Seedling-lethal phenotypes of Arabidopsis (Arabidopsis thaliana) mutants that are defective in early steps in the sterol biosynthetic pathway are not rescued by the exogenous application of brassinosteroids. The detailed molecular and physiological mechanisms of seedling lethality have yet to be understood. Thus, to elucidate the underlying mechanism of lethality, we analyzed transcriptome and proteome profiles of the cyp51A2 mutant that is defective in sterol 14α-demethylation. Results revealed that the expression levels of genes involved in ethylene biosynthesis/signalizing and detoxification of reactive oxygen species (ROS) increased in the mutant compared with the wild type and, thereby, that the endogenous ethylene level also increased in the mutant. Consistently, the seedling-lethal phenotype of the cyp51A2 mutant was partly attenuated by the inhibition of ethylene biosynthesis or signaling. However, photosynthesis-related genes including Rubisco large subunit, chlorophyll a/b-binding protein, and components of photosystems were transcriptionally and/or translationally down-regulated in the mutant, accompanied by the transformation of chloroplasts into gerontoplasts and a reduction in both chlorophyll contents and photosynthetic activity. These characteristics observed in the cyp51A2 mutant resemble those of leaf senescence. Nitroblue tetrazolium staining data revealed that the mutant was under oxidative stress due to the accumulation of ROS, a key factor controlling both programmed cell death and ethylene production. Our results suggest that changes in membrane sterol contents and composition in the cyp51A2 mutant trigger the generation of ROS and ethylene and eventually induce premature seedling senescence.

Sterols are isoprenoid-derived molecules that play essential roles in all eukaryotes. The squalene production pathway is well conserved in all organisms synthesizing sterols de novo; however, post-squalene pathways differ among biological kingdoms and lead to the production of different end products. Ergosterol and cholesterol are major sterol forms in fungi and mammals, respectively. Higher plants synthesize a complex mixture of sterols in which sitosterol, campesterol, and stigmasterol are predominant forms (Benveniste, 2002, 2004). As integral components of the membrane lipid bilayer, sterols not only play a functional role in regulating membrane fluidity and permeability but also modulate the activity and distribution of membrane-bound proteins such as receptors, enzymes, and components of the signaling pathway (Hartmann, 1998). Sterols are also precursors for the synthesis of diverse bioactive compounds involved in important developmental processes, such as steroid hormones (in animals), antheridiol (in fungi), and ecdyson (in insects). Especially in higher plants, campesterol is a direct precursor for synthesis of the plant hormone brassinosteroids (BRs; Hartmann, 1998), which function in postembryonic growth and development (Clouse and Sasse, 1998). Previous results also demonstrated that sterols play a crucial role in...
in cellulose biosynthesis during cell wall formation (Peng et al., 2002; Schrick et al., 2004).

In addition to their structural function as membrane components and their role as biosynthetic precursors, sterols have been known to play various regulatory functions in biological systems. In animal systems, sterols have been implicated in transcriptional and posttranscriptional regulation, control of cholesterol biosynthesis, meiosis, developmental patterning, and protein cleavage and degradation (Edwards and Ericsson, 1999). For instance, SCAP bearing sterol-sensing domain is an escort protein for endoplasmic reticulum-bound transcription factors, the sterol regulatory element-binding proteins (SREBPs) that activate genes for cholesterol synthesis and uptake after their proteolytic maturation in the Golgi apparatus. Cholesterol has been known to play a key role in regulating the trafficking of SREBPs between the endoplasmic reticulum and the Golgi (Goldstein et al., 2006). Cholesterol also plays important roles in the maturation of HEDGEHOG proteins, which transduce signals to adjacent cells and regulate many developmental processes, including neuronal specification and embryo development (Edwards and Ericsson, 1999).

In plant systems, phytosterols are also believed to act as signaling molecules to regulate diverse plant developmental processes (Clouse, 2000, 2002). The sterol/lipid-binding (Steroidogenic Acute Regulatory Transfer [START]) domain has been found in a number of signaling proteins, including homeodomain proteins (Kallen et al., 1998; Ponting and Aravind, 1999). For example, the START domain was found in the homeodomain HD-ZIP family of putative transcription factors, including PHABULOSA (ATHB14) and GLABRA2, which are involved in leaf morphogenesis and trichome and root hair development, respectively (Ponting and Aravind, 1999; Schrick et al., 2004). He et al. (2003) showed that sterols affect the expression of genes involved in cell expansion and cell division. Similarly, the transcription of a number of genes has been shown to be activated in response to exogenous cholesterol treatments in animal systems (Edwards and Ericsson, 1999), indicating that sterols could perform common regulatory functions in both animal and plant development.

The identification and characterization of Arabidopsis (Arabidopsis thaliana) mutant lines for the genes encoding structural enzymes of the post-squalene sterol biosynthetic pathway have revealed that the pathway can be divided into two domains, in terms of mutant phenotypes and BR responsiveness: BR-independent (sterol-specific) and BR-dependent pathways (Clouse, 2002; Lindsey et al., 2003; Schaller, 2004). Mutants defective in the BR-dependent pathway, from 24-methylene lophenol to the pathway end products (i.e., sitosterol, campesterol, and stigmasterol), typically display a BR-deficient dwarf phenotype that can be rescued by an exogenous BR application. However, detailed analyses of Arabidopsis mutants defective in the sterol-specific pathway, from mevalonate-derived squalene to 24-methylene lophenol, have revealed that sterols play essential roles in embryogenesis, hormone signaling, pattern formation, organized cell division and expansion, chloroplast biogenesis and plant viability, and the correct localization of membrane-bound proteins (Lindsey et al., 2003; Willemsen et al., 2003; Schaller, 2004; Kim et al., 2005; Babychkuk et al., 2008; Posé et al., 2009). The functional roles of the aforementioned sterols have not been assigned to those of BRs, indicating the BR-independent function of plant sterols.

Arabidopsis mutants defective in the early sterol biosynthetic pathway have displayed an early seedling-lethal phenotype as well as altered developmental patterns (Clouse, 2002; Lindsey et al., 2003; Schaller, 2004; Kim et al., 2005). Studies on underlying molecular mechanism of the seedling lethality still have been limited. Recently identified Arabidopsis sterol biosynthetic mutant lines might give us plausible clues to the fundamental mechanism of seedling lethality. The Arabidopsis cycloartenol synthase1 (cas1) mutant defective in CAS1 showed an albino or cell-death phenotype with severe defects in chloroplast biogenesis. Chloroplasts of the cas1 mutant displayed a disintegration of internal thylakoid membrane structures, with an accumulation of electron-dense lipid droplets called plastoglobuli. Chlorophyll and carotenoid pigment levels were also reduced in the cas1 mutant (Babychkuk et al., 2008). Changes in sterol content and composition due to a mutation on the Arabidopsis SQUALENE EPOXIDASE1 (SQE1) gene resulted in the mislocalization of membrane-targeted RDH2/Atrboh NADPH oxidase, which is responsible for the generation of apoplastic space-derived reactive oxygen species (ROS); these changes thereby induced an ectopic accumulation of ROS in root hair cells (Posé et al., 2009). ROS is one of the key regulators controlling the oxidative stress-induced programmed cell death (PCD) pathway (Overmyer et al., 2003).

Previously, we isolated T-DNA insertion mutants for the Arabidopsis CYP51A2 gene that mediates the 14α-demethylation step in the de novo sterol biosynthetic pathway. The loss-of-function mutant displayed a postembryonic seedling-lethal phenotype (Kim et al., 2005). Although multiple developmental defects, including reduced cell elongation and abnormal skotomorphogenesis, were also observed in the mutants, these phenotypes could be largely attributed to BR deficiency stemming from the depletion of its biosynthetic precursor campesterol (Kim et al., 2005); these defects were also observed in BR biosynthetic/signaling mutants (Choe, 2004). To gain insight into the underlying molecular mechanism of seedling lethality in the Arabidopsis cyp51A2 mutant, we performed further detailed molecular, biochemical, and physiological analyses on the basis of comparative transcriptome and proteome profilings between the wild type and the cyp51A2 mutant. According to the results, changes in sterol content and composition in the mutant likely activated the production of two key
players involved in senescence-associated PCD (i.e., ethylene and ROS). These changes in the sterol-deficient mutant thereby triggered a genetically encoded senescence program and eventually induced premature PCD.

RESULTS

Global Gene Expression in the cyp51A2 Mutant

According to our previous results (Kim et al., 2005), the Arabidopsis cyp51A2 mutant that was defective in the 14α-demethylation step of the sterol biosynthetic pathway displayed a seedling-lethal phenotype with reduced cell elongation. To gain insight into these phenotypic characteristics, we performed three independent whole-genome microarray experiments using total RNA from 9-d-old light-grown seedlings.

Campesterol, one of the major plant sterols, is a direct precursor for the production of BRs. The expression of BR biosynthetic genes, including DWF4 and CPD, was up-regulated in the biosynthetic/signaling mutants and down-regulated by an exogenous BR application (Choe, 2004, 2007; Goda et al., 2004). Our microarray data revealed that the expression of sterols and BR biosynthetic genes was increased in the cyp51A2 mutant (Table I; i.e., a homolog of sterol-C5-desaturase \([\text{HDF7}]\), C-22 sterol desaturase \([\text{CYP710A4}]\), steroid 22α-hydroxylase \([\text{DWF4}]\), and BR-6 oxidase \([\text{CYP85A1} \text{ and CYP85A2}]\)). Up-regulation of DWF4 and CPD genes in the cyp51A2-3 mutant was further confirmed by GUS histochemical analyses for promoter-GUS lines crossed to the mutant (Supplemental Fig. S1). This gene expression pattern also further supports the previous data indicating that the negative feedback regulation system normally operates in the mutant, even under conditions of defective membrane integrity caused by sterol deficiency (Kim et al., 2005).

However, the expression level of the CYP51A2 gene (At1g11680) encoding obtusifoliol 14α-demethylase was greatly decreased in the mutant background (Supplemental Table S1), demonstrating the fidelity of the microarray experiment.

### Table 1. Up- or down-regulation of selected genes in the cyp51A2-3 mutant

| Functional Category | Gene Description | AGI Code | Fold Change ± SE |
|---------------------|------------------|----------|------------------|
| Sterol/BR biosynthesis | C-22 sterol desaturase \([\text{CYP710A4}]\) | At2g28860 | 3.1 ± 1.27 |
|                     | C-6 oxidase \([\text{CYP85A1}]\) | At5g38970 | 3.3 ± 1.53 |
|                     | C-6 oxidase \([\text{CYP85A2}]\) | At3g30180 | 6.1 ± 2.87 |
|                     | Δ²-Sterol-C5-desaturase \([\text{HDF7}]\) | At3g02590 | 2.9 ± 0.28 |
|                     | Steroid 22α-hydroxylase \([\text{DWF4}]\) | At3g50660 | 4.4 ± 0.33 |
| Ethylene biosynthesis | ACC synthase \([\text{ACS7}]\) | At4g26200 | 6.5 ± 1.28 |
|                     | ACC synthase \([\text{ACS11}]\) | At4g08040 | 4.8 ± 0.54 |
|                     | ACC oxidase \([\text{ACO4}]\) | At1g05010 | 2.4 ± 0.64 |
|                     | ACC oxidase \([\text{ACO2}]\) | At1g62380 | 2.4 ± 0.50 |
| Ethylene signaling | Ethylene receptor \([\text{ETR2}]\) | At3g23150 | 4.0 ± 0.79 |
|                     | Ethylene response sensor \([\text{ERS1}]\) | At2g40940 | 3.5 ± 0.29 |
|                     | Ser/Thr protein kinase \([\text{CTR1}]\) | At5g03730 | 2.8 ± 0.25 |
| ROS detoxification | Catalase \([\text{CAT3}]\) | At1g20620 | 3.6 ± 0.88 |
|                     | Superoxide dismutase [Fe] | At4g25100 | 2.8 ± 0.41 |
|                     | GST | At2g02930 | 4.3 ± 1.80 |
|                     | GST | At2g29440 | 4.3 ± 0.50 |
|                     | GST | At4g02520 | 3.3 ± 0.41 |
|                     | GST | At3g43800 | 2.5 ± 0.53 |
|                     | GST | At1g78170 | 2.4 ± 0.44 |
|                     | Thioredoxin | At1g69880 | 19.2 ± 2.09 |
|                     | Peroxiredoxin | At1g48130 | 4.0 ± 0.98 |
| Photosynthetic related | CAB-binding protein | At1g76570 | 0.6 ± 0.24 |
|                     | CAB-binding protein | At3g54890 | 0.4 ± 0.18 |
|                     | CAB-binding protein \([\text{LHCB2:4}]\) | At3g27690 | 0.2 ± 0.06 |
|                     | CAB-binding protein \([\text{LHCA3:1}]\) | At1g61520 | 0.5 ± 0.14 |
|                     | CAB-binding protein \([\text{CP26}]\) | At4g10340 | 0.5 ± 0.20 |
|                     | PSI reaction center subunit IV | At4g28750 | 0.6 ± 0.04 |
|                     | PSI 5-kD protein | At1g51400 | 0.5 ± 0.04 |
|                     | PSI reaction center W \([\text{PsbW}]\) | At2g30570 | 0.6 ± 0.22 |
|                     | Oxygen-evolving complex-related | At1g76450 | 0.6 ± 0.10 |
|                     | Oxygen-evolving enhancer protein \([\text{PsbQ}]\) | At1g14150 | 0.6 ± 0.10 |
|                     | Rubisco activase | At2g39730 | 0.5 ± 0.18 |
|                     | Thylakoid lumenal 29.8-kD protein | At1g77090 | 0.5 ± 0.06 |
 Auxin-responsive genes, including \textit{AUX/IAA} and \textit{Small Auxin Up RNAs} (SAUR), are known to be specifically or commonly regulated by indole-3-acetic acid (IAA) and BR (Goda et al., 2004). Those genes were generally down-regulated in the \textit{cyp51A2} mutant (Supplemental Table S1); the down-regulation of auxin-responsive genes such as \textit{AUX/IAA} and SAUR could be attributed to a reduction in BR level or a defect in the auxin signaling pathway in the mutant. The expression of anthranilate synthase and anthranilate phosphoribosyltransferase involved in the biosynthesis of Trp, an auxin precursor (Normanly et al., 2004), was up-regulated in the \textit{cyp51A2} mutant (Supplemental Table S1).

One of the gene groups up-regulated in the \textit{cyp51A2} mutant is ethylene biosynthesis/signaling-related genes. These include 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (\textit{ACS11} and \textit{ACS7}), ACC oxidase (\textit{ACO4} and \textit{ACO2}), two ethylene receptors (\textit{ETR2} and \textit{ERS1}), and a signaling component (\textit{CTR1}; Table I). The transcription levels of ethylene-inducible defense-related genes such as chitinase and defensin were also increased in the mutant compared with the wild type (Supplemental Table S1), indicating that the endogenous ethylene level could be increased in the mutant seedlings.

The transcription levels of genes involved in ROS detoxification generally increased in the mutant compared with the wild type (Table I), although several genes were down-regulated. The up-regulated genes included catalase, Fe-superoxide dismutase (Fe-SOD), glutathione S-transferase (GST), thioredoxin, and peroxiredoxin (Mittler et al., 2004; Ahmad et al., 2008).

The down-regulation of photosynthesis-related genes was a prominent molecular feature culled from the global gene expression profiling between the wild type and the \textit{cyp51A2} mutant. These included a number of \textit{CAB} genes encoding chlorophyll \textit{a/b}-binding proteins (Table I; Supplemental Table S1), genes encoding components of the PSI and PSII reaction centers (Table I; Supplemental Table S1), genes encoding proteins comprising an O\textsubscript{2}-evolving complex, a gene encoding Rubisco activase, and a gene encoding a thylakoid lumenal protein (Table I).

### Proteome Analysis of the \textit{cyp51A2} Mutant

To better understand the molecular features of sterol deficiency with regard to plant growth and development, we compared the protein profiles of wild-type and \textit{cyp51A2} mutant seedlings using two-dimensional (2-D) gel electrophoresis and then identified the protein spots up- and down-regulated in the mutant compared with the wild type using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF).

Microarray data showed that ethylene biosynthetic genes, including two genes encoding ACC oxidase, were up-regulated in the \textit{cyp51A2} mutant compared with the wild type. One of them, the protein level of ACC oxidase (\textit{ACO4}), one also increased in the mutant (Table II). Another protein group that increased in the mutant included ROS-scavenging and -detoxifying enzymes such as GST, ascorbate peroxidase, Fe-SOD, ferritin, and thioredoxin (Table II; Mittler et al., 2004; Ahmad et al., 2008). Some of the genes encoding GSTs, Fe-SOD, and thioredoxin were also transcriptionally activated in the mutant (Table I; Supplemental Table S1). Salvage pathway-related proteins were also abundant in the \textit{cyp51A2} mutant compared with the wild type. These include proteasome subunits and enzymes involved in the mobilization of lipids (pyruvate, orthophosphate dikinase), proteins (Gln synthetase, aspartate aminotransferase [AAT], etc.), and nucleic acids (adenine phosphoribosyltransferase). Among them, mRNA levels of pyruvate, orthophosphate dikinase, and AAT were increased in the mutant (Supplemental Table S1).

### Ethylene Biosynthesis and Signaling in the \textit{cyp51A2} Mutant

Microarray and proteome data revealed that ethylene biosynthesis and signaling could be enhanced in the \textit{cyp51A2} mutant compared with the wild type. To further confirm these data, we performed semiquantitative and/or quantitative reverse transcription (qRT)-PCR analysis for the selected genes involved in ethylene biosynthesis and signaling, such as \textit{ETR1}, \textit{ETR2}, and \textit{ERS1} (for ethylene signaling) and \textit{ACS7}, \textit{ACS11}, and \textit{ACO4} (for ethylene biosynthesis). We also verified the expression level of \textit{BASIC CHITINASE} as an ethylene-responsive marker gene. The expression levels of all the genes investigated in the experiment increased in the mutant compared with the wild type (Fig. 1).

These results strongly suggest that both ethylene signaling and biosynthesis are activated in the sterol-deficient \textit{cyp51A2} mutant. In this context, we hypothesized that inhibition of either ethylene biosynthesis or signaling may partially alleviate seedling lethality of the mutant. To test this hypothesis, we used two approaches: treatment with chemical inhibitors that block ethylene biosynthesis or signaling, and genetic blocking of the ethylene signaling pathway. Treatment with a biosynthesis inhibitor (AVG) or a signaling
inhibitor (silver nitrate [AgNO$_3$]) on the cyp51A2 mutant not only delayed early seedling senescence but also resulted in increases in fresh and dry weight and chlorophyll content, both in a dose-dependent manner, compared with the mock treatment control (Fig. 2). These results further support the assertion that sterol deficiency induces the activation of ethylene biosynthesis/signaling. To investigate the effect of genetic blocking of the ethylene signaling pathway in the cyp51A2 mutant, we genetically crossed cyp51A2-3 heterozygote plants to an ethylene signaling mutant, etr1-1. Seedlings of the double homozygous mutant cyp51A2-3/etr1-1 were dark green in color, whereas the cyp51A2-3 mutant seedlings showed pale green

Table II. Up- or down-regulated proteins in the cyp51A2-3 mutant
*Arabidopsis Genome Initiative (AGI) code numbers with asterisks indicate that the transcription level was also changed in the cyp51A2-3 mutant compared with the wild type. Proteins down-regulated in the mutant are listed in Functional Categories photosynthesis related and ATPDX1.1 under nitrogen metabolism.

| Functional Category          | Identified Protein                                                                 | AGI Code     | Fold Change ± se |
|------------------------------|------------------------------------------------------------------------------------|--------------|------------------|
| ROS related                  | ATGSTF2, GST (belonging to the Ψ class)                                            | A4g02520*    | 1.83 ± 0.69      |
|                              | ATGSTF6, GST                                                                        | A1g02930     | 4.82 ± 1.43      |
|                              | ATGSTF7, GST (belonging to the Ψ class)                                            | A1g02920     | 3.58 ± 1.66      |
|                              | ATGSTU20, GST (belonging to the τ class)                                           | A1g78370*    | 2.26 ± 0.6       |
|                              | FSD1, Fe-superoxide dismutase                                                      | A4g25100*    | 2.19 ± 0.67      |
|                              | APX1, l-ascorbate peroxidase                                                       | A1g07890     | 1.59 ± 0.89      |
|                              | ATER1, ferritin (chloroplast-localized)                                            | A5g01600     | 2.13 ± 0.08      |
|                              | ATTRX3, thioredoxin                                                                | A5g42980     | 2.75 ± 4.31      |
| Hormone biosynthesis         | ACO4, ACC oxidase                                                                   | A1g05010*    | 3.32 ± 1.32      |
|                              | Rooty/superroot 1/hookless 3 (transaminase activity)                               | A2g20610     | 2.13 ± 0.06      |
|                              | CORI3, cystine lyase                                                                | A4g23600     | 2.56 ± 0.75      |
| Salvage pathway related      | NADP-malic enzyme 4, malate dehydrogenase                                         | A1g79750     | 1.81 ± 0.13      |
|                              | Pyruvate,orthophosphate dikinase                                                   | A4g15330*    | 1.33 ± 0.51      |
|                              | GDH2, α-subunit of the Glu dehydrogenase                                           | A5g07440     | 3.73 ± 1.83      |
|                              | AAT2, Asp aminotransferase, cytoplasm                                              | A5g19550*    | 3.55 ± 1.24      |
|                              | Gin synthetase, cytosolic                                                          | A5g37600     | 1.76 ± 0.76      |
|                              | PAC1, 2OS proteasome α-subunit C1                                                  | A3g22110     | 1.61 ± 0.88      |
|                              | PBD2, 2OS proteasome β-subunit                                                     | A4g14800     | 2.21 ± 0.66      |
|                              | APT1, adenine phosphoribosyltransferase 1                                          | A1g27450     | 1.8 ± 0.64       |
| Carbon metabolism            | Putative fructokinase, pKβ-type carbohydrate kinase family protein                | A2g31390     | 2.62 ± 1.66      |
|                              | SSP2; Suc-phosphatase                                                               | A3g52340*    | 42.98 ± 4.66     |
|                              | Sedoheptulose-1,7-bisphosphatase (SBPase)                                          | A3g55800     | 3.78 ± 0.43      |
| Nitrogen metabolism          | ATCYS1C, Cys synthase                                                               | A3g61440     | 5.79 ± 1.35      |
|                              | 3-Isopropylmalate dehydrogenase (Leu biosynthesis), plastid                        | A5g14200     | 158.02 ± 52.23   |
| Others                       | ANNA1, annexin                                                                     | A1g35720     | 3.02 ± 1.77      |
|                              | APFI, γ-carbonic anhydrase (mitochondrial)                                          | A1g47260     | 5.5 ± 2.04       |
|                              | P40, acidic protein associated with 40S ribosomal subunit of ribosomes             | A1g72370     | 2.3 ± 1.17       |
|                              | Sulforotransferase family protein                                                   | A1g74090     | 6.32 ± 7.28      |
|                              | Putative alcohol dehydrogenase, ald/keto reductase family protein                  | A2g37760*    | 3.14 ± 2.11      |
|                              | Universal stress protein (USP) family protein                                       | A3g17020     | 2.83 ± 0.63      |
|                              | Carboxyesterase                                                                   | A1g48690     | 2.17 ± 0.82      |
|                              | Unknown protein                                                                    | A4g19970     | 3.48 ± 1.05      |
|                              | Major latex-related/MLP-related                                                     | A4g23680     | 6.89 ± 4.27      |
|                              | Cinnamyl-alcohol dehydrogenase, putative (CAD)                                     | A5g19440     | 8.37 ± 6.59      |
|                              | NADH-cytochrome b, reductase, putative                                             | A5g20080     | 2.45 ± 0.91      |
|                              | Methylthioalkylmalate synthase-like protein                                        | A5g23020*    | 3.97 ± 0.68      |
|                              | COP1-interacting protein-related                                                  | A5g43310     | 7.34 ± 7.79      |
| Photosynthesis related       | PSAG, PSI reaction center subunit V, chloroplast precursor                         | A1g55670     | 0.61 ± 0.04      |
|                              | RbcL, Rubisco large subunit                                                        | ACG00490     | 0.53 ± 0.13      |
|                              | Glyceraldehyde-3-phosphate dehydrogenase B subunit, chloroplast localized          | A1g42970     | 0.61 ± 0.27      |
|                              | Fru-bisphosphate aldolase, putative                                                | A2g21330     | 0.25 ± 0.15      |
|                              | Fru-bisphosphate aldolase, putative                                                | A4g26330*    | 0.34 ± 0.12      |
|                              | CA1, carbonic anhydrase 1 (chloroplast precursor)                                  | A3g01300*    | 0.16 ± 0.02      |
| Nitrogen metabolism          | ATPDX1.1, pyridoxine biosynthesis 1.1                                              | A2g38230     | 0.52 ± 0.09      |
color (Fig. 3). These genetic crossing data are consistent with the results of the signaling inhibitor treatment, further supporting the assertion that the ethylene signaling pathway is activated in the sterol-deficient cyp51A2 mutant.

RT-PCR data for ethylene biosynthetic genes (ACS and ACO) and an ethylene-responsive gene (BASIC CHITINASE) indicated that ethylene content might be increased in the mutants compared with the wild type (Fig. 1). This notion was further supported by the data that treatment with ethylene biosynthesis inhibitor partly delayed the early seedling senescence of the mutant compared with the nontreatment control (Fig. 2). In this context, we measured the ethylene content of

![Figure 1](image1.png)

**Figure 1.** Expression levels of ethylene signaling/biosynthesis-related genes in the cyp51A2-3 mutant. A, RT-PCR analysis of ethylene signaling/biosynthesis-related genes in cyp51A2-3 mutants. Total RNA was isolated from 9-d-old light-grown seedlings and used for RT-PCR. Actin-2 transcripts were amplified as a positive control for PCR. B, qRT-PCR analysis of selected ethylene signaling/biosynthesis-related genes in cyp51A2-3 mutants. Ws-2, Wassilewskija-2.

![Figure 2](image2.png)

**Figure 2.** Effect of ethylene biosynthesis or signaling inhibition on a seedling-lethal phenotype of the cyp51A2-3 mutant. A, Morphological change of the cyp51A2-3 mutant treated with AVG or AgNO₃. Ten-d-old seedlings were transferred to each treatment condition and further cultured for 15 d under a dark/light cycle. Bar = 0.5 cm. B, Changes in fresh weight (left), dry weight (middle), and chlorophyll (Chl.) content (right) in cyp51A2-3 mutants treated with AVG or AgNO₃.
the wild type, the weak mutant allele cyp51A2-2, and the strong mutant allele cyp51A2-3. The strong mutant allele produced approximately 4-fold more ethylene than the wild type (Table III), whereas the weak mutant allele produced only slightly more than the wild type.

Accumulation of ROS in the cyp51A2 Mutant

Microarray and proteomic data showed that ROS-scavenging and -detoxifying enzymes were transcriptionally and/or translationally activated in the cyp51A2 mutant compared with the wild type (Tables I and II). Based on these results, we hypothesized that ROS could accumulate to higher levels in the mutant than in the wild type. To test this hypothesis, we performed nitroblue tetrazolium chloride (NBT) histochemical staining to detect superoxide radicals in light-grown seedlings. A dark blue insoluble formazan compound was hardly detected in the wild type, whereas the mutants were densely stained with dark blue at the whole-plant level (Fig. 4). The staining level was denser in the strong mutant allele cyp51A2-3 than in the weak mutant allele cyp51A2-2. This result strongly suggests that ROS highly accumulate in the sterol-deficient cyp51A2 mutant.

Photosynthetic Activity in the cyp51A2 Mutant

The sterol-deficient cyp51A2 mutant displayed a seedling-lethal phenotype (Kim et al., 2005). According to our microarray and proteomic data (Tables I and II), the seedling-lethal phenotype could be partially attributed to the greatly reduced photosynthetic activity in the mutant compared with the wild type. To verify the results, we performed several experimental approaches: qRT-PCR analysis for photosynthesis-related genes, western analysis for the proteins involved in photosynthesis, electron microscopy analysis for chloroplast structure, and the measurement of direct photosynthetic activities.

qRT-PCR analyses were performed for two genes encoding either the PSI reaction center subunit VI (At1g52230) or the chlorophyll a/b-binding protein (At5g54890). The expression levels of both genes were remarkably reduced in the mutant alleles compared with the wild type; the reduction was much more pronounced in the strong mutant allele than in the weak allele (Fig. 5, A and B).

Western analysis using antibodies against RbcL, components of the light-harvesting complex, and photosystem components clearly showed that the levels of these photosynthesis core proteins were greatly down-regulated in the sterol-deficient cyp51A2 mutant compared with the wild type (Fig. 5C). The levels of these proteins were much lower in the strong mutant allele cyp51A2-3 than in the weak mutant allele cyp51A2-2 (Fig. 5C).

Electron microscopy analysis revealed that the cyp51A2 mutant displayed an aberrant plastid developmental pattern compared with the wild type. The chloroplasts of the wild type showed a highly ordered arrangement of thylakoid membrane structures and had smaller electron-dense lipid droplets, plastoglobuli (Fig. 6A), whereas the number of thylakoid membrane structures was reduced in the weak allele cyp51A2-2 mutant, which had an arrangement similar to that of the wild type (Fig. 6B). However, the chloroplasts of the strong mutant allele cyp51A2-3 lacked intact thylakoid membrane structures. Plastoglobuli in the strong mutant allele were larger than those in both the wild type and the weak mutant allele (Fig. 6C).

The expression levels of photosynthesis-related genes were transcriptionally and/or translationally reduced in the cyp51A2 mutant compared with the wild type (Tables I and II; Fig. 5). Microscopy analysis also revealed that chloroplast development was severely affected by sterol deficiency (Fig. 6). In this context, we

Table III. Ethylene contents of the wild type and cyp51A2-2 and cyp51A2-3 mutants

| Plants                  | Ethylene Productiona |
|-------------------------|----------------------|
| Wild-type Wassilewskija-2 | 5.51 ± 0.50          |
| cyp51A2-2               | 6.85 ± 0.68          |
| cyp51A2-3               | 22.36 ± 2.40         |

*aEthylene production was repeatedly measured for three independent samples (total of six measurements) using 10-d-old light-grown seedlings.*
compared photosynthetic activity between the wild-type and mutant plants. First, the wild-type and mutant seedlings were measured for their chlorophyll content. Total chlorophyll content was slightly decreased in the weak mutant allele compared with the wild type but noticeably reduced in the strong mutant allele (Fig. 7A). We also verified photosynthetic oxygen evolution as a measurable photosynthetic activity. In the weak mutant, the photosynthetic oxygen evolution value was comparable to that of the wild type, whereas the oxygen evolution rate of the strong mutant was less than 50% of that of the wild type (Fig. 7B).

DISCUSSION

Arabidopsis mutants defective in the early sterol biosynthetic pathway that produces sterol intermediates between squalene and 24-methylene lophenol displayed a seedling-lethal phenotype as well as an altered developmental pattern (Lindsey et al., 2003; Schaller, 2004; Kim et al., 2005). These mutants include fackel/ hydra2, hydra1, and cyp51A2. Recently, the Arabidopsis cas1 mutant defective in cycloartenol synthase, which mediates the first committed step of the post-squalene pathway, displayed albino or cell-death phenotypes, depending on mutation. The cas1 mutant also exhibited severe defects in chloroplast development (Babiychuk et al., 2008). However, the underlying molecular mechanisms of the lethality observed in sterol-deficient mutants have not yet been studied. In this paper, to address the mechanism of seedling lethality in sterol biosynthetic mutants, we performed molecular, biochemical, and physiological analyses using the Arabidopsis cyp51A2 mutant, which is defective in the 14α-demethylation step of the early sterol pathway (Kim et al., 2005). In those analyses, we found that the production of ethylene and ROS, key factors of senescence-associated PCD, was activated in the cyp51A2 mutant and that the ethylene signaling pathway might also be influenced in the mutant. Based on these findings, we conclude that the production of ethylene and ROS was perturbed in the sterol-deficient cyp51A2 mutant and that changes in these endogenous factors partially caused premature seedling senescence and ultimately led to cell death.

Figure 4. Accumulation of ROS in cyp51A2 mutants. In situ histochemical detection of ROS production in the wild type (left), a weak allele cyp51A2-2 mutant (middle), and a strong allele cyp51A2-3 mutant (right). Nine-d-old seedlings were used for staining with NBT, which reacts with superoxide to produce a dark blue insoluble formazan compound. Bars = 2 mm (left and middle) and 1 mm (right). Ws-2, Wassilewskija-2.

Figure 5. Expression levels of photosynthesis-related genes in cyp51A2 mutants. A, RT-PCR analysis of photosynthesis-related genes in cyp51A2 mutants. B, qRT-PCR analysis of selected photosynthesis-related genes in the cyp51A2-3 mutant. C, Western analysis of photosynthesis-related proteins in cyp51A2 mutants. Ws-2, Wassilewskija-2.
Transcriptome and Proteome Analyses Reveal Activation of Ethylene Biosynthesis/Signaling in the cyp51A2 Mutant

A comparison of transcriptomes of the wild type and the cyp51A2 mutant revealed that the genes involved in ethylene biosynthesis and perception/signaling were generally up-regulated in the mutant (Table I; Fig. 1). The protein level of ACO4, one of the ethylene-forming enzymes, was also increased in the mutant compared with the wild type (Table II). These molecular data were further confirmed by the inhibition of either ethylene biosynthesis or signaling (Figs. 2 and 3) and more directly by the measurement of endogenous ethylene levels (Table III). The activation of ethylene biosynthesis and its signaling presumably resulted in the up-regulation of defense-related genes such as defensin and chitinase (Fig. 1A; Supplemental Table S1). Previous microarray analyses showed that the expression levels of ethylene receptor/signaling genes increased in wild-type plants that had been exogenously treated with ethylene and/or the constitutive ethylene-responsive mutant ctr1-1 (Schenk et al., 2000; Alonso et al., 2003; Zhong and Burns, 2003; De Paepe et al., 2004), indicating that ethylene regulates its own signaling. Hua et al. (1998) also previously showed that expression of the ethylene receptor genes ERS1, ERS2, and ETR2 was induced by ethylene.

Ethylene biosynthesis can be altered by developmental or environmental factors (Hartmann, 1998). Several pieces of direct and indirect evidence have demonstrated that changes in plant sterol content and composition affect the activities of H^+-ATPase, NADPH oxidase, hormone signaling, and the polar distribution of auxin efflux carriers (Grandmougin-Ferjani et al., 1997; Souter et al., 2002; Willemsen et al., 2003; Men et al., 2008; Posé et al., 2009). Arabidopsis hydra1 and fackel/hydra2 mutants displayed phenotypes reminiscent of abnormal ethylene signaling (i.e., an abnormal root hair formation pattern and reduced root growth). Inhibition of ethylene signaling through the application of silver ions, or the genetic crossing to the ethylene-resistant mutant etr1-1, resulted in the rescue of root hair formation and root growth. Based on these findings, the authors proposed that the ethylene receptor ETR1 is altered constitutively in activity to promote the ethylene signaling process.

Figure 6. Plastid morphology in cyp51A2 mutants. A, The wild type. B, cyp51A2-2 mutant. C, cyp51A2-3 mutant. Cotyledons from 9-d-old seedlings were used for transmission electron microscopy. Bars = 1 \( \mu \)m (A) and 0.5 \( \mu \)m (B and C).

Figure 7. Comparison of photosynthetic activity between the wild type and cyp51A2 mutants. A, Chlrophyll (Chl.) contents. B, Photosynthetic oxygen evolution (Pmax value). Ws-2, Wassilewskija-2.
pathway as a consequence of changes to the membrane environment, which is caused by reduction in sterol content and incorrect sterol composition (Souter et al., 2002). In the cyp51A2 mutant described here, the inhibition of ethylene signaling by chemical treatment or genetic crossing to etr1-1 partially suppressed the early seedling-lethal phenotype (Figs. 2 and 3). Therefore, our data also serve as indirect evidence that correct sterol composition is essential for regulated ethylene signaling, as demonstrated previously in hydra mutants (Souter et al., 2002). The effects of altered sterol content and composition on the activity of ethylene receptors need to be directly demonstrated.

Accumulation of ROS in the cyp51A2 Mutant

ROS are continuously generated as toxic by-products of many essential metabolic reactions, including photosynthesis. Because they are highly reactive, ROS cause irreversible damage to major cellular components such as lipids, proteins, and nucleic acids (Apel and Hirt, 2004; Ahmad et al., 2008). A reduction in RbcL protein level was observed in the cyp51A2 mutant (Fig. 5C; Table II; Supplemental Fig. S2) and could be partially attributed to the breakdown of the abundant protein by highly reactive ROS. Evidence is accumulating that ROS also play important roles as signaling molecules in controlling various biological processes, including PCD, stomatal closure, and defense responses to environmental stresses (Apel and Hirt, 2004; Mittler et al., 2004). Chloroplasts have been shown to be one of the major sites of ROS production. Recent studies revealed that nucleus-encoded plastid proteins EXECUTER1 and EXECUTER2 mediate the retrograde transfer of the ROS signal from the plastid to the nucleus and thereby modulate ROS-dependent nuclear gene expression (Lee et al., 2007; Kim et al., 2008).

To avoid oxidative damage to cellular components, the cellular ROS level should be stringently maintained at nontoxic levels by enzymatic and nonenzymatic antioxidant systems in several subcellular compartments (Apel and Hirt, 2004; Ahmad et al., 2008). However, the cellular ROS level can be acutely or chronically changed by environmental stimuli or during the execution of normal developmental processes. The imbalance between ROS production and scavenging results in the triggering of oxidative stress, thereby perturbing production at the cellular level of plant hormones such as ethylene and ultimately promoting cell death. NBT staining results showed accumulation of ROS such as superoxide radical in the mutant compared with low levels in the wild type (Fig. 4). This result indicates that the cyp51A2 mutant is certainly under oxidative stress due to an imbalance between ROS production and its removal. As a compensatory mechanism against challenged oxidative stress, the enzymes or genes involved in ROS detoxification were up-regulated in the cyp51A2 mutant (Tables I and II; Supplemental Table S1), as has been observed in oxidatively stressed plants (Desikan et al., 2001; Vranová et al., 2002; Apel and Hirt, 2004).

Among the diverse sites of ROS production, plasma membrane-localized NADPH oxidases are responsible for ROS generation in the apoplastic space. Several reports have demonstrated that sterols modulate activity or the asymmetric distribution of membrane-bound proteins such as receptors, carriers, and enzymes (Grandmougin-Ferjani et al., 1997; Souter et al., 2002; Willemsen et al., 2003; Men et al., 2008). In the Arabidopsis dry2/sqe1-5 mutant defective in SQE1, out of six putative SQE genes (Benveniste, 2002), the mislocalization of RHD2/AtrbohC NADPH oxidase resulted in an ectopic ROS accumulation in the root hairs of the mutant. Based on this result, Posé et al. (2009) proposed that sterols play an essential role in the localization of NADPH oxidases in root hair cells. Changes in sterol composition and content in the cyp51A2 mutant could modify the membrane environment and thereby alter the stability or activity of NADPH oxidases localized in the plasma membrane, by which stringent maintenance of the cellular ROS level was broken and ROS accumulated in the mutant (Fig. 4).

Premature Seedling Senescence of the cyp51A2 Mutant Resembles Leaf Senescence

Arabidopsis mutants defective in the early sterol biosynthetic pathway displayed premature seedling senescence or a cell-death phenotype (Lindsey et al., 2003; Schaller, 2004; Kim et al., 2005; Babychuk et al., 2008). Plant senescence is a highly regulated developmental process and a type of PCD, rather than a simple and passive type of degeneration. PCD can occur at the cell, tissue, organ, or organism level and can be initiated by environmental, developmental, or hormonal factors (Dangl et al., 2000). Leaf senescence is a well-characterized PCD that is accompanied by changes in cell structure, metabolism, and gene expression. These changes include the redifferentiation of chloroplasts into gerontoplasts, a reduction of chlorophylls and carotenoids, the activation of salvage pathways, and the down-regulation of photosynthesis-related genes (Buchanan-Wollaston, 1997; Biswal et al., 2003; Lim and Nam, 2007; Lim et al., 2007). The molecular, cellular, and physiological changes observed in the cyp51A2 mutant are quite similar to those of the senescing leaf; for example, a number of genes involved in the structure of the photosynthetic apparatus and photosynthetic pathway were down-regulated in the mutant (Tables I and II; Supplemental Table S1). During senescence, chloroplasts redifferentiate into premortal plastid-type gerontoplasts, which have lost thylakoid membrane structures and accumulated electron-dense lipid droplets known as plastoglobuli (Biswal et al., 2003). These gerontoplasts were observed in the cyp51A2 mutant, whereas wild-type plants have a normal chloroplast structure containing intact grana (Fig. 6). This change in chloroplast structure
was also observed in a sterol-specific cas1 mutant (Babiychuk et al., 2008). However, the Arabidopsis cas1 mutant is not seedling lethal, probably due to the presence of a homologous gene (At3g45130) sharing 62% identity to the cas1 gene (Benveniste, 2002). Considering that the BR biosynthetic dwf4 mutant had normal plastid structure (Azpiroz et al., 1998), we can assume that sterols might play specific roles in plastid development. The changes in chloroplast structure in the cyp51A2 mutant were also accompanied by a loss of chloroplast proteins, including RbcL, CAB, photosystem components, and photosynthetic pathway enzymes (Table II; Fig. 5C); a reduction in chlorophyll level (Fig. 7A); and ultimately a severe reduction in photosynthetic oxygen evolution (Fig. 7B). These changes indicate that premature senescence occurred in the sterol-deficient mutant.

During the degenerative senescence process, the salvage pathway is highly geared toward the remobilization of hydrolyzed macromolecules such as lipids, proteins, and nucleic acids (Buchanan-Wollaston, 1997; Dangl et al., 2000; Lim and Nam, 2007). Gluconeogenesis leading from lipids to sugars is known to be activated during the senescence of photosynthetic tissues. mRNA or protein levels for malate synthase, phosphoenolpyruvate carboxy kinase, and pyruvate, orthophosphate dikinase were activated in the cyp51A2 mutant (Table II; Supplemental Table S1). Enzymes for the biosynthesis of amide forms of amino acids were also up-regulated in the cyp51A2 mutant. These include Glu dehydrogenase, AAT, and Gln synthetase (Table II; Supplemental Table S1). The induction of these enzymes is related to the remobilization of amino acids from the breakdown of proteins during leaf senescence. The breakdown of nucleic acids, in particular rRNA, occurs in senescing cells. An RNase gene (RNS1) responsible for the breakdown of nucleic acids during senescence was induced in the cyp51A2 mutant (Supplemental Table S1).

Among the plant hormones, ethylene is a strong promoter of leaf senescence, although it is not an essential factor for progression of the highly organized developmental process (Lim and Nam, 2007; Lim et al., 2007). Treatment with exogenous ethylene induces senescence, whereas inhibition of ethylene biosynthesis, perception, or signal transduction increases leaf longevity, primarily due to the delayed onset of the senescence program. As a promoter of senescence, ethylene inhibits photosynthesis, which is achieved by decreasing the expression of photosynthesis-related genes and increasing the expression of senescence-associated genes (Dangl et al., 2000; Gan, 2004). Several microarray approaches have revealed that ethylene down-regulates genes involved in the structure of the photosynthetic apparatus and the photosynthetic pathway (Schenk et al., 2000; Alonso et al., 2003; Zhong and Burns, 2003; De Paeppe et al., 2004). This molecular feature was also observed in the microarray and proteomic data for the cyp51A2 mutant (Tables I and II; Supplemental Table S1). Therefore, chronic overproduction of ethylene in the sterol-deficient cyp51A2 mutant led to the early activation of the senescence pathway and resulted in a reduction in photosynthetic activity due to the down-regulation of photosynthesis-related genes. Ultimately, this led to premature senescing leaves in the mutant.

**Ethylene and ROS Mediate Premature Seedling Senescence in the cyp51A2 Mutant**

ROS closely interacts with plant hormones, including ethylene, to control the PCD pathway. In the oxidative cell-death pathway, ROS not only activates its own generation but also increases ethylene synthesis by inducing ethylene biosynthetic genes such as ACS and ACO; an increased ethylene level serves as a positive feedback signal for ROS production (Overmyer et al., 2003). According to previous results, environmental factors that destroy the cellular redox state induce ROS production and consequently activate ethylene production (Wang et al., 2002). Based on previous reports, oxidative stress induced by an imbalance between ROS accumulation and scavenging could be one of the causal agents that promote increased ethylene production in the cyp51A2 mutant, by up-regulating the ACS and ACO genes (Tables I–III). Oxidative stress causes a rapid inhibition of photosynthetic activity, which is reflected by changes in the activity or synthesis of chloroplast proteins. A reduction in chloroplast proteins was observed in the cyp51A2 mutant (Table II; Fig. 5C). Recent microarray data further support the assertion that oxidative stress can suppress the transcription level of photosynthesis-related genes such as CAB and photosystem components (Desikan et al., 2001; Vranová et al., 2002; Mahalingam et al., 2006), as reported in wild-type Arabidopsis that had been exogenously treated with ethylene or the ethylene-responsive mutant ctr1-1 (Schenk et al., 2000; Alonso et al., 2003; Zhong and Burns, 2003; De Paeppe et al., 2004). Therefore, down-regulation in photosynthesis-related genes could be caused by oxidative stress-mediated ethylene production. Microarray and proteome data for the cyp51A2 mutant also showed that a number of genes involved in the photosynthetic apparatus, as well as the resulting biochemical reactions, were down-regulated in the mutant (Tables I and II; Supplemental Table S1). In comparison with previous reports, these changes in gene expression might result from the accumulation of ROS in the mutant, which causes an increase in ethylene biosynthesis that is, in turn, followed by a reduction in the expression of photosynthesis-related genes and, eventually, by seedling lethality.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

The Arabidopsis (Arabidopsis thaliana) ecotype Wassilewskija-2 was used as the wild type. T-DNA-tagging mutants for CYP51A2, cyp51A2-2, and...
were washed once in 2× Hybridization Chamber Lids (BioMicro Systems) for 18 h at 60°C. The Qiagen-Operon Arabidopsis Genome Array Ready Oligo Set version 3.0 (Genisphere). The cDNA probes were hybridized to a 29,000-element Arabidopsis Oligonucleotide Microarray printed at the University of Arizona using an Etta MALDI-TOF (Amersham Biosciences). Peptides were evaporated with a nitrogen laser at 337 nm and accelerated with 20 kV injection pulse for time-of-flight analysis. Each spectrum was the cumulative average of 300 laser shots. The search program ProFound, developed by The Rockefeller University (http://129.85.19.192/profound_bin/WebProFound.exe), was used for protein identification by peptide mass fingerprinting. Spectra were calibrated with trypsin autodigestion ion peak mass-to-charge ratios 842.510 and 2,211.1046 as internal standards.

Microarray Hybridization
cDNAs were prepared from 15 μg of total RNA per sample using SuperScript II reverse transcriptase (Invitrogen), and microarray probes labeled with Cy3 and Cy5 were prepared from the cDNA using the 3DNA Array 900 DNA-labeling kit according to the manufacturer’s instructions (Genisphere). The cDNA probes were hybridized to a 29,000-element Arabidopsis Oligonucleotide Microarray printed at the University of Arizona using the Qiagen-Operon Arabidopsis Genome Array Ready Oligo Set version 3.0 (http://www.ag.arizona.edu/microarray/). All cDNA and fluorescent dye hybridizations were performed in a volume of 35 μL using the SDS-based hybridization buffer provided by the manufacturer. The cDNA hybridizations were performed on a MAUI Hybridization System and MAUI Mixer AO Hybridization Chamber Lids (BioMicro Systems) for 18 h at 60°C. The slides were washed once in 2× SSC for 5 min and twice in 0.2× SSC for 3 min.

Scanning and Data Analysis
After hybridizations, the slides were scanned with GenePix 4000B (Axon Instruments) and the spots were quantified using GenePix Pro 4.0 (Axon Instruments). The scanned microarray results were imported into Acuity analysis software 3.0 (Axon Instruments) and normalized using global Lowsess normalization (Yang et al., 2002). Data files were then created for each experiment that satisfied the following filter: Sum of Medians ≥ 100 and (Flags ≥ 0) and (FSD5% Sat < 3) and (FSQ32% Sat < 3) and (RgnR2 < 85/522 > 0.6) and (SNR635 > 3) and (SNR532 > 3). This filter eliminates data points that were flagged as bad by Genepix or that had sum of medium < 100 (very weak) or that had pixels less than those of background (not likely to be real spots). The spots that passed these criteria for at least 66% out of the used slides were analyzed. For the comparison of wild-type and cyp51A2-3 lines, the average of median ratios for spots that matched these criteria in each data set were calculated. Since the distribution of the difference may not follow a normal distribution, we performed a permutation t-test. We randomly permuted the expression values for each gene 1,000 times by group-wise shuffling at a significance level of 0.01 by q values of false discovery rate to correct for multiple comparisons.

Proteome Analysis
For 2-D PAGE and MALDI-TOF analysis, we used a commercial proteome analysis service that was supplied by Genomine (http://www.genomine.com). Total soluble proteins were extracted from 9-d-old seedlings using the sample buffer (7 M urea, 2 M thiourea containing 4% [w/v] CHAPS, 1% [w/v] dithiothreitol, 2% [v/v] Phomalate, and 1 mM benzamidine). Protein samples (each 200 μg) were loaded onto equilibrated immobilized pH gradient strips.

Isometric focusing was performed using a Multiphor II electrophoresis unit and EPS 3500 XL power supply (Amersham Biosciences). After equilibration, immobilized pH gradient strips were inserted onto SDS-PAGE gels (20–24 cm, 10%–16%). SDS-PAGE was performed using the Hoefer DALT 2-D system (Amersham Biosciences). 2-D gels were silver stained as described by Oakley et al. (1980).

Quantitative analysis of digitized images was carried out using the PDQuest software (version 7.0; Bio-Rad). The quantity of each spot was normalized by total valid spot intensity. Protein spots were selected for the significant expression variation deviated over 2-fold in its expression level compared with control or normal sample.

Protein spots were enzymatically digested in-gel in a manner similar to that described previously by Shevchenko et al. (1996). Gel pieces were washed with 50% acetonitrile, dried to remove solvent, rehydrated with trypsin, and incubated 8 to 10 h at 37°C. Trypsin peptides were recovered by combining the aqueous phase from several extractions of gel pieces with 50% aqueous acetonitrile. After concentration, the peptide mixture was desalted using C18 ZipTips (Millipore), and peptides were eluted in acetonitrile. An aliquot of this solution was mixed with an equal volume of a saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% aqueous acetonitrile, and the mixture was spotted onto a target plate. Protein analysis was performed using an Etta MALDI-TOF (Amersham Biosciences). Peptides were evaporated with a nitrogen laser at 337 nm and accelerated with 20 kV injection pulse for time-of-flight analysis. Each spectrum was the cumulative average of 300 laser shots. The search program ProFound, developed by The Rockefeller University (http://129.85.19.192/profound_bin/WebProFound.exe), was used for protein identification by peptide mass fingerprinting. Spectra were calibrated with trypsin autodigestion ion peak mass-to-charge ratios 842.510 and 2,211.1046 as internal standards.

Western-Blot Analysis
Total proteins were extracted from 9-d-old light-grown seedlings using an extraction buffer (125 mM Tri-HCl, pH 8.8, 1% [w/v] SDS, and 10% [v/v] glycerol). Approximately 20 μg of protein per lane was separated using 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (QRICOAT SDI). After blocking with 6% skim milk in Tris-buffered saline plus Tween 20, the membrane was incubated with antibodies at 2001 in the blocking buffer for 2 h at room temperature, incubated with the secondary antibody at 1:10,000 in the blocking buffer for 1 h at room temperature, and visualized using the Immuno-Star HRP Substrate Kit (Bio-Rad). Antibodies used in this experiment were purchased from Agrisera (http://www.agrisera.se).

Genetic Crossings
The ethylene signaling mutant etr1-1 was genetically crossed to cyp51A2-3 heterozygotes. PCR genotyping was performed to identify heterozygous cyp51A2-3 from the F1 seedlings. The seedlings were self-crossed to achieve an F2 generation. F2 seedlings were grown on MS medium containing 5 μM ACC under dark conditions to determine segregation of the etr1-1 phenotype and on MS medium under light conditions to determine segregation of the cyp51A2-3 homozygotes. F3 lines homozygous for etr1-1 and heterozygous for cyp51A2-3 were established and used in phenotypic analysis.

Ethylene Analysis
For ethylene measurements, Arabidopsis seedlings were grown for 10 d on 1× MS solid medium containing 1% Suc under light/dark conditions (18 h of light at 24°C, 6 h of dark at 20°C). Ethylene was measured by placing the seedlings on 25 mL of solid MS medium in a 45 mL air-tight glass vial sealed with a rubber stopper in an ambient atmosphere at room temperature. Gas samples were taken from each tube with a 1-mL gas-tight syringe and injected into a gas chromatograph (Hewlett Packard 5890 series II) equipped with a flame ionization detector and a packed stainless steel column (2 m × 0.18 cm, 3% Restek’s SE-54 or Porapak Q, and 10% Carbowax 20M) and water, 1% [w/v] CHAPS, 1% [w/v] dithiothreitol, 2% [v/v] Phomalate, and 1 mM benzamidine). Protein samples (each 200 μg) were loaded onto equilibrated immobilized pH gradient strips.
biosynthesis inhibitor) or AgNO₃, (as an ethylene signaling inhibitor) and further cultured for 15 d under light/dark conditions (16 h of light at 24°C/8 h of dark at 20°C).

Measurements of Chlorophyll Content and Photosynthetic Activity

Total chlorophylls were extracted by incubating the seedlings in 1 mL of 95% ethanol (v/v) under 80°C for 30 min. Contents of chlorophylls a and b were measured following the method of Lichtenthaler (1987). The photosynthetic oxygen evolution in the seedlings was measured at 25°C using an oxygen electrode chamber (model LD2; Hansatech) according to the methodology of Oquist et al. (1992). After the seedlings were dark adapted for 15 min in the oxygen electrode chamber, we applied a continuous actinic light for 4 min in air enriched with 1% CO₂, CO₂ for photosynthesis was supplied via 1 M carbonate/bicarbonate buffer solution (pH 9.0). Actinic light (600 μmol m⁻² s⁻¹) was provided by a KL1500 lamp (Walz) equipped with a Schott RG 630 red filter.

Transmission Electron Microscopy

For electron microscopy, cotyledons from 9-d-old Arabidopsis seedlings were prefixed in 1% osmium tetroxide (in 0.05 M sodium cacodylate buffer, pH 7.8). Chlorophyll was removed from the seedlings by incubating with ethanol: acetic acid (9:1) and ethanol.

Supplemental Data

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

Supplemental Figure S1. GUS histochemical analysis of DWF4::GUS and CPD::GUS in the cyp9/12A2-3 mutant.

Supplemental Figure S2. Comparison of 2-D gel electrophoresis pattern between the wild type and the cyp9/12A2-3 mutant.

Supplemental Table S1. Differentially expressed genes in microarray experiments.

Supplemental Table S2. Primers for RT-PCR or qRT-PCR used in this experiment.

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