently identi
induce changes in the HMGR transcript levels (Wentzinger et al.,
hindrance of squalene epoxidase (SQE) activity in tobacco (#
pathway has been obtained (Nieto et al., 2009). Similarly, in-
ition in shoots, indicating an essential role for SQE1 in root
altered sterol composition in roots but wild-type sterol compo-
ent (Hemmerlin et al., 2012). In fact, evidence of post-
fis signals, and it has been proposed that major changes in HMGR
modulated by a variety of developmental and environmental
es (Boutté and Grebe, 2009; Clouse, 2002).

The enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR)
is considered the major rate-limiting enzyme controlling the
metabolic flux in the early steps of the MVA pathway (Hemmerlin
et al., 2012). The genome of Arabidopsis thaliana contains two dif-
fierentially expressed HMGR genes, HMG1 and HMG2 (Enjuto
et al., 1994), encoding three HMGR isoforms: HMGR1S (short
form), HMGR1L (long form), and HMGR2. HMGR1S and
HMGR1L are both encoded by the HMG1 gene and are iden-
tical in sequence, except for an N-terminal extension of
50 amino acid residues in HMGR1L (Lumbrañas et al., 1995).
HMGR1S has been proposed to have a housekeeping role,
whereas HMGR1L and HMGR2 have a more specialized
function, which might be required in particular cell types or at
specific developmental stages (Suzuki et al., 2004, 2009). All
plant HMGR variants are targeted to the endoplasmic reticulum
(ER) and have the same topology in the membrane (Campos
and Boronat, 1995). The diverged N-terminal region and the
conserved catalytic domain are located in the cytosol, whereas
only a short stretch of amino acids connecting the two trans-
membrane (TM) segments is in the ER lumen. Plant HMGR is
modulated by a variety of developmental and environmental
signals, and it has been proposed that major changes in HMGR
activity are determined at the transcriptional level, whereas
posttranscriptional regulation allows a finer and faster adjust-
ment (Hemmerlin et al., 2012). In fact, evidence of post-
translational regulation of HMGR in Arabidopsis plants with
enhanced or depleted flux through the sterol biosynthetic
pathway has been obtained (Nieto et al., 2009). Similarly, in-
hibition of squalene epoxidase (SQE) activity in tobacco (#
icotiana tabacum) Bright Yellow-2 cells using terbina

RESULTS

Phenotypic Characterization of the dry2 Suppressors

To identify undescribed elements that regulate the isoprenoid
biosynthetic pathway, we performed a suppressor screening
based on the recovery of the extreme drought hypersensitive
phenotype of the previously characterized dry2 mutant affected
in SQE1 (Posé et al., 2009; see Supplemental Figure 1 online).
As a result, four independent mutants were selected and named
sud, which maintained the reversion of the dry2 drought hy-
persensitive phenotype across multiple generations. Identification
of the gene affected in the four suppressors indicated that
the mutations were allelic (see below), and the mutants were
subsequently designated dry2/sud1-1 to dry2/sud1-4.

The recovery of the multiple dry2 phenotypic defects was
further analyzed using the dry2/sud1-1 and dry2/sud1-2 alleles.
The dry2/sud1-1 and dry2/sud1-2 mutants showed a restoration
of leaf size and color observed in dry2 and rendered the mutant
shoots undistinguishable from those of the wild type (Figure 1A).
This phenotypic recovery of the shoots in the suppressors
correlated with the reestablishment of hydrogen peroxide and
O2− (superoxide) accumulation to wild-type levels (Figure 1B).
Since the identification of the suppressors was based on the
restoration of the dry2 extreme drought hypersensitivity, we
expected that the defective abscisic acid (ABA) stomatal re-
sponses observed in dry2 would also be restored in the sup-
pressors. As shown in Figure 1C, exogenous application of 20
μM ABA only caused an ~20% reduction in the stomatal con-
ductance of dry2 compared with the ~80% reduction that oc-
curred in wild-type, dry2/sud1-1, and dry2/sud1-2 plants. In
addition, the Pro content in dry2/sud1-1 and dry2/sud1-2 was
more similar to that of wild-type plants (Figure 1D). These results
confirm that the recovery of the dry2 shoot phenotypes was
associated with a restoration of the water relations in the sup-
pressors.

The primary root length of the dry2/sud1-1 and dry2/sud1-2
alleles was double that of dry2, reaching ~70% of the wild-
type seedlings (Figures 2A and 2B). dry2/sud1-1 and dry2/sud1-2
also exhibited a decreased number of lateral roots compared

2010), cellulose accumulation (Schrick et al., 2004), reactive
oxygen species (ROS) production (Posé et al., 2009), and normal
microRNA function (Brodersen et al., 2012). Still, little is known
about the mechanisms and downstream targets by which iso-
openoids in general, and sterols in particular, influence these
processes (Boutté and Grebe, 2009; Clouse, 2002).

In plants, SQEs catalyze the conversion of squalene, the first
committed precursor of essential MVA-derived isoprenoids, to
2,3-oxidosqualene (Rasbery et al., 2007; Posé et al., 2009;
Schaller, 2010). The Arabidopsis drought hypersensitive2 (dry2/
sqe1-5) mutant was identified by its extreme hypersensitivity to
drought stress, altered stomatal responses, and root defects.
Chemical analysis indicated that the dry2/sqe1-5 mutant has altered
sterol composition in roots but wild-type sterol compo-
sition 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
ROS, establishing a previously unknown link between the
MVA pathway and ROS (Posé et al., 2009).

The dry2/sqe1-5 allele contains a point mutation in the 4th
exon that produces a substitution of a conserved Gly by an Arg,
with dry2 (Figures 2A and 2C). The striking defects in root hair length and morphology observed in dry2 (Posé et al., 2009) were also substantially restored in the suppressors (Figures 2D and 2E). Consistent with the rescue of the root hair growth defects, dry2/sud1-1 and dry2/sud1-2 showed wild-type ROS production at the bulge of the root hair tip (Figure 2F), in contrast with the aberrant dry2 ROS production caused by an ectopic localization of the NADPH oxidase C (AtrbohC) (Posé et al., 2009).

All Four dry2 Suppressors Harbor Mutations in the SUD1 Gene

As a first step to determine the gene(s) affected by the sud mutations, we crossed dry2/sud1-1 and dry2/sud1-2 and performed an allelism test. As shown in Figure 3A, reciprocal crosses rendered progenies with wild-type phenotypes, suggesting that the sud1-1 and sud1-2 mutations were allelic. However, F1 plants from the backcross between the suppressors and dry2 showed an intermediate phenotype, indicating that the mutations were semidominant (Figures 3A and 3B). Based on these results, we could not directly infer whether the sud1-1 and sud1-2 mutations were allelic (Koomneef et al., 2006).

Next, we used a combination of map-based cloning and high-throughput sequencing to identify the sud1-1 mutation. For that purpose, the dry2 mutant allele (Landsberg erecta [Ler] ecotype) was crossed over seven generations into Columbia-0 (Col-0) background (dry2Col-0). Molecular markers demonstrated that dry2Col-0 was a near isogenic Col-0 line with the dry2 mutation, and this line displayed similar phenotypes as the original dry2 mutant (see Supplemental Figures 2A to 2C online). Since the dry2Col-0 line was a suitable parental line for map based cloning, an F2 population from the cross between dry2/sud1-1 and dry2Col-0 was generated. Fine-scale map-based cloning delimited the region harboring the sud1-1 mutation in chromosome IV between the AT4G33970 and AT4G34250 loci.
High-throughput sequencing and analysis of the region containing \textit{sud1-1} determined that the second-site mutation responsible for the \textit{dry2/sud1-1} suppression phenotype was a G-to-A substitution at nucleotide 652 relative to the ATG of the \textit{AT4G34100} gene (hereafter named \textit{SUD1}). This nucleotide change caused a Gly218Arg substitution in the predicted SUD1 amino acid sequence (Figure 3C; see Supplemental Figure 2D online). Rough mapping of the other \textit{sud} mutations using markers linked to \textit{sud1-1} indicated that all four \textit{sud} mutations were located in the same region, and targeted sequencing of the \textit{SUD1} gene identified additional mutations on the three \textit{sud} alleles. Thus, \textit{dry2/sud1-2} caused a Gly360Glu substitution in the predicted SUD1 amino acid sequence (Figure 3C; see Supplemental Figure 2D online). Rough mapping of the other \textit{sud} mutants using markers linked to \textit{sud1-1} indicated that all four \textit{sud} mutations were located in the same region, and targeted sequencing of the \textit{SUD1} gene identified additional mutations on the three \textit{sud} alleles. Thus, \textit{dry2/sud1-2} caused a Gly360Glu substitution.
Mechanisms underlying SUD1 activity. The Arabidopsis SUD1 locus AT4G34100 has been recently reported as ECERIFERUM9, a gene involved in cuticular wax biosynthesis (Lü et al., 2012), but little is known about the molecular mechanisms underlying SUD1 activity. SUD1 is predicted to encode a large protein of 1108 amino acids with a molecular mass of ~123 kD. SUD1 contains a Really Interesting New Gene-variant (RING-v) domain (C4HC3 RING-finger domain) near the N terminus (Stone et al., 2005; Lü et al., 2012) and 14 putative TM domains (Lü et al., 2012) (Figure 4A). The SUD1 RING-v domain shares high similarity to that of the E3 ubiquitin ligases TEB4 (57% amino acid identity) and Doa10 (49% amino acid identity). TEB4 and Doa10 are components of the ERAD complex involved in the quality control of ER proteins in human and yeast, respectively (Hassink et al., 2005; Kreft et al., 2006; Kreft and Hochstrasser, 2011). SUD1 also displays a high degree of similarity in an internal conserved segment of ~130 residues called TD (TEB4-Doa10) present in all Doa10 orthologs (Swanson et al., 2001). Thus, the TD domain (TMs 5, 6, and 7) of SUD1 has 45 and 31% amino acid identity to those of TEB4 and Doa10, respectively (Lü et al., 2012).

Suppression of the dry2 Defects Occurs without Recovery in the Composition of Major Sterols

Next, we generated a topological model for SUD1 using sequence alignments and data from the experimental validation available for the homologous Doa10 (Kreft and Hochstrasser, 2011). For that purpose, we selected multiple SUD1 homologous proteins that complied with the following criteria: (1) the conserved N terminus RING-v domain, (2) the internal conserved TD domain, and (3) at least 10 predicted TM domains (Swanson et al., 2001). As a result of the topological analysis, the N terminus RING-v domain (and hence the putative ligase activity of SUD1) and the C terminus were predicted to face the cytosol (Figure 4), a similar disposition to that of Doa10 (Kreft and Hochstrasser, 2011). This model was also used to locate the putative position of the amino acid residues affected in the different sud1 mutant alleles. Thus, the mutations in the sud1-1 and sud1-2 alleles affected residues located at the transition between a TM domain and a hydrophilic loop. The mutation in sud1-3 was located in the second cytosolic loop, and the mutation in sud1-4 produced a premature stop codon at the end of the TM5 domain (Figure 4B).

Additionally, an alignment between SUD1 and SUD1 homologous proteins of several plant species was performed using the plant comparative genomics resource PLAZA database (http://bioinformatics.psb.ugent.be/plaza/; Proost et al., 2010). As shown in Supplemental Figure 3 online, the alignment of Arabidopsis SUD1 protein with the most homologous SUD1 proteins from several dicots (Vitis vinifera, Populus trichocarpa, Medicago truncatula, Lotus japonicus, and Glycine max) and monocots (Brachypodium distachyon, Oryza sativa, and Zea mays) showed striking sequence conservation. From the alignment, we inferred that the amino acid substitutions in all suppressors occurred in conserved residues among monocots and dicots (see Supplemental Figure 3 online).

SUD1 Is Homologous to E3 Ubiquitin Ligases from Yeast and Mammals Involved in ERAD

The Arabidopsis SUD1 locus AT4G34100 has been recently reported as ECERIFERUM9, a gene involved in cuticular wax biosynthesis (Lü et al., 2012), but little is known about the molecular mechanisms underlying SUD1 activity. SUD1 is predicted to encode a large protein of 1108 amino acids with a molecular mass of ~123 kD. SUD1 contains a Really Interesting New Gene-variant (RING-v) domain (C4HC3 RING-finger domain) near the N terminus (Stone et al., 2005; Lü et al., 2012) and 14 putative TM domains (Lü et al., 2012) (Figure 4A). The SUD1 RING-v domain shares high similarity to that of the E3 ubiquitin ligases TEB4 (57% amino acid identity) and Doa10 (49% amino acid identity). TEB4 and Doa10 are components of the ERAD complex involved in the quality control of ER proteins in human and yeast, respectively (Hassink et al., 2005; Kreft et al., 2006; Kreft and Hochstrasser, 2011). SUD1 also displays a high degree of similarity in an internal conserved segment of ~130 residues called TD (TEB4-Doa10) present in all Doa10 orthologs (Swanson et al., 2001). Thus, the TD domain (TMs 5, 6, and 7) of SUD1 has 45 and 31% amino acid identity to those of TEB4 and Doa10, respectively (Lü et al., 2012).

Suppression of the dry2 Defects Occurs without Recovery in

Dry2 and the wild type have similar sterol compositions in shoots but significantly different sterol composition in roots (Posé et al., 2009) (Table 1). Since it has been proposed that the developmental defects in sterol biosynthetic mutants are the result of structural defects due to sterol depletions (Babiychuk et al., 2008; Men et al., 2008), we determined whether the suppression of dry2 phenotypes was associated with a recovery in sterol content in roots. Sterol profiling using gas chromatography–mass spectrometry analysis was performed separately in the shoots and roots of wild-type, dry2, and dry2/sud1-1 seedlings. As shown in Table 1, wild-type, dry2, and dry2/sud1-1 shoots presented similar bulk sterol compositions. By contrast, the bulk sterol composition in dry2/sud1-1 roots was similar to that of dry2 and showed significant differences relative to the wild type. We therefore concluded that the reversion of the dry2/sud1-1 root defects was not due to a recovery of major sterols to wild-type levels.

The recovery of the dry2 defects by the sud1-1 mutation without a recovery of sterol composition was further investigated by analyzing whether sud1-1 was able to suppress the developmental defects of more severe sterol-deficient mutants. The selected mutants were cpi1-1, which causes the loss of function
of the cyclopropylsterol isomerase gene (Lovato et al., 2000; Men et al., 2008), and fackel, which is mutated in a sterol C-14 reductase (Jang et al., 2000; Schrick et al., 2000). As shown in Supplemental Figure 4 online, no phenotypic recovery was observed when sud1-1 was introduced in the cpi1-1 and fk-x224 mutants. These combined results support the notion that the reversion of the dry2 phenotype by sud1 is not concomitant with changes in the sterol content.

A Root-Derived Long-Distance Signal Causes the dry2 Shoot Phenotypic Defects

Consistent with the identification of SQE1 as the main SQE enzyme in roots and with the sterol profiling results for dry2 (Rasbery et al., 2007; Posé et al., 2009), we observed a dramatic accumulation of the substrate squalene in roots but not shoots of the dry2 mutant (Table 1). Interestingly, dry2/sud1-1 roots showed a significant reduction of squalene relative to dry2 (Table 1), suggesting that dry2 root defects could be caused by an accumulation of squalene and/or isoprenoid intermediates upstream of SQE1.

Since dry2 shoots showed no differences in terms of bulk sterols or squalene accumulations with the wild type, we questioned whether squalene or other isoprenoid intermediates generated in the dry2 roots could move toward the shoots, causing the observed phenotypes. In order to investigate this possibility, we performed micrografting experiments using wild-type, dry2, and dry2/sud1-1 seedlings. The root and shoot of the grafted plants were genotyped by sequencing the corresponding DRY2 and SUD1 alleles. As expected, control grafted plants (i.e., Ler scion/Ler rootstock and dry2 scion/dry2 rootstock) showed wild-type and dry2 phenotypes, respectively (Figure 5A). Importantly, dry2 scion onto both Ler rootstock (Figure 5A) and dry2/sud1-1 rootstocks (Figure 5B) showed a wild-type phenotype, suggesting that a root-derived signal was causing the dry2 shoot defects. Despite multiple attempts, we were unable to obtain a viable graft using wild-type or sud1-1 scions.
onto dry2 rootstocks. We therefore could not evaluate the effect of the dry2 root-derived signal in healthy scions.

**sud1 Mutations Suppress dry2 Root Defects by Downregulating HMGR Activity**

It is known that in addition to the accumulation of squalene, the reduction of SQE activity causes a compensatory increase of HMGR activity (Wentzinger et al., 2002; Posé et al., 2009). As shown in Figure 6A, dry2 roots increased the HMGR activity approximately twofold, while no differences were found in shoots. Importantly, in the roots of dry2/sud1-1, the HMGR activity returned to near wild-type levels, whereas in shoots, HMGR activity decreased to ~0.7-fold that in shoots of both the wild type and dry2 (Figure 6A).

To further investigate whether the reduction of HMGR activity could cause the recovery of the dry2 roots, we used atorvastatin, a specific inhibitor of HMGR activity. After testing several

<table>
<thead>
<tr>
<th>Sterol</th>
<th>Wild Type</th>
<th>dry2</th>
<th>dry2/sud1-1</th>
<th>Wild Type</th>
<th>dry2</th>
<th>dry2/sud1-1</th>
</tr>
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<tbody>
<tr>
<td>Cycloartenol</td>
<td>37 ± 6a</td>
<td>104 ± 22b</td>
<td>82 ± 23a</td>
<td>28 ± 2a</td>
<td>28 ± 6a</td>
<td>26 ± 5a</td>
</tr>
<tr>
<td>24-Methylenecycloartenol</td>
<td>46 ± 1a</td>
<td>86 ± 12a</td>
<td>102 ± 8a</td>
<td>48 ± 2a</td>
<td>44 ± 3a</td>
<td>47 ± 9a</td>
</tr>
<tr>
<td>Isolucosterol</td>
<td>63 ± 4a</td>
<td>41 ± 9a</td>
<td>51 ± 25a</td>
<td>63 ± 3a</td>
<td>69 ± 4a</td>
<td>66 ± 13a</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>2579 ± 14^b</td>
<td>1518 ± 58a</td>
<td>1617 ± 91a</td>
<td>2234 ± 101^ab</td>
<td>2052 ± 34a</td>
<td>2302 ± 148^b</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>904 ± 10^a</td>
<td>304 ± 53a</td>
<td>317 ± 19a</td>
<td>60 ± 3a</td>
<td>65 ± 13a</td>
<td>69 ± 6a</td>
</tr>
<tr>
<td>Campesterol</td>
<td>407 ± 3^ab</td>
<td>499 ± 75a</td>
<td>263 ± 107^a</td>
<td>422 ± 27a</td>
<td>439 ± 38a</td>
<td>423 ± 50a</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>32 ± 12a</td>
<td>18 ± 6a</td>
<td>24 ± 7a</td>
<td>46 ± 4a</td>
<td>45 ± 1a</td>
<td>48 ± 4a</td>
</tr>
<tr>
<td>Squalene</td>
<td>10 ± 4a</td>
<td>1206 ± 207^b</td>
<td>75 ± 13a</td>
<td>10 ± 2a</td>
<td>15 ± 4a</td>
<td>13 ± 5a</td>
</tr>
</tbody>
</table>

Values are given in μg g⁻¹ dry weight. Mean ± so, n = 3; values with the same letter are not significantly different at P < 0.05.

**Figure 5. Suppression of dry2 Shoot (Scion) Defects by Wild-Type and dry2/sud1-1 Rootstocks.**

A total of 20 viable plants per graft combination were analyzed, and a representative plant per combination is depicted after 18 d of growth in soil. Bottom panels show the plants after soil removal to visualize the root phenotype. Bar = 2 cm. (A) dry2 shoot (scion) recovers the wild-type phenotype when grafted onto wild-type Ler rootstock. Self-grafted wild-type (Ler) and dry2 were used as controls. (B) dry2 shoot (scion) recovers the wild-type phenotype when grafted onto dry2/sud1-1 rootstock. Self-grafted dry2/sud1-1 and dry2 were used as controls. [See online article for color version of this figure.]
concentrations, we selected 10 nM atorvastatin. As shown in Figure 6B, this concentration did not have apparent effects on wild-type root growth or branching but substantially improved both phenotypes in dry2. This result supports the notion that a reduction in HMGR activity in the dry2/sud1-1 suppressor was responsible for the recovery of the dry2 root phenotypic defects. We next treated wild-type, dry2, and dry2/sud1-1 seedlings with MVA, the product of the reaction catalyzed by HMGR. As shown in Figure 6C, a 5 mM MVA treatment caused a mild reduction in wild-type root elongation, while no visible changes were observed in dry2 roots. However, the same treatment abolished the sud1-1 suppressive effect of the dry2 root defects, phenocopying dry2 roots (Figure 6C). This result indicates that bypassing HMGR activity by adding the product of the HMGR reaction prevents the recovery of the dry2 phenotype in a dry2/ sud1-1 background.

Genetic Analysis of the Regulation of HMGR Activity by SUD1

To further investigate the link between HMGR activity and SUD1, the dry2/sud1-1 mutant was crossed with transgenic lines overexpressing the catalytic domain of the HMGR1 (HMGR1-CD) and the short isoform of HMGR1 containing the TM domains (HMGR1S) (see Supplemental Figure 5 online). HMGR1-CD and HMGR1S lines show an ~10-fold and an approximately threefold increase in HMGR activity compared with the wild type, respectively (Manzano et al., 2004). As shown in Figure 7, the enhanced HMGR activity in the wild-type background only caused slight growth inhibition in the HMGR1-CD line and no visible effect in the HMGR1S line (Manzano et al., 2004). By contrast, the HMGR1-CD/dry2 combination greatly enhanced the growth inhibition defects of dry2 generating dwarf plants (Figure 7). The HMGR1S/dry2 combination also showed enhanced growth inhibition compared with dry2, but this effect was less drastic than that observed in the HMGR1-CD/dry2. In fact, viable seeds were obtained in this genotype, while HMGR1-CD/dry2 plants died before reaching maturity (Figure 7). These results indicate that the increase in HMGR activity in the presence of a dry2 mutation accounts for the severity of the observed developmental phenotypes. Importantly, when the sud1-1 mutation was introduced into HMGR-CD/dry2 and HMGR1S/dry2, there was an important recovery of the defective phenotypes to such an extent that HMGR-CD/dry2/sud1-1 plants were fertile (Figure 7).

Based on these results, we propose a model in which the increase of HMGR activity concomitant to the reduction of SQE1 activity is mainly responsible for the observed developmental phenotypes in the dry2 background. In this scenario, mutations in the positive regulator of HMGR SUD1 cause the reversion of the dry2 developmental defects by decreasing HMGR activity. An obvious question was whether the regulation of HMGR activity by SUD1 was dependent on the presence of the dry2 mutation. Therefore, the sud1-1 mutation was segregated from dry2 by backcrossing dry2/sud1-1 to the wild type. As shown in Figure 8A, the single sud1-1 mutant did not show any obvious phenotypic difference compared with wild-type plants, except by a glossy-like phenotype in shoots reminiscent of the phenotypes described for the loss-of-function cer9-1 and cer9-2 mutants allelic to sud1 (Lü et al., 2012; see Supplemental Figure 6 online). As shown in Figure 6A, HMGR activity in the sud1-1 single mutant was ~0.75-fold lower than that of the wild type in both shoots and roots, indicating that SUD1 is a positive regulator of HMGR activity acting independently of the dry2 mutation.

Regulation of HMGR Activity by SUD1 Does Not Involve Changes in Protein Content

Because SUD1 contains a RING-v domain putatively involved in ubiquitination (Stone et al., 2005), we used protein gel blot analysis to investigate whether the regulation of HMGR activity mediated by SUD1 involves changes in HMGR protein content. The immunospecific antibodies used in the analysis were raised against the catalytic domain of HMGR1 (Manzano et al., 2004; Leivar et al., 2005). As shown in Figure 8B, the analysis of HMGR protein content in root samples revealed two bands of ~63 and 69 kD, corresponding to the HMGR1S and HMGR1L isoforms, respectively, whereas in shoot samples, only the 63-kD protein band was detected. In both cases, no significant differences in HMGR protein content among the wild type, dry2, dry2/sud1-1, and sud1-1 were observed (Figure 8B). This result, together with results from previous pharmacological studies (Wentzinger et al., 2002; Nieto et al., 2009), indicates that the variations in HMGR activity in the different genetic backgrounds occur without changes in the total HMGR protein content.

DISCUSSION

Mutations in the sterol biosynthetic SQE1 gene produce multiple developmental defects, but in contrast with null alleles of SQE1 (Rasbery et al., 2007), the hypomorphic sqe1-5 allele is fully fertile (Pose et al., 2009). This characteristic lends itself to the use of dry2/sqe1-5 as a genetic tool to identify processes that otherwise would be concealed. To find components regulating isoprenoid biosynthesis and/or signaling in Arabidopsis, we performed genetic screening for suppressors of dry2. Here, we report the analysis of four suppressors and show that all mutations affect the At4g34100 gene encoding a protein with a RING-v domain, found in ubiquitin E3 ligases, subsequently named SUD1. Based on phylogenetic and structural similarities, it is proposed that SUD1 is an Arabidopsis orthologous protein of yeast Doa10 and mammalian TEB4, which are involved in the ERAD-C pathway. Our physiological, molecular, biochemical, and genetic analyses strongly support that sud1 recovers the dry2 defects through the reversion of the enhanced HMGR activity of dry2 to wild-type levels. Thus, our study uncovers SUD1 as a regulator of HMGR activity in the plant isoprenoid biosynthetic pathway.

The Accumulation of an MVA-Derived Signal in Roots Causes the dry2 Phenotypes

Despite its dramatic phenotypic defects, sterol analysis of dry2 showed only moderate changes in major bulk sterols in roots and no significant changes in shoots, compared with the wild type. Interestingly, sterol analysis of dry2/sud1-1 roots revealed a similar composition to that of dry2, indicating that the dry2
Figure 6. HMGR Activity Changes Correlate with the Phenotypes Observed in dry2 and dry2/sud1-1.
Wild-type (WT), dry2, and dry2/sud1-1 plants are in the Ler background ecotype. The transgenic HMGR1-CD or HMGR1S plants are in Col glabrous background. The resulting F2 from the crosses are Col-Ler ecotype. The transgenic HMGR1-CD or HMGR1S plants are in Col glabrous background. The resulting F2 from the crosses are Col-Ler hybrids. Plants were grown for 3 weeks in soil under long-day conditions. All plants were fertile with the exception of CD/dry2 plants. Bar = 1 cm. [See online article for color version of this figure.]

Phenotypes cannot simply be explained by structural defects caused by the reduction in bulk sterols. Moreover, dry2 shoots display wild-type characteristics when grafted onto wild-type rootstocks, suggesting that a toxic mobile signal that originated in the dry2 roots is responsible for the observed dry2 shoot phenotypes.

Although it is tempting to speculate that the squalene accumulated in dry2 roots is the mobile signal responsible for the phenotypes, we argue against this notion because plants deal with excess endogenously produced or exogenously added squalene by storing it as remobilizable cytosolic lipid droplets without obvious phenotypic defects (Wentzinger et al., 2002; Bouvier-Navé et al., 2010). Thus, we propose an alternative model whereby the accumulation of toxic intermediates, or derivatives acting upstream of squalene, is responsible for the observed dry2 developmental phenotypes. In fact, three independent experiments, including (1) the inhibition of HMGR with atorvastatin that partially improved the dry2 root defects, (2) the HMGR bypass with MVA that caused dry2/sud1-1 (but not the wild type) to phenocopy dry2, and (3) the overexpression of HMGR that enhanced the dry2 phenotypes, suggest that the dry2 mobile signal(s) is not only triggered by the reduction of SQE1 activity but also by the concomitant upregulation of HMGR activity.

Supporting our model, the presence of toxic MVA-derived intermediates associated with HMGR activity changes has been reported in Insig double knockout mice that show developmental defects linked to enhanced HMGR activity (Engelking et al., 2006). As was true for dry2, the developmental defects of the mice were ameliorated with the use of HMGR inhibitors (Engelking et al., 2006). Interestingly, the Insig knockout mice are not an isolated example. Nonsterol MVA-derived compounds upstream of squalene have been linked to the regulation of HMGR protein content in mammals, yeast, and plants. Thus, the degradation of mammalian HMGR is accelerated by the addition of farnesol, geranylgeraniol, and its precursor geranylgeranyl diphasphate (Correll et al., 1994; Meigs et al., 1996; Räikkönen et al., 2010). Geranylgeranyl diphasphate is also known to regulate the degradation of HMGR2p in yeast (Garza et al., 2000). Surprisingly, the effect of farnesol on plant HMGR activity seems to be different from that in mammals because the addition of subtoxic concentrations of farnesol to tobacco Bright Yellow-2 cells had a drastically stimulatory effect on HMGR activity (Hemmerlin and Bach, 2000).

Despite the similarities in the regulation of HMGR by a nonsterol MVA-derived molecule in different species, the mechanisms that regulate HMGR activity in Arabidopsis seem to operate at a different level from those in yeast and animals. Thus, our study and a previous report (Nieto et al., 2009) have shown that both the genetic and pharmacological block of Arabidopsis SQE activity leads to upregulation of HMGR activity without changing HMGR protein amounts, while in yeast and animals, HMGR activity depends on protein stability.

Figure 7. Phenotypic Analysis of Plants Overexpressing HMGR1S and HMGR1-CD in dry2 and dry2/sud1-1 Backgrounds.

Wild-type (WT), dry2, and dry2/sud1-1 plants are in the Ler background ecotype. The transgenic HMGR1-CD or HMGR1S plants are in Col glabrous background. The resulting F2 from the crosses are Col-Ler hybrids. Plants were grown for 3 weeks in soil under long-day conditions. All plants were fertile with the exception of CD/dry2 plants. Bar = 1 cm. [See online article for color version of this figure.]

Figure 6. (continued).

(A) HMGR activity measurement in roots and shoots of 15-d-old wild-type (WT), dry2, dry2/sud1-1, and sud1-1 seedlings (mean ± so, n = 3; each measurement corresponds to a pool of ≥100 seedlings). Values with the same letter are not significantly different at P < 0.05. The experiment was repeated at least three times with similar results.

(B) Inhibition of HMGR activity partially recovers dry2 roots defects. Wild-type and dry2 seeds were germinated and grown on MS plates for 4 d. Seedlings were then transferred to MS supplemented with 10 nM of the HMGR inhibitor atorvastatin and grown for additional 2 weeks (mean ± so, n = 30; values with the same letter are not significantly different at P < 0.05). The experiment was repeated three times with similar results. Bar = 1 cm.

(C) dry2/sud1-1 roots phenocopy dry2 in the presence of MVA. The wild type, dry2, and dry2/sud1-1 were germinated and grown on MS plates for 4 d. Seedlings were then transferred to MS medium supplemented with MVA and grown for two additional weeks (mean ± so, n = 30; values with the same letter are not significantly different at P < 0.05). The experiment was repeated three times with similar results. Bar = 1 cm. [See online article for color version of this figure.]
are orthologous proteins (Kreft et al., 2006). Successful despite the fact that yeast Doa10 and human TEB4 contain the known sterol sensing domain motif. ERAD-regulated HMGR proteins, such as those from yeast (Hampton et al., 1996). The HRD pathway (ERAD-L and ERAD-M) is involved in the degradation of misfolded ER-luminal and intramembrane domains; HRD genes were identified in a genetic screening for regulators of HMGR degradation (hence the name HRD, for HMGR reductase degradation) (Hampton et al., 1996). The finding that feedback regulation of sterol synthesis in mammalian and yeast cells uses the ERAD machinery (Hampton, 2002) illustrates co-option of the basic quality control mechanism for regulatory processes and reveals potential functions in cell-to-cell signaling. ERAD-regulated HMGR proteins, such as those from yeast and mammals, contain the known sterol sensing domain motif consisting of five consecutive TM spans (Goldstein et al., 2006; Thesfeld et al., 2011). However, HMGR from plants contain two predicted TM domains (see Supplemental Figure 5 online).

### Structural Characteristics of SUD1

All sud1 alleles show a similar phenotypic recovery of dry2 phenotypes, including sud1-4, which is caused by a premature stop codon at the fifth TM domain. The sud1 alleles also show a glossy-like phenotype in leaves reminiscent of the cer9 mutants in the same locus. Because cer9 has been reported to be a loss-of-function mutant (Rashotte et al., 2004; Lü et al., 2012), and the premature stop codon is much more downstream in cer9-2 than in sud1-4, we presume that sud1 are also loss-of-function alleles. Interestingly, the cer9 alleles are recessive while all sud1 alleles are semidominant with respect to the dry2 mutation, which suggests that SUD1 regulation of HMGR activity is dose dependent. Thus, the heterozygous, SUD1/sud1 genotype is unable to produce enough SUD1 protein to fully reproduce the dry2 phenotypes.

The phylogenetic analysis and the structural features of SUD1 suggest that this protein might function as one ortholog of yeast Doa10 and human TEB4 in Arabidopsis (Carvalho et al., 2006; Kreft et al., 2006; Kreft and Hochstrasser, 2011); these proteins are involved in the quality control that degrades misfolded ER proteins (Swanson et al., 2001). However, despite multiple attempts, we failed to complement the yeast doa10 mutant with SUD1 because this protein is highly unstable in yeast. This result is not entirely surprising because efforts to perform complementation of yeast doa10 with TEB4 have also been unsuccessful despite the fact that yeast Doa10 and human TEB4 are orthologous proteins (Kreft et al., 2006).

When we analyze SUD1 plant homologs, we find a striking conservation of SUD1 sequence with homologous proteins from dicots and monocots. Indeed, amino acid substitutions in all suppressors occur in plant conserved residues. Thus, sud1-1 and sud1-2 result in Gly218Arg and Gly360Glu substitutions that change small nonpolar residues for basic and acidic residues, respectively. Interestingly, both residues are located at the transition between a TM segment and a hydrophylic loop. Interruption of TM helices by a short nonhelical segment containing Pro, Gly, and/or Ser residues has also been observed in many classes of transporters, including amino acid antiporters (Gao et al., 2009), neurotransmitter-sodium symporters (Yamashita et al., 2005), and sodium-independent transporters (Schulze et al., 2010). Interruption of helical structures exposes main-chain carbonyl oxygen and nitrogen atoms for hydrogen bonding and ion coordination, aspects that are essential for proper function (Yamashita et al., 2005). The mutation in sud1-3 results in the Arg244Lys substitution. These two amino acids are chemically related, and it would be expected that its substitution did not cause important changes. However, phylogenetically distant plant species, such as monocots and dicots, maintain a conserved Arg around position 244, suggesting an important role for this specific residue in SUD1 function.

### Figure 8. sud1-1 Mutation Produces No Visible Phenotype and No Changes in HMGR Protein Content.

(A) Phenotype of wild-type (WT), dry2, dry2/sud1-1, and sud1-1 seedlings grown for 15 d on MS medium under long-day conditions. Bar = 1 cm. (B) Protein gel blot analysis of HMGR protein in 15-d-old roots and shoots of the wild type, dry2, dry2/sud1-1, and sud1-1. Intensities of the HMGR protein bands (top panel) and the Coomassie blue–stained gel (bottom panel) were quantified using ImageJ software (http://rsb.info.nih.gov/ij). The normalized HMGR protein levels expressed as relative abundance to the amount of the HMGR1S isoform in wild-type plants (arbitrarily set at 1) is shown at the top of each lane. Image shows the results from one representative experiment. Four independent experiments were performed with similar results. [See online article for color version of this figure.]
(Campos and Boronat, 1995), therefore lacking any potential sterol sensing domain motif. Surprisingly, following a non-targeted screening for plant HMGR regulators, we identified SUD1, a likely ERAD component. Our first explanation for this was that HMGR stability was regulated by ERAD, either directly by SUD1 or through a compensatory increase of the HRD pathway in sud1 mutants. However, protein gel blot analyses indicated that SUD1 did not exert its function by regulating HMGR proteins levels. Because SUD1 likely encodes an E3 ubiquitin ligase, another plausible explanation is that a negative regulator of HMGR is being degraded in a SUD1-dependent manner in dry2, so the loss of SUD1 function would impair this degradation, leading to the recovery of HMGR activity to wild-type levels.

Transcriptional versus Translational Regulation of HMGR

It has been proposed that major changes in HMGR activity in plants would be determined at the transcriptional level, whereas posttranslational control would allow a finer and faster adjustment (Chappell, 1995). Whereas transcriptional modulation of HMGR has been demonstrated in many plant systems, evidence for posttranslational control would allow a faster adjustment and faster adjustment of the flux through the sterol pathway in Arabidopsis causes a compensatory response in HMGR activity, without changes in transcript or protein levels, and Flores-Pérez et al. (2010) reported that the inactivation of the Arabidopsis WD protein PRL1 leads to reduced HMGR activity with no changes in transcript and protein levels. This effect could be related to the ability of PRL1 to interact and inhibit the activity of the Arabidopsis SNF1-related protein kinases (SnRK1) AKIN10 and AKIN11 (Bhalerao et al., 1999), presumably targeting them for ubiquitination and proteasomal degradation (Lee et al., 2008). Since plant SnRK1 phosphorylates and inactivates HMGR (Dale et al., 1995; Sugden et al., 1999), the loss of PRL1 function would result in increased SnRK1 activity followed by HMGR phosphorylation and the subsequent reduction of HMGR activity. It has also been demonstrated that HMGR activity is negatively regulated by PP2A-mediated dephosphorylation (Leivar et al., 2011). Therefore, SnRK1 and/or PP2A, regulators of Arabidopsis HMGR activity, are candidates to act as mediators of SUD1 regulation of HMGR activity. An alternative possibility is that SUD1 might produce the direct monoubiquitination of HMGR, thereby increasing its activity, as has been reported for other proteins (Schnell and Hicke, 2003).

Overall, using genetic, physiological, biochemical, and molecular approaches, we show that SUD1, a likely component of the Arabidopsis ERAD-C pathway, is a positive regulator of HMGR activity. Future research should help clarify the mechanistic basis for the ERAD regulation of HMGR activity in plants and what signals are implicated in this regulation.

METHODS

Plant Material and Growth Conditions

Unless stated otherwise, the Arabidopsis thaliana plants used in this study were either grown on soil or in Petri dishes using an environmental chamber set for long-day lighting conditions (16 h light/8 h dark) and a temperature of 22°C. For in vitro assays, surface-sterilized and cold-stratified Arabidopsis seeds were sown onto Murashige and Skoog (MS) phytagel-solidified medium (MS salts, 30 g L⁻¹ Suc, and 7 g L⁻¹ phytagel [Sigma-Aldrich], pH 5.7). For chemical treatments, the appropriate amounts of filter sterilized chemical stock solutions were added to cooled autoclaved growth medium. The dry2 (Posé et al., 2009), cpi1-1 (Schrick et al., 2000), and fk-x224 (Men et al., 2008) mutants and the HMGR1-CD and HMGR1S overexpressing lines (Manzano et al., 2004) have been previously described.

Genetic Screen for Second-Site Suppressor Mutations of dry2

The dry2 seeds were mutagenized by imbibition in 75 mM ethyl methanesulfonate (Sigma-Aldrich) for 4 h at room temperature. After washing thoroughly with water for complete methanesulfonate removal, the mutagenized seeds (M1) were sown on soil and grown under high humidity conditions. The M2 seeds were harvested as 131 independent pools (each pool corresponding to 50 M1 plants). For the identification of dry2 suppressors, the M2 seeds were grown on soil under low watering conditions. Suppressors with enhanced drought tolerance compared with dry2 plants were visually identified and selected for further analysis. The SQE1-DRY2 gene of each candidate suppressor was amplified by PCR using DRY2-specific primers and sequenced in order to confirm the presence of the dry2 mutation as described by Posé et al. (2009). The primers sequences for genotyping were DRY2 SEQ F, 5'-ATTGTCTCCGGTTGGGTGAG-3', and DRY2 SEQ R, 5'-GATTGCA-GTTCTCTAGGACCAA-3', and internal primer to sequence DRY2 SEQ2, 5'-TCAAAGATGCGGGGAAAG-3'.

Detection of ROS

Hydrogen peroxide was visually detected in leaves using the 3,3′-diaminobenzidine (DAB; Sigma-Aldrich) substrate as described previously (Orozco-Cardenas and Ryan, 1999). DAB was also used for in situ detection of hydrogen peroxide in roots from seedlings grown on phytagel-solidified medium (Carol et al., 2005). For in situ detection of superoxide in leaves, the nitroblue tetrazolium (NB1 Color Development Substrate; Promega) staining method (Jabs et al., 1996) was used. In all cases, stained leaves were imaged under dark-field illumination using a Leica MZ FLII stereomicroscope.

Whole-Plant Stomatal Conductance and Determination of Pro Content

Leaf stomatal conductance to water vapor was measured in 25-d-old leaves grown under short-day lighting conditions (8 h light/16 h dark) using a Leaf Porometer Model SC-1 (Decagon Services). Measurements were performed after spraying the leaves with 0, 0.2, 2, or 20 μM of ABA (Sigma-Aldrich) dissolved in a 0.1% Tween 20 solution. Pro was extracted and quantified as described previously (Borsani et al., 2002).

Root Measurements

Root measurements were performed according to the procedure described by Posé et al. (2009). Briefly, seeds were grown vertically on phytagel-solidified MS medium for 5 and 10 d for roots hair and root elongation measurements, respectively. For root elongation assays, primary root length pictures were taken daily using a Nikon Coolpix 4500 camera attached to a M2 FLII stereomicroscope (Leica). Quantitative measurements were made using Image J software (http://rsb.info.nih.gov/ij/). Root branching was determined by counting the number of root tips per length unit (cm) of primary root. Root hair length was measured in the differentiation zone as described by Posé et al. (2009).
Identification of the sud1-1 Suppressor Mutation

The dry2 mutant in the Ler background was crossed into the Col-0 eco-
type for seven generations to generate a nearly isogenic dry2-1/sud1-1
line for map-based cloning. An F2 mapping population was created
from a cross between the dry2/sud1-1 (Ler) and the introgressed dry2-1/sud1
lines. A total of 120 F2 plants displaying the suppression phenotypes
conferred by the dry2/sud1-1 mutations were used for rough mapping. For
fine mapping, a total of 2,000 chromosomes were analyzed to locate the
SUD1 locus in a 117-kb region at the bottom of chromosome IV with 36 can-
didate genes. All information regarding the genetic markers used in
the map-based cloning was obtained from The Arabidopsis Information
Resource (http://www.Arabidopsis.org/). The entire genome of dry2/sud1-1
was sequenced using high-throughput sequencing with the Illu-
mina platform. Reads were filtered with Fastx-Toolkit software (http://
hannonlab.cshl.edu/fastx_toolkit/index.html) and mapped with the Arab-
idopsis genome sequence version TAIR10 using the Burrows-Wheeler
Alignment Tool (Li and Durbin, 2009). Polymorphisms for the 117-kb
candidate region were analyzed with Samtools and Bcftools using regions
with at least 5X depth coverage (Li et al., 2009) and filtered using the
sequence information for the Ler ecotype available at 1001 Genomes
(http://1001genomes.org/). After filtering, two nonsynonymous mutations
in the AT4G34100 and AT4G34135 loci were identified. The identification of
four independent suppressor alleles with mutations in the AT4G34100
locus confirmed the identity of the AT4G34100 locus as SUD1.

Informatic Tools Used for Functional Characterization of SUD1

The InterPro database (http://www.ebi.ac.uk/Tools/pfa/prsscan/) was
used to search for conserved domains of SUD1 protein. The National
nih.gov/Blast.cgi?PAGE=Proteins) was used to identify putative SUD1
orthologs using the predicted proteome from Saccharomyces cerevisiae
(txid4932), Homo sapiens (txid9606), Mus musculus (txid10090),
Drosophila melanogaster (txid7227), and Caenorhabditis elegans
(txid6239).

The TMHMM2.0 program (www.cbs.dtu.dk/services/TMHMM/) was
used to predict the putative TM domain topology of SUD1 based on the
hydrophobicity plot (Krogh et al., 2001). The plant comparative genomics
resource PLAZA (http://bioinformatics.psb.ugent.be/plaza/) was used to
search for SUD1 homologous protein sequences in different plant species
with homologous proteins from other plant species was performed with
the software ClustalW2 available online from the European Bioinformatics
Institute (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Default values were
used for all parameters, including Gonnet protein weight matrix, gap
open of 10, gap extension of 0.20, gap distance of 5, no end gaps, should
read: no iteration, numiter of 1, and clustering neighbor-joining no iter-
ation, numiter of 1, and clustering neighbor-joining (Gonnet et al., 1992).

Sterol and Squalene Analysis and Determination of HMGR Activity

Fifteen-day-old seedlings grown in phytagel-solidified MS medium were
used for sterol, squalene, and HMGR activity measurements. A pool of
≥100 seedlings per genotype was used per each measurement. Since
HMGR activity and isoprenoid biosynthesis are regulated by light con-
ditions (Learned, 1996; Rodriguez-Concepcion et al., 2004), shoot and
roots were collected and measured separately at 3 h from the start of the
light period. Quantification of total sterol content and determination of
sterol profiles were performed as previously reported (Masferrer et al.,
2002). HMGR activity was assayed as described (Nieto et al., 2009). In our
assays, one unit of HMGR activity is defined as the amount of enzyme that
converts 1 μM of 3-hydroxy-3-methylglutaryl CoA into MVA per min and
mg of protein at 37°C.

Arabidopsis Grafting

Four-day-old seedlings grown vertically were transferred to a 0.22-μm
sterile filter (Millipore) in contact with half-strength MS medium containing
0.65% (w/v) phytagel (Sigma-Aldrich). After 3 d, seedlings were grafted in
a wedge graft (Y shape) under sterile conditions, as described by Turnbull
et al. (2002). Afterwards, the grafted plants were grown for seven addi-
tional days under humid conditions. Successful grafts were transferred to
soil, and the grafting unions were confirmed by sequencing analysis of the
dry2 mutant allele of shoots and roots as described above.

Determination of HMGR Protein Levels

HMGR protein levels were determined by immunoblot analysis using a
rabbit polyclonal antibody raised against the catalytic domain of Arab-
idopsis HMGR1 (Manzano et al., 2004; Leivar et al., 2005). The antibody
was used at 1:1,000 dilution, and the secondary antibody (horse-sera
peroxidase anti-rabbit IgG; Sigma-Aldrich) was diluted at 1:14,000. For
immunoblot analysis, total root and shoot protein were loaded onto 10%
acrylamide SDS gels. Immunoblot images were developed with Advanced
ECL (GE Healthcare) and exposed to an x-ray film for 30 s to 1 min.
Coomassie Brilliant Blue staining was used to confirm equal loading.

Statistical Analysis

Statistical analysis was performed using the Statgraphic Centurion
program (Statpoint Technologies). The significance of differences was
determined by analysis of variance (for three or more samples) or a t test
(for two samples).

Accession Numbers

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in
this article are as follows: SGE1 (At1g58440), SUD1 (At4g34100), CPI1
(At5g50375), FK (At3g52940), and HMGR1 (At1g76490).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Identification of Suppressor Lines Recovering
the dry2 Drought Hypersensitivity.

Supplemental Figure 2. Map-Based Cloning of sud1-1.

Supplemental Figure 3. Protein Sequence Alignment of SUD1 with
Homologs from Other Plant Species.

Supplemental Figure 4. The sud1-1 Mutation Does Not Improve the
Phenotypic Defects of the Sterol Biosynthesis Mutants cpi1-1 and
fKx224.

Supplemental Figure 5. Schematic Representation of the HMGR
Protein Versions Overexpressed in the HMGR1S and HMGR1-CD
Transgenic Lines.

Supplemental Figure 6. sud1-1 Leaves but Not the Wild Type Show
Glossy-Like Appearance.

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AUTHOR CONTRIBUTIONS

V.G.D. and V.A.-S. performed the physiological, biochemical, and genetic experiments. D.P. identified the suppressors and performed an initial characterization. M.A. performed sterols and squalene analysis and HMGR activity measurements. A.B. conducted the genomic analysis for SUD1 identification. H.A. performed the ROS analyses. A.E. performed genetic crosses for SUD1 identification. O.B. designed and performed the atorvastatin experiments. V.G.D., A.R., R.M.T., and M.A.B. designed the research. V.G.D., V.A.-S., A.R., A.F., and M.A.B. wrote the article.

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