Arabidopsis thaliana Squalene Epoxidase 1 Is Essential for Root and Seed Development

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Squalene epoxidase converts squalene into oxidosqualene, the precursor of all known angiosperm cyclic triterpenoids, which include membrane sterols, brassinosteroid phytohormones, and non-steroidal triterpenoids. In this work, we have identified six putative Arabidopsis squalene epoxidase (SQE) enzymes and used heterologous expression in yeast to demonstrate that three of these enzymes, SQE1, SQE2, and SQE3, can epoxidize squalene. We isolated and characterized Arabidopsis sqe1 mutants and discovered severe developmental defects, including reduced root and hypocotyl elongation. Adult sqe1–3 and sqe1–4 plants have diminished stature and produce inviable seeds. The sqe1–3 mutant accumulates squalene, consistent with a block in the triterpenoid biosynthetic pathway. Therefore, SQE1 function is necessary for normal plant development, and the five SQE-like genes remaining in this mutant are not fully redundant with SQE1.

Plants are estimated to produce more than 500,000 secondary metabolites (1). These compounds have many functions, including attracting pollinators, communicating with neighboring plants, and defending against pathogens and herbivores (2, 3). The importance of secondary metabolites is highlighted by the extensive resources that plants invest in producing these compounds. Although once thought to be metabolically simple, more than 170 secondary metabolites have been identified in Arabidopsis thaliana (reviewed in Ref. 4).

Triterpenoids are the 30-carbon subset of terpenoids, the largest class of secondary metabolites. Triterpenoid biosynthesis is diagramed in Fig. 1. Isopentenyl diphosphate and dimethylallyl diphosphate are synthesized from mevalonate and farnesyl diphosphate (FPS). Farnesyl diphosphate is dimerized to squalene by squalene synthase. Squalene epoxidase (SQE)-mediated oxidation then produces oxidosqualene, which triterpene synthases cyclize to >80 triterpene skeletons (5, 6). Further metabolism of these compounds produces membrane sterols, brassinosteroid phytohormones, saponins, other defense compounds, cuticular waxes, and numerous triterpenoids that have not been functionally characterized.

The yeasts and mammals that have been investigated each encode a single squalene epoxidase. In contrast, several plants have multiple genes predicted to encode squalene epoxidases, a diversity suggesting that this step may be subject to additional or unique regulation in plants. Two Medicago truncatula SQE enzymes have been biochemically characterized (7). The Brassica napus (8), Populus trichocarpa, and Oryza sativa genomes each have multiple predicted SQE enzymes. Despite the likely importance of SQE to plant growth and development, no plant mutants with defects in these enzymes have been reported.

In this work, we heterologously expressed the six Arabidopsis putative SQE enzymes in Saccharomyces cerevisiae lacking squalene epoxidase to determine which have squalene epoxidase activity. We isolated Arabidopsis sqe1 loss-of-function mutants and found that these mutants display severe developmental defects and accumulate squalene. Our results demonstrate that SQE1 is particularly important for oxidosqualene production in the roots and reproductive tissues of Arabidopsis.

EXPERIMENTAL PROCEDURES

Chemicals—Bis(trimethylsilyl)trifluoroacetamide, pyridine, and potassium hydroxide were from Aldrich Chemical Company (Milwaukee, WI). Lovastatin (mevinolin), squalene, epibrassinolide, hemin, ergosterol, raffinose, TRI Reagent, and hexane (OmniSolv grade) were from EM Science (Gibbstown, NJ). Ammonium glufoisinate (Basta) was from Sigma-Aldrich and Crescent Chemical (Augsburg, Germany). Sitosterol was obtained from wild-type (Col-0, Columbia) Arabidopsis plant extracts by high pressure liquid chromatography purification and was identified by NMR and GC-MS. Oxidosqualene was synthesized according to previously published procedures (9).

Yeast Expression—SQE1 (At1g58440), SQE3 (At4g37760), SQE5 (At5g24150), and SQE6 (At5g24160) were PCR-amplified...
Mutant plants were identified by PCR-amplifying genomic DNA prepared from a leaf or seedling with primers designed either to span the insert location (to identify the wild-type locus) or to amplify a genomic fragment, including the left border of the T-DNA (to identify the mutant locus). Insert locations were confirmed by sequencing PCR products spanning the junction between the gene and the T-DNA. The sqe1–1 mutant allele was identified by PCR amplification with a modified Lb1 oligonucleotide (Lb1-Salk, 5’-CAAAAACAGCTTGAGCCGTCGTCGAACTC-3’) and At1g58440-10 (5’-CGCGATTGTGTGAAACAAAAATTGTTA-3’), which yielded a 500-bp product. The SQE1 allele was identified by amplification with Salk016111-1 (5’-AGTTTTATTTGATTTGATCTACTGAATAG-3’) and At1g58440-10, which yielded a 917-bp product. The sqe1–2 mutant allele was identified by PCR amplification with Lb1-Salk and Salk016111-1, which yielded a 285-bp product. The SQE1 allele was identified by amplification with Salk016111-1 and Salk016111-2 (5’-AAAC-AACGAGAAGGAGAAGATATATAAG-3’), which yielded a 370-bp product. The sqe1–3 mutant allele was identified by amplification with Lb1-Salk and At1g58440-8 (5’-AACACCGTGGCGAATGCTTTT-3’), which yielded a 408-bp product, and the SQE1 allele was identified by amplification with At1g58440-7 (5’-GCTGGGAAACACCATCTGCTTACT-3’) and At1g58440-8, which yielded a ~450-bp product. sqe1–4 mutants were identified using an insert-specific primer (CSHL_Ds3-2, 5’-CGATTACCGTTATTATCCGCTT-3’) and SQE44–4R2 (5’-GTTGACACAGAAACACCATTACT-3’) and SQE44-4R2, which yielded a 526-bp product.

**Plant Growth Conditions**—Seeds were surface-sterilized in a 30% bleach solution with 0.01% Triton X-100 for 12 min, rinsed extensively with sterile water, and grown aseptically on plant nutrient (PN) medium (19) supplemented with 0.5% sucrose (hereafter referred to as PNS) and solidified with 0.1% agar under continuous white light at 22 °C unless otherwise indicated. Seedlings were transferred to soil (Metro-Mix 200, Scotts, Marysville, OH) and grown under continuous white light at 22 °C.

For hydroponic growth, seedlings were transferred from agar-based medium to 250-ml Erlenmeyer flasks containing 1/2× liquid PN medium. Aerial portions were suspended above the liquid by threading roots through a hole in a piece of Parafilm over the top of the flask.

For hypocotyl elongation assays, seeds from an SQE1/sqe1–3 heterozygote were incubated in the light for 1 day before transfer to the dark at 22 °C for five additional days. Hypocotyl lengths were measured and seedlings were transferred to PN medium for an additional 2 weeks in the light. PCR analysis of seedling DNA was used to determine the genotype (SQE1/ SQE1, SQE1/sqe1–3, or sqe1–3/sqe1–3) of each plant.

For root elongation assays, seeds from an SQE1/sqe1–3 heterozygote were grown vertically on PNS medium at 22 °C for 11 days. Root lengths were measured daily, and PCR analysis of seedling DNA was used to determine the genotype of each plant.
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RNA Analysis—Seven- and 14-day-old seedlings and 54-day-old rosette leaves, stems, inflorescences, and siliques from wild type and 14-day-old sqe1–3 seedlings were collected for RNA isolation. RNA was isolated using TRI Reagent according to the manufacturer’s instructions. Reverse transcription with Superscript III reverse transcriptase (Invitrogen) used a 0.6-μg total RNA template and random hexamer primers. PCR amplification of the resultant cDNA was performed using the following oligonucleotides: sqe1–3 (spanning the location of the T-DNA), At1g58440-7 and At1g58440-8; SQE1 (upstream of the T-DNA), SQE1cDNA-1 (5′-CTCTCCTCACATCTCTCTGCTGTTTCTAT-3′) and SQE1cDNA-2 (5′-ATGCGGTTTCTCTCTCTGTTTATTTCTT-3′); SQE2, SQE2probe-1 (5′-GCGCGGTTCCGCTCTGGTCTCAT-3′) and SQE2probe-2 (5′-ACCCGTGTTTTTCAGATCATTTGCAATTCTCA-3′); SQE3, SQE3-3 (5′-TGATTATCCGGTTTCTTCAACCA-3′), and SQE3probe-3 (5′-GTATAGAAGAGACTTAACG-3′); SQE4, SQE4probe-1 (5′-AATAAGCGCGAGGAAAGAATG-3′) and SQE4probe-2 (5′-ATTGGCTTGCGGATGATGTAAAA-3′) and SQE5 probe-3 (5′-GCCGCCTAGCAAAGCCATCATC-3′); SQE5 probe-4 (5′-AGCTTGAGGGTA-3′), SQE5probe-1 (5′-AGAGACTTAACG-3′); and SQE2probe-1 (5′-AATAAGCGCGAGGAAAGAATG-3′) and SQE2probe-2 (5′-ATTGGCTTGCGGATGATGTAAAA-3′) and SQE5 probe-3 (5′-GCCGCCTAGCAAAGCCATCATC-3′); SQE6, SQE6probe-1 (5′-TAAAGAAGCGCAAAGAACAACAGC-3′) and SQE6probe-2 (5′-AGAGACTTAACG-3′); and TUB4, TUB4-P1 (5′-TTTGCCATTTCA-3′) and TUB4-P2 (5′-ATGCGGTTTGTTCCTCTCCTGCTTTATTCTT-3′).

FIGURE 1. Triterpenoid biosynthesis. Isopentenyl diphosphate (IPP) and dimethallyl diphosphate (DMAPP) are condensed to farnesyl diphosphate (FPP) by farnesyl diphosphate synthase (FPS). Squalene synthase (SQE) converts farnesyl diphosphate into squalene. Squalene is oxidized by squalene epoxidase (SQE) to produce 2,3-oxidosqualene, which is further metabolized to produce triterpenoids including membrane sterols and brassinosteroids. Dashed arrows indicate multiple reactions. OPP, diphosphate.

sae1–3 Mutant Rescue—Arabidopsis SQE1, SQE4, and SQE5 cDNAs were excised from the pRS426Gal-SQE vectors described above using Sall and NotI and subcloned into the 35S promoters of pBARN (10) vector cut with XhoI and NotI. Plasmids were electroporated into Agrobacterium tumefaciens GV3101 (20), which was used to transform SQE1/sqe1–3 plants using the floral dip method (21). Transformants were selected on PN medium supplemented with 7.5 g/ml ammonium glufosinate. Homozygous sqe1–3/sqe1–3 mutants were identified in the T1 or T2 generation using PCR as described above, and lines homozygous for the 35S transgene were selected by examining the pattern of ammonium glufosinate resistance in the T3 generation.

Microscopy—Seedlings, roots, and developing seeds were visualized using a Leica MZ FLIII dissecting microscope.

Plant Lipid Extraction—Roots and aerial tissues from 15-day-old sqe1–3 and wild type (Col-0) grown on PNS medium were harvested for chemical analysis. For these experiments, sqe1–3/sqe1–3 plants were selected from the progeny of a SQE1/sqe1–3 parent by visually inspecting the root phenotype at 15 days. For trial 1, ~50 sqe1–3/sqe1–3 seedlings were selected from ~500 segregating plants, and for trials 2 and 3, ~100 sqe1–3/sqe1–3 seedlings were selected from ~1000 segregating plants. Tissue from ~50 (trial 1) or 86 (trials 2 and 3) wild-type Col-0 plants was used for comparison. Fresh plant tissue (trial 1, 454.5 mg aerial and 54.2 mg root for sqe1–3; 452 mg aerial and 58.2 mg root for Col-0; trial 2, 644.8 mg aerial and 118.3 mg root for sqe1–3; 623.2 mg aerial and 90.8 mg root for Col-0; trial 3, 606.0 mg aerial and 125.8 mg root for sqe1–3; 632.0 mg aerial and 106.5 mg root for Col-0) was extracted with 1:1 CH₂Cl₂/MeOH (3 × 8 ml for aerial tissues; 3 × 3 ml for roots). Extracts were dried under a N₂ stream, washed with water, and weighed. Recovered non-polar extracts in trial 1 were 4.2 mg of aerial and 1 mg of root for sqe1–3; 2.6 mg of aerial and 0.6 mg of root for Col-0. In trial 2, recovered non-polar extracts were 5.8 mg of aerial and 0.3 mg of root for sqe1–3; 2.8 mg of aerial and 0.4 mg of root for Col-0. In trial 3, recovered non-polar extracts were 17.1 mg of aerial and 2 mg of root for sqe1–3; 11 mg of aerial and 1.4 mg of root for Col-0. An aliquot of the redissolved extracts (4% of aerial; 20–25% of root) was removed for GC-MS analysis (with 1.2 μg of cholesterol ethyl ether standard added to each). Sitosterol was quantified in this crude sample for use as an internal standard to correct for losses during subsequent purification required for squalene quantification.

Saponification—To the remainder of the extracts dissolved in MeOH (96% of aerial; 75–80% of roots), KOH solution was added to make 12.5% KOH in 75:25 MeOH/water (1.2 ml for roots; 6 ml for aerial). These solutions were saponified at 70 °C under N₂ for 2 h. The samples were then allowed to cool to room temperature and extracted with methyl t-butyl ether (5 × 3 ml for aerial; 5 × 1 ml for roots) and washed with
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FIGURE 2. Arabidopsis encodes multiple squalene epoxidases. Phyllogenetic relationships of plant, yeast, and animal squalene epoxidases. Sequences from A. thaliana (At), B. napus (BnSE1,1, O65727; BnSE1,2, O65728), Canis familiaris (XM845447), Homo sapiens (HsERG1, NM003129), Macaca fascicularis (AB220561), Medicago truncatula (MtSE1,1, CAD23249; MtSE1,2, CAD23248), Mus musculus (NM009270), O. sativa (Os03g12900; Os03g12910), Rattus norvegicus (RnERG1, NM058832), and S. cerevisiae (ScERG1, CAA97201) were from the GenBankTM database; P. trichocarpa sequences (Populus 1, eugene3.04180003; Populus 2, estExt_Genewise1_v1.C_LG_I00395; Populus 3, estExt_fgenesh1.pg_v1.C.LG.V00167; Populus 4, fgenesh1.pg.C.LG.XIX000144) were accessed through the Department of Energy Joint Genome Institute website (genome.jgi-psf.org/Poptr1/Poptr1.home.html). Sequences were aligned using MegAlign (DNAStar) and the ClustalW method. The PAUP 4.0b5 program (50) was used to generate a phylogenetic tree (Fig. 2). The bootstrap method was performed for 1000 replicates with distance as the optimality criterion and all characters weighted equally. Bootstrap values are indicated at the tree nodes. Proteins with confirmed squalene epoxidase activity are underlined, including Arabidopsis SQE1, SQE2, and SQE3 (this work), the two Medicago enzymes (7), and the yeast (51), human (52), and rat (53) ERG1 enzymes. Asterisks mark SQE sequences with predicted mitochondrial targeting sequences.

RESULTS

Arabidopsis Squalene Epoxidase Genes—Six putative squalene epoxidase genes were identified in the Arabidopsis genome using a BLAST search (22) with the characterized Medicago SQE enzymes (7). We identified additional plant epoxidases using a BLAST search with the Arabidopsis SQE1 sequence and used these sequences to generate a phylogenetic tree (Fig. 2). Three putative Arabidopsis epoxidase enzymes (SQE1, SQE2, and SQE3) are 71–75% identical to characterized Medicago SQE enzymes. In contrast, the other three putative Arabidopsis epoxidases (SQE4, SQE5, and SQE6) are only 44–48% identical to characterized Medicago epoxidases but are more closely related to two Brassica putative SQE enzymes (8). SQE1, SQE2, and SQE3 are 44–46% identical to
FIGURE 3. Protein sequence alignment of characterized SQE enzymes. The Arabidopsis SQE sequences were aligned with other characterized SQE sequences using MegAlign (DNAStar) and the ClustalW method. Identical residues in at least six sequences are boxed in black. Similar residues are boxed in gray. Triangles indicate the locations of T-DNA inserts in sqe1–3 and sqe1–4. The black rectangle indicates a predicted flavin adenine dinucleotide binding domain (53). Asterisks indicate cysteine residues (42), and filled circles indicate hydrophobic residues (41) that are important for R. norvegicus ERG1 enzymatic activity. Open circles indicate residues that alter the catalytic efficiency of R. norvegicus ERG1 for squalene versus oxidosqualene substrates (41).
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the characterized SQE enzymes from yeast and humans, whereas SQE4, SQE5, and SQE6 are only 27–38% identical to these enzymes (Fig. 3).

To determine whether any of the Arabidopsis SQE homologs have squalene epoxidase activity, we expressed each Arabidopsis cDNA in a S. cerevisiae erg1 mutant, which lacks the ERG1 squalene epoxidase and is therefore a sterol auxotroph (23), and assayed the growth of the transformed yeast on medium lacking exogenous sterols. We found that the Arabidopsis SQE1, SQE2, and SQE3 cDNAs complemented the yeast erg1 null mutant to a similar extent as the yeast ERG1 gene (Fig. 4, A and B), suggesting that these cDNAs encode functional squalene epoxidases. In contrast, Arabidopsis SQE4, SQE5, and SQE6 failed to complement the erg1 mutant (Fig. 4, A and B), consistent with the possibility that these putative SQE enzymes are catalytically distinct, as suggested by the phylogenetic analysis. As we do not have antibodies to the SQE proteins, we could not explore the alternate possibility that SQE4, SQE5, and SQE6 do not accumulate when expressed in yeast.

To examine the products of the SQE enzymes, we expressed the Arabidopsis epoxidases in an erg1 erg7 double mutant, which lacks both the ERG1 squalene epoxidase and the ERG7 lanosterol synthase (24), the enzyme that would normally metabolize any oxidosqualene made following heterologous SQE expression. Thin layer chromatography analysis of extracts from these strains revealed that oxidosqualene failed to accumulate to detectable levels in erg1 erg7 yeast expressing SQE4, SQE5, or SQE6 but accumulated to similar levels in erg1 erg7 yeast expressing ERG1, SQE1, SQE2, or SQE3 (data not shown). GC-MS analysis confirmed that erg1 erg7 yeast expressing SQE4, SQE5, or SQE6 but accumulated to similar levels in erg1 erg7 yeast expressing ERG1, SQE1, SQE2, or SQE3 all produce oxidosqualene (Fig. 4, C–J, and supplemental Fig. 1), as expected from the ability of these genes to rescue the

FIGURE 4. SQE1, SQE2, and SQE3 encode functional squalene epoxidase enzymes. Arabidopsis SQE cDNAs or the S. cerevisiae ERG1 open reading frame were expressed from a galactose-inducible promoter in a S. cerevisiae erg1 mutant. Transformants were selected on synthetic medium lacking uracil and grown for 6 days at 30 °C in the presence (A) and absence (B) of ergosterol supplementation. Gas chromatographs of squalene (Sq) derivatives produced the following heterologous expression of Arabidopsis SQE cDNAs in the S. cerevisiae erg1 erg7 mutant. The GC traces for oxidosqualene (Os) (C), dioxidosqualene (dOs) (D), untransformed SMY8 (erg1 erg7) (E), and RXY6 (erg1 erg7 transformed with pRS426Gal (vector)) (F) are shown as controls. GC traces for erg1 erg7 transformed with the S. cerevisiae ERG1 (G) and Arabidopsis SQE1 (H), SQE2 (I), or SQE3 (J) revealed accumulation of oxidosqualene and dioxidosqualene (ERG1, SQE1, SQE3) or oxidosqualene (SQE2). The presence of oxidosqualene and dioxidosqualene was confirmed by MS analysis of the corresponding GC peaks (supplemental Fig. 1).
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yeast erg1 mutant (Fig. 4B). Together, the yeast complementation and GC-MS data demonstrate that SQE1, SQE2, and SQE3 encode bona fide squalene epoxidases but do not illuminate the activity of SQE4, SQE5, and SQE6.

In addition to 2,3-oxidosqualene, we found that Arabidopsis SQE1 and SQE3 produced easily detectable amounts of 2,3:22,23-dioxidosqualene (Fig. 4, G and J, and supplemental Fig. 1) when expressed in yeast. Similarly, the yeast ERG1 enzyme oxidized 2,3-oxidosqualene to 2,3:22,23-dioxidosqualene (Fig. 4, E and G). In contrast, SQE2 expression in erg1 erg2 yeast yielded oxidosqualene, but dioxidosqualene was not detected (Fig. 4I). These data suggest that the Arabidopsis SQE enzymes may have distinct substrate preferences.

To examine the expression of the Arabidopsis SQE genes, we compiled data from the Genevestigator expression data base (25). This analysis revealed substantial SQE1 and SQE3 transcript levels in most plant tissues, whereas SQE2 and SQE4 appear to be more weakly expressed (Fig. 5A). SQE5 appears to be expressed most highly in adult plant tissues, and SQE6 is most highly expressed in seeds, hypocotyls, and rosettes (Fig. 5A). We confirmed these general patterns using RT-PCR analysis of SQE transcripts in several tissues from wild-type plants (Fig. 5B).

Developmental Defects of sqe1–3—

We sought to explore the in vivo roles of the SQE genes with confirmed squalene epoxidase activity (SQE1, SQE2, and SQE3) by examining the phenotypes of loss-of-function mutants from various T-DNA insertion collections. For SQE1, we isolated three T-DNA insertion mutants from the Salk Institute collection (17) and one enhancer trap insertion line from the Cold Spring Harbor Laboratory collection (18, 26). The T-DNAs in sqe1–1 and sqe1–2 contain T-DNAs 292 and 152 bp upstream of the SQE1 coding region, respectively. sqe1–3 is located in SQE1 exon 7 (2775 bp), and the insert in sqe1–4 is in SQE1 exon 6 (2301 bp) (Fig. 6A). RT-PCR with gene specific primers revealed that intact SQE1 mRNA was not detectable in sqe1–3 mutant seedlings. However, the locus is transcribed in the mutant, as mRNA upstream of the insert was still detected (Fig. 6B).

Although we did not note morphological defects in the sqe1–1 or sqe1–2 alleles (data not shown), which contain T-DNAs upstream of the SQE1 coding region, homozygous sqe1–3 and sqe1–4 mutants were small plants that invariably died within a few weeks of transfer to soil. To determine when sqe1–3 defects are first apparent, we grew progeny from a heterozygous SQE1/sqe1–3 plant on agar-based medium for 14 days and then transferred the plants to soil and removed a leaf for genotype determination. For the first 14 days of development, sqe1–3/sqe1–3 shoots were indistinguishable from those of wild type (Fig. 7, A–D). Only after the production of several leaves was the small stature of the sqe1–3 plants apparent (Fig. 7K).

In contrast to the normal development of sqe1–3 aerial tissues in light-grown seedlings, hypocotyls of sqe1–3 seedlings grown in the dark were shorter than wild-type hypocotyls (Fig. 8A). This elongation defect is shared with mutants with reduced brassinosteroid (BR) levels, such as det2 (de-etiolated2) (27, 28). To determine whether the sqe1–3 hypocotyl elongation defects could be attributed to reduced BR production, we grew seedlings (including det2-1 as a control) on medium supplemented with 100 nM epibrassinolide. We found that the short hypocotyl of det2-1 was partially rescued by BR supplementation (Fig. 8B), as expected from previous studies (29). However, sqe1–3 short hypocotyls did not elongate substantially with BR supplementation (Fig. 8B), indicating that the...
brassinosteroid deficiency is not the primary cause of poor sqe1–3 elongation.

In addition to shoot defects, sqe1–3 plants had abnormal roots, which we could examine by growing plants hydroponically or on agar-based medium. sqe1–3 mutants produced short, highly branched roots (Figs. 7, E and F, and 8C). Unlike the det2-1 mutant, which also displays a short root, the sqe1–3 short root was not rescued by exogenous brassinolide (data not shown). The short root phenotype may contribute to the small stature of sqe1–3 plants grown hydroponically. Seed pods of sqe1–3 mutants produced long, highly branched roots (Figs. 7, A–D). The 35S-SQE1 transformants that were fertile and displayed wild-type morphology (Fig. 7K). The 35S-SQE1 construct also rescued the short root and short hypocotyl phenotypes of sqe1–3/sqe1–3 plants (Fig. 8D and data not shown). These results indicate that the sqe1–3 phenotypes result from disrupted SQE1 function.

We also tested other potential SQE enzymes for their ability to rescue the sqe1–3 phenotypes. Unlike SQE1, ectopic expression of SQE4 or SQE5 from the 35S promoter did not rescue the sqe1–3 defects (data not shown), consistent with the failure of the corresponding SQE enzymes to complement the yeast erg1 mutant (Fig. 4B).

Pleiotropic Phenotypes Resulting from SQE1 Disruption—Although several Arabidopsis sterol biosynthetic mutants with severe developmental defects have been identified (31–35), plant sqe mutants have not been reported previously. Here, we have demonstrated that one of the six putative Arabidopsis SQE genes, SQE1, is essential for normal plant development. Similar to previously identified sterol biosynthetic mutants, the sqe1–3 and sqe1–4 mutants have multiple developmental defects.

The sqe1–3 developmental defects resemble other triterpenoid biosynthetic mutants. Similar to the hmg1 mutant (31)
blocked earlier in the pathway, sqe1–3 has stem elongation and fertility defects. The cvp/smt1/cpd/hydra1 mutants, which are defective in a sterol methyltransferase, produce short stunted roots (35–39), and the fackel/hydra2 mutant, defective in a sterol C-14 reductase, has severe stem and root elongation defects (32, 33, 35). BR-deficient mutants with elongation and fertility defects also have been identified in several plants (40). Although sqe1–3 plants had short hypocotyls when grown in the dark, this defect was not as dramatic as that of BR-deficient mutants. Moreover, sqe1–3 mutants displayed severe defects in root elongation, producing very short roots (Figs. 7, E, and F, and 8C). However, exogenous BR application did not rescue sqe1–3 defects (Fig. 8B and data not shown), suggesting the sqe1–3 elongation defects are not solely due to a lack of BR.

In contrast to other triterpenoid biosynthetic mutants, which display marked defects in stem elongation (28, 31, 33, 34), sqe1–3 growth defects were most notable in roots (32) and developing seeds (33). Indeed, when sqe1–3 plants were grown hydroponically, the mutant stems elongated nearly normally (Fig. 7H), suggesting that hydroponic growth conditions may compensate in part for the limited root mass of the mutant.

In addition to the importance of SQE1 for elongation, our results demonstrate an essential role for SQE1 in seed development. Although sqe1–3/sqe1–3 embryos developed normally on a heterozygous parent plant, sqe1–3/sqe1–3 embryos developing on sqe1–3/sqe1–3 plants were completely inviable (Fig. 7, I and J). This result suggests that maternal tissue contributes SQE1 product(s) to developing embryos.

Chemical analysis of sqe1–3 plants showed dramatically increased squalene levels compared with wild-type plants (Fig. 9). However, a corresponding sqe1–3 reduction in sitosterol, the predominant Arabidopsis oxidosqualene metabolite, was not observed when data were normalized to tissue fresh weight. Thus, the remaining intact SQE enzymes in sqe1–3 can provide sufficient oxidosqualene for nearly normal sitosterol production, at least when the analysis is averaged over an entire tissue. A possible explanation for this apparent anomaly is that only a subset of cells in a particular tissue depends on SQE1 and that, when these cells accumulate squalene, it is easily detected above the normally low squalene levels. However, if this hypothetical subset of cells has a corresponding reduction in sitosterol, this deficit may be masked by sitosterol in the overall tissue. Although current analytical techniques do not allow us to monitor triter-

FIGURE 7. Developmental defects of sqe1–3. Shown is a comparison of wild-type (A and C) and sqe1–3 (B and D) seedlings. Seedlings were grown on PN medium supplemented with 0.5% sucrose and photographed at 4 (A and B) and 7 (C and D) days of development. Scale bar = 1 mm. E, roots of 26-day-old wild-type and sqe1–3 plants grown hydroponically. Scale bar = 1 cm. F, closer view of sqe1–3 roots shown in E. Scale bar = 5 mm. G, wild-type and sqe1–3 siliques from 38-day-old hydroponically grown wild-type and sqe1–3 plants. Scale bar = 1 cm. H, stems of 38-day-old wild-type and sqe1–3 plants grown hydroponically. Wild-type (I) and sqe1–3 (J) siliques from 38-day-old plants grown hydroponically were opened and photographed. Scale bars = 1 mm. I, 25-day-old wild-type (Col-0 and Ler), sqe1–3, sqe1–4, and sqe1–3 (35S-SQE1) soil-grown plants.
penoids in individual cells, it will be interesting to examine the precise localization of the various SQE enzymes in different tissues.

The sqe1 disruptions that confer these dramatic phenotypes are near the C terminus of the protein (Fig. 3). We could not detect intact SQE1 mRNA by RT-PCR in sqe1–3 seedlings; however, mRNA upstream of the sqe1–3 insertion was still present (Fig. 6B), suggesting that a truncated sqe1–3 protein missing the C-terminal 100 amino acids might accumulate in sqe1–3 plants. The missing region in sqe1–3 is the region in rat ERG1 that includes four cysteine residues and several aromatic residues that are important for enzyme activity (41, 42), sug-

![FIGURE 8. Elongation defects of sqe1–3. Shown are sqe1–3 hypocotyl elongation defects. Seeds from a SQE1/sqe1–3 heterozygote and a det2-1 control plant were grown at 22 °C in the dark for 5 days on medium without (A) or with (B) 100 nM epibrassinolide. Hypocotyls were measured and SQE1 genotypes were determined using PCR following completion of the experiment. Error bars represent S.D. of mean hypocotyl lengths (n = 12).](image)

![FIGURE 9. Squalene and sitosterol accumulation in sqe1 tissues. Roots and aerial tissues from 15-day-old seedlings from three biological replicates of Col-0 and sqe1–3 were harvested for analysis of squalene (A and B) and sitosterol (C and D) levels. Squalene was quantified in crude extracts by using GC-MS analysis. Squalene was quantified in extracts of non-saponifiable lipids (root tissue) or non-saponifiable lipids that were partially purified by solid phase extraction (aerial tissue) prior to GC-MS analysis. Squalene levels were corrected for losses during purification by comparing sitosterol levels in the crude and purified samples and adjusting appropriately. Data were normalized to both wet weight of starting tissue (A and C) and dry weight of non-polar extracts (B and D).](image)
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gesting that, even if sqe1–3 protein accumulated, it would be catalytically inactive.

Implications of Multiple Arabidopsis SQE Genes—The presence in Arabidopsis of six SQE homologs raises the question of whether these genes have redundant or unique functions. The dramatic sqe1–3 and sqe1–4 developmental defects and sqe1–3 squalene accumulation demonstrate that none of the five remaining SQE genes fully compensates for impaired SQE1 function in the endogenous context. Using heterologous expression in yeast, we found that three of the six Arabidopsis SQE-like enzymes, SQE1, SQE2, and SQE3, have SQE activity and can epoxidize squalene to 2,3-oxidosqualene. Further MS analysis revealed SQE1 and SQE3 can produce, not only oxidosqualene, but also 2,3:22,23-dioxidosqualene (Fig. 4, H and J, and supplemental Fig. 1), whereas SQE2 appears to produce primarily oxidosqualene (Fig. 4I). Interestingly, single amino acid changes can either increase or decrease the ability of rat ERG1 to metabolize oxidosqualene to dioxidosqualene (41). Moreover, certain Arabidopsis oxidosqualene cyclases can metabolize, not only oxidosqualene, but also dioxidosqualene (43), suggesting that dioxidosqualene derivatives may have functions in plants.

In addition to possible catalytic diversity, multiple SQE genes may allow organ- or tissue-specific expression of certain isozymes. In Arabidopsis, FPS1 is expressed in most plant organs, whereas FPS2 expression is restricted to inflorescences (44). Microarray (25) and RT-PCR experiments indicate that SQE1 and SQE3 have largely overlapping expression patterns, whereas SQE4 and SQE6 expression may be more restricted (Fig. 5). In addition to tissue specificity, SQE enzymes may be localized to particular subcellular compartments or participate in substrate channeling and thereby provide substrate to a subset of oxidosqualene cyclases. Arabidopsis SQE2 and one putative rice squalene epoxidase (Os03g12910) have predicted mitochondrial targeting sequences (Fig. 2) and Arabidopsis FPS1 is localized to mitochondria (45), suggesting the possibility that some triterpenoid biosynthesis is associated with mitochondria.

In addition to tissue and developmental differences, the various SQE genes may be differentially responsive to environmental stimuli, and certain SQE isozymes may be produced in conjunction with other triterpenoid biosynthetic enzymes to make specific products in response to biotic or abiotic challenges. In Medicago, one SQE is up-regulated upon methyl-jasmonate treatment, whereas a second SQE is unaffected (7). In Arabidopsis, SQE1 and SQE3 appear to be widely expressed, whereas SQE2 and SQE4 appear to be expressed at low basal levels (Fig. 5) but may be induced under certain conditions.

Plant Triterpene Diversification—Several mechanisms contribute to the remarkable diversity of plant triterpenes. As in animals, plant triterpenes are decorated at numerous positions by alkylation, hydroxylation, and glycosylation resulting in combinatorial diversity of modified skeletons. Although animals are limited to decorating a single initial cyclization product (lanosterol), plants amplify this diversity by producing >100 triterpene skeletons (5). For example, although the eight characterized Arabidopsis oxidosqualene cyclases all can accept oxidosqualene as a substrate, several of these enzymes are multifunctional, cyclizing oxidosqualene to a variety of distinct triterpene skeletons with 1–6 rings (6).

In addition to the product diversity generated by the oxidosqualene cyclase family, the presence of six SQE homologs in Arabidopsis hints that squalene epoxidation may represent an additional divergence point in triterpenoid biosynthesis. The three epoxidases that fail to function in yeast (Fig. 4, A and B) also are phylogenetically distinct (Fig. 2) and may have adopted novel functions. SQE4, SQE5, and SQE6 are more similar to Brassica SQE enzymes than to Arabidopsis SQE1, SQE2, SQE3, and putative SQE enzymes from other plants (Fig. 2). Ectopic expression of SQE4 and SQE5 fails to rescue the Arabidopsis sqe1–3 mutant (data not shown) or the yeast erg1 mutant (Fig. 4, A and B), consistent with the possibility that SQE4 and SQE5 (and the closely related SQE6) may have adopted different functions. Certain fern triterpenoids do not require an oxidosqualene intermediate and may be cyclized directly from squalene (46, 47). It will be interesting to identify the substrates and products of members of the SQE4–6 subfamily.

Analysis of the rice receptor kinase family has suggested that recent duplication and divergence of genes in families function in plant defense (48, 49). Recent divergence among SQE isozymes in the flowering plant lineage (Fig. 2) may suggest a similar occurrence for SQE4, SQE5, and SQE6.

CONCLUSIONS

Triterpenoid biosynthetic mutants often have severe pleiotropic defects, highlighting the importance of an intact triterpenoid biosynthetic pathway for plant survival. We have determined that three of the six Arabidopsis SQE homologs are bona fide squalene epoxidases and demonstrated that SQE1 is essential for plant development. sqe1 mutants have elongation defects and fail to produce viable seeds. In addition, sqe1 mutants accumulate squalene, suggesting that triterpenoid biosynthesis is blocked. Intriguingly, the Arabidopsis SQE homologs that fail to complement the Arabidopsis sqe1 mutant also fail to complement the yeast erg1 mutant, consistent with the possibility that a subset of Arabidopsis SQE enzymes may have adopted unique functions. Further genetic and biochemical analysis will provide insight into the functions of the complete family of SQE enzymes in plants.

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