Plant Science 209 (2013) 46–57

Contents lists available at SciVerse ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

A chemical genetics approach reveals a role of brassinolide and cellulose synthase in hypocotyl elongation of etiolated Arabidopsis seedlings☆

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ARTICLE INFO

Article history:
Received 8 August 2012
Received in revised form 17 April 2013
Accepted 18 April 2013
Available online 28 April 2013

Keywords:
ACC synthase
Acsinones
CESA6
Chemical genetics
DET2
ETO1

ABSTRACT

The development of juvenile seedlings after germination is critical for the initial establishment of reproductive plants. Ethylene plays a pivotal role in the growth of seedlings under light or dark during early development. Previously, we identified small molecules sharing a quinazolinone backbone that suppressed the constitutive triple response phenotype in dark-grown *eto1-4* seedlings. We designated these small molecules as ACS inhibitor quinazolinones (acsinones), which were uncompetitive inhibitors of 1-aminocyclopropane-1-carboxylic acid synthase. To explore the additional roles of acsinones in plants, we screened and identified 19 Arabidopsis mutants with reduced sensitivity to acsinone7303, which were collectively named *revert to eto1* (*ret*) because of their recovery of the *eto1* phenotype. A map-based cloning approach revealed that *CELLULOSE SYNTHASE6* (*CESA6*) and *DE-ETIOLATED2* (*DET2*) were mutated in *ret8* (*cesa6^{ret8};eto1-4*) and *ret41* (*det2^{ret41};eto1-5*), respectively. Etiolated seedlings of both *ret8* and *ret41* exhibit short hypocotyls and roots. Ethylene levels were similar in etiolated *cesa6^{ret8}* and *det2-1* and in *eto1* mutants treated with acsinone7303. Chemical inhibitors of ethylene biosynthesis and perception did not significantly suppress the etiolated phenotype of *cesa6^{ret8}* and *det2^{ret41}*. However, together with *eto1*, *cesa6^{ret8}* and *det2^{ret41}* exhibited an enhanced phenotype in the hypocotyls and apical hooks of etiolated seedlings. These results confirm that, in addition to ethylene, cellulose synthesis and brassinolides can independently contribute to modulate hypocotyl development in young seedlings.

1. Introduction

Plant growth between seed germination and emergence of the first true leaves is pivotal for survival. When germinated in the dark (soil), seedlings undergo etiolated growth, namely skotomorphogenesis, characterized by an elongated hypocotyl with an apical hook, closed cotyledons, and a reduced root system [1]. This mode of growth helps seedlings reach the light for photosynthesis in the shortest time, with the apical meristem protected within the curved downward and closed cotyledons. Once in contact with the light, the seedlings start an irreversible process called photomorphogenesis, with reduced elongation of hypocotyls, expanding cotyledons (which are active in photosynthesis), and well-developed roots [2]. Timely switching to the proper mode of growth according to environmental cues is the key for seedlings to successfully reach maturity [3].

Plant hormones are important growth factors in regulating seedling development in response to environmental stimulation and developmental signals. For hook formation, ethylene coordinates with auxin [4–6] and gibberellins (GA) [7–9]. Hypocotyl growth in developing seedlings at least involves auxin [10], GA [11], brassinolides, and ethylene [12]. Root development is modulated by auxin, ethylene [13,14] and the GA response repressor DELLA [15]. The regulation of seedling growth by hormones is frequently caused by cell expansion or cell division, which requires a change in cell wall structure or synthesis of new cell wall. Conversely, defective cell-wall conformation sometimes results in seedlings with reduced hypocotyls and short roots [16].

Ethylene has an opposite effect on hypocotyl growth in the dark and under the light [17]. Arabidopsis seedlings germinated in the dark on agar medium treated with ethylene show an unmistakable phenotype called the triple response: shortened and swollen hypocotyls, exaggerated apical hooks and short roots [18]. In the light, ethylene promotes hypocotyl elongation [19]. In addition, ethylene plays a crucial role in the transition from skotomorphogenesis to photomorphogenesis by facilitating greening of etiolated

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http://dx.doi.org/10.1016/j.plantsci.2013.04.005

seedlings [20]. Ethylene is gaseous and can be synthesized in almost all plant tissues [21]. Major components in the ethylene response pathway were identified and characterized primarily by genetics approaches [22]. Five genes, *ETHYLENE RESPONSE1* (*ETR1*), *ETR2*, *ETHYLENE RESPONSE SENSOR1* (*ERS1*), *ERS2*, and *ETHYLENE INSENSITIVE4* (*EIN4*), encode ethylene receptors that are related to bacterial two-component histidine kinase sensors [23] and are localized on the endoplasmic reticulum membrane [24]. In the absence of ethylene, the receptors maintain a negative regulation of the ethylene pathway by activating CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) to suppress the ethylene response that requires EIN2 [25,26]. CTR1 is inactivated with the binding of ethylene to the receptors, which results in de-repression of EIN2 to initiate the ethylene signaling pathway by stabilizing the transcriptional factors EIN3 and EIL1 (EIN3-LIKE1) via an unknown mechanism. EIN3 and EIL1 switch on a transcriptional cascade mediating ethylene response [27,28].

The triple-response phenotype has been effectively used to isolate ethylene response mutants [18,29], which were subsequently used in genetic and biochemical studies to construct the current model of the ethylene response pathway [30]. Several refined genetic screening strategies were also used to uncover additional elements potentially involved in the ethylene response pathway. *Weak ethylene-insensitive* (*wei*) mutants were identified by screening Arabidopsis mutants exhibiting reduced response to a low concentration of 1-aminocyclopropane-1-carboxylic acid (ACC), which is the immediate precursor of ethylene and is widely used to induce the triple response phenotype in the wild-type seedlings [31]. Characterization of the *wei* mutants confirmed the functional role of transcriptional factor EIL1 in the ethylene response and the hormone crosstalk between ethylene and auxin in regulating the ethylene-induced phenotype [31–33]. In contrast, *enhanced ethylene response* (*eer*) mutants were identified based on the exaggerated triple response phenotype in the etiolated *Arabidopsis* seedlings that were treated with saturated ethylene [12,34–37]. EER4 and EER5 physically interact with EIN3 [36] and EIN2 [37], respectively, and likely play a role in dampening or resetting the ethylene signal. Identification of *wei* and *eer* mutants based on different degrees of the altered triple response phenotype revealed new genes contributing to the ethylene response, which highlighted the use of alternative strategies to uncover additional components involved in the ethylene pathway.

Chemical genetics, which consists of chemical screening and genetics studies, provides an alternative approach to conventional genetic screens for discovery of novel components in a biological pathway [38,39]. Use of small molecules has the advantage of determining reversible, conditional and kinetic effects for functional studies in organisms in which lethality of genetically null mutants is an issue. In addition, small molecules can be agonists or antagonists to a group of proteins sharing conserved functions. Thus, using small molecules may provide a solution to the issues of gene redundancy and genetic lethality [38,40]. Chemical genetics has been increasingly used in plant hormone research. The long-sought abscisic acid (ABA) receptor was convincingly revealed by initial identification of a synthetic small molecule, pyrabactin, as an ABA agonist, followed by cloning the gene encoding PYRABACTIN RESISTANCE1 (PYR1) as the ABA receptor [41]. Small-molecule compounds that interfered with the biosynthesis or response of several phytohormones, including auxin, ABA, brassinosteroids (BRs), and ethylene, were identified as potential chemical tools for phytohormone research [42–47]. However, the targets of most of these identified small molecules affecting hormone biology remain unidentified.

We previously screened a collection of 10,000 synthetic chemical compounds to identify small molecules that suppressed the triple response phenotype in etiolated Arabidopsis seedlings. We successfully used a phenotype-based screening strategy to identify a group of small molecules that interfered with the triple response phenotype in the *ethylene overproducer1* (*eto1*) mutant, which generated 5–10 fold higher ethylene levels than the wild type [46]. The identified small molecules suppressed the triple response phenotype by reducing ethylene level in etiolated *eto1* seedlings. Genetic and biochemical analyses confirmed that the small molecules acted as novel uncompetitive inhibitors of 1-aminocyclopropane-1-carboxylate synthase (ACC synthase or ACS), which catalyzed the rate-limiting and committed step of ethylene synthesis [48]. Because of their commonly shared quinazolinone backbone, we named them ACS inhibitor quinazolinones, acsinones. To uncover additional roles of acsinones in plant physiology, we used genetic screening to identify Arabidopsis mutants with reduced response to acsinones and revealed the *revert to eto1* (*ret*) phenotype so named because of its recovery of the *eto1* phenotype.

Here, we report on the identification and characterization of 2 *ret* mutants that are insensitive to acsinone7303. Using a map-based cloning approach, we identified the genes mutated in *ret8* and *ret41* and found that they encoded *CELLULOSE SYNTHASE CATALISTIC SUBUNIT6* (CESA6) and *DE-ETIOLATED2* (DET2), respectively. We discuss the relevant roles of *CESA6* and *DET2* in the ethylene response pathway in regulating hypocotyl development in etiolated seedlings.

2. Materials and methods

2.1. Plant materials and growth conditions

All plants were derived from the wild-type Arabidopsis (*Arabidopsis thaliana*) Columbia ecotype (Col-0) and cultivated in a controlled room at 22 °C and light intensity of 100–150 microeinsteins m^{-2} s^{-1} under long-day conditions (16 h light/8 h dark). Seeds of *det2-1* (CS6159), *cbb1* (CS291), and *cesa6*$^{prc1-1}$ (CS297) were obtained from ABRC and were described previously [16,49,50]. Seeds of *GFP-CESA6* were kindly provided by Dr. Samantha Vernhettes [51]. Seeds were sterilized with solutions of 30% bleach and 0.1% Tween 20 for 7 min, sown in half-strength Murashige and Skoog (MS) agar medium (0.7%) supplemented with chemicals as indicated in experiments, and stratified in the dark at 4 °C for 2–5 days to synchronize germination. Cold-treated seeds germinated in the dark (etiolated) at 22 °C for 3 days were used to score the triple response phenotype. In general, 5 μM aminoethoxyvinylglycine (AVG) (Sigma; water as solvent), 10 μM sodium thiosulfate (STS) (Sigma; prepared by mixing silver nitrate and sodium thiosulfate at a 1:4 molar ratio immediately before use), 10 μM acsinone7303, or 15 μM acsinone9393 [46; DMSO as solvent] were used unless otherwise indicated. For brassinolide assays, 0.1 μM 24-epibrassinolide (EBL; Sigma; DMSO as solvent) was used, and hypocotyl length was measured 4 days after germination [modified from 12].

2.2. Screening procedure for ret mutants

eto1-5 plants were mutagenized by an activation tagging approach [52]. pSKI015 was transferred into *eto1-5* by Agrobacterium (GV3101)-mediated transformation, and primary transformants (T1 plants) were identified by scoring for Basta (BAYER; 1:2000 dilution) resistance and produced T2 seeds by self-pollination. Ethane methyl sulfonate (EMS) mutagenesis involved soaking seeds (~100,000) of *eto1-4*, which carried a reporter construct *5xEBS::LUC* described previously [46], in 0.2% EMS for 16 h. EMS was subsequently washed away with water. Seeds (M2) were generated from self-fertilized plants (M1) mutagenized by EMS. We used T2 seeds from approximately 11,000 T1 plants (in 51 pools; transformed with

pSKI015) and M2 seeds from approximately 58,000 M1 plants (in 48 pools; mutagenized with EMS) to screen for putative *ret* mutants.

Seeds from mutagenized plants were germinated in the dark for 3 days on MS medium supplemented with the ACS inhibitors 10 μM acsinone9393 or 15 μM acsinone7303 and were screened for etiolated seedlings resistant to chemical treatment. While the triple response phenotype in etiolated *eto1* seedlings is suppressed by acsinones [46], the putative *ret* mutants are expected to retain the triple response phenotype in the presence of acsinones, which is comparable to that in *eto1* seedlings without chemical treatment. The putative *ret* mutants were grown under long-day conditions in soil to propagate seeds by self-pollination for subsequent phenotype analysis in the M3 and M4 generations.

2.3. Map-based cloning of ret mutants

To generate a mapping population, we pollinated the ovules of *ret* mutants (Col-0, Columbia) with Landsberg *erecta* (L*er*) to produce F1 plants and collected F2 seeds for mapping the *ret* mutations. Genomic DNA was extracted from each of the F2 seedlings with a *ret* phenotype. Markers for mapping *ret8* and *ret41* were searched in the Monsanto Arabidopsis Polymorphism collection (http://www.arabidopsis.org/browse/Cereon/index.jsp) and Arabidopsis Mapping Platform (http://amp.genomics.org.cn/), respectively. Primers (Table S1) were designed to isolate candidate genes by PCR with use of Phusion DNA polymerase (Finnzymes). The PCR products were cloned into pJET1.2/blunt (Fermentas) and sequenced by Sanger sequencing. The mutation was identified by comparing the sequences in *ret* mutants from independent plants with those in the Arabidopsis genome database.

2.4. Genetic analyses

The *ret* mutants with *eto1-5* (activation-tagged mutants) or *eto1-4* (EMS mutants) were backcrossed at least twice, and homozygous F2 or F3 plants were used for phenotypic characterization. For allelic analyses, *ret8* and *ret41* were crossed with *cesa6*$^{prc1-1}$ and *det2-1*, respectively. The *ret* mutants were crossed with Col-0 to remove the *eto1* mutation from the genetic background. In addition, *cesa6*$^{prc1-1}$ was crossed with *eto1-4* (*5xEBS::LUC*) and *det2-1* with *eto1-5* to introduce the *eto1* mutation into plants. Plants with the desired genotype were selected from F1 or F2 progeny of crosses by PCR-based genotyping with dCAPS primers (Table S1) after scoring the phenotype of etiolated seedlings.

2.5. Complementation of ret8 and ret41

For functional complementation in *ret8*, plants of *CESA6pro::GFP-CESA6* in a *cesa6*$^{prc1-1}$ background [51] were crossed with *cesa6*^{ret8}. The phenotype of F1 seedlings, harboring *CESA6pro::GFP-CESA6* in a *cesa6*$^{prc1-1}$;*cesa6*^{ret8} background, was scored after germination in the dark for 3 days. For functional complementation in *ret41*, a 2.3-kb genomic construct containing a 660-bp upstream sequence, the *DET2* locus, and a 600-bp downstream sequence was generated by PCR (see Table S1 for primers) and cloned into the region between EcoRI and HindIII sites of pCHF3 [53] to generate the plasmid pCHF3-gDET2, which was transformed into *ret41* heterozygote plants by Agrobacterium strain GV3101. The primary transformants were identified by scoring resistance to an antibiotic (kanamycin). The *ret41* homozygous plants were confirmed by genotyping (Table S1), and the complementation phenotype was confirmed in the next generation (T2) with co-segregation of the wild-type phenotype and kanamycin resistance.

2.6. Measurement of hypocotyl length

Images of Arabidopsis seedlings were obtained by use of a digital camera (Canon Powershot A620) attached to a stereomicroscope (Zeiss Discovery V8). The same magnification and distance to the objects were used at each imaging time. Lengths of hypocotyls in images were measured by use of ImageJ (http://rsbweb.nih.gov/ij/). We measured 20 to 30 individuals for each line or treatment. Because homozygous *det2*^{ret41} and *ret41* (*eto1-5*;*det2*^{ret41}) plants are nearly sterile, we could only collect very limited seeds from self-pollinated heterozygous plants for use in phenotype assays. Genetic analysis revealed *det2*^{ret41} as a single recessive mutation, so we expected that one-quarter of the seedlings would exhibit a phenotype resembling homozygous plants. For seedlings germinated from seeds of heterozygous plants (i.e., *det2*^{ret41}/+ and *eto1-5*;*det2*^{ret41}/+), we measured hypocotyl length for approximately 100 individuals. The hypocotyl length of three-quarters of the individuals was similar to that of the wild type (Col-0, in the case of *det2*^{ret41}/+) or *eto1-5* (in the case of *eto1-5*;*det2*^{ret41}/+) in response to acsinone7303 and were classified as not carrying the mutant allele; the rest of seedlings were considered homozygous for *det2*^{ret41} or *eto1-5*;*det2*^{ret41} (Fig. 4A).

2.7. Measurement of ethylene production

Rate of ethylene production was measured as previously described [46] with minor modification. Seeds were sown in 10-ml gas chromatography (GC) crimp-top vials (30 seeds per vial for each treatment) containing MS agar media and grown in the dark for 3 days. Ethylene level was measured from the headspace of the GV vials by a GC machine (HP6890, Hewlett Packard) equipped with a capillary column (CP7381, Varian) and an autosampler (HP7694, Agilent Technologies).

2.8. Imaging and quantification of luciferase activity

Live imaging of luciferase activity was performed as previously described [46] with minor modification. For each treatment, approximately 15 seeds were sown and grown vertically on MS agar media. After 3 days in the dark, seedlings were sprayed with luciferin (2 mM, Biosynth International) and kept in the dark for 5 min before imaging by the Xenogen IVIS System (Caliper Life Sciences). Quantification of luciferase activity involved the Luciferase Assay System (Promega). Approximately 750 3-day-old dark-grown seedlings were quick-frozen in liquid nitrogen, ground to powder, and resuspended in Luciferase Cell Culture Lysis Reagent. Protein concentration of lysates was estimated by use of a Bio-Rad Protein Microassay kit. The emission was read from a Synergy Mx 3M Microplate Reader (BioTek) right after mixing 10 μl lysates and 50 μl Luciferase Assay Reagent in each well of a 96-well plate.

3. Results

3.1. Screening and identification of ret mutants

To screen for genetic mutants with altered response to acsinones, we first generated 2 mutant populations, one was (in *eto1-4* background) mutagenized with a chemical mutagen, EMS, and the other (in *eto1-5* background) by T-DNA insertional mutagenesis with the binary vector pSKI015 [52]. Subsequently, approximately 350,000 (M2) and 50,000 (T2) seeds from self-pollinated plants of EMS-mutagenized and activation-tagged mutant populations, respectively, were germinated in the dark on agar medium supplemented with acsinone7303. The *ret* mutants were scored for

Table 1
Genetic analyses of *ret8* and *ret41*.

Strains and crosses (♂ × ♀)	Generation	Total	Response to acsinone7303		
			Yes[a]	No	χ^{2}[b]
eto1-4/eto1-4		43	43	0	
eto1-4/eto1-4 × *ret8/ret8*[c]	F_1	51	51	0	
	F_2	215	166	49	0.56
cesa6$^{prc1\text{-}1}$/*cesa6*$^{prc1\text{-}1}$[c]		32	0	32	
ret8/ret8 × *cesa6*$^{prc1\text{-}1}$/*cesa6*$^{prc1\text{-}1}$	F_1	62	0	62	
eto1-5/eto1-5		25	25	0	
eto1-5/eto1-5 × *ret41/ret41*[d]	F_1	119	119	0	
	F_2	1099	808	291	1.28
det2-1/det2-1[d]		37	0	37	
ret41/ret41 × *det2-1/det2-1*	F_1	49	0	49	

[a] The hypocotyl length of the 3-day-old etiolated seedlings is at least twice longer with acsinone7030 treatment than without (MS medium), and seedling hypocotyls are shorter than those of Col-0 in MS medium.
[b] The calculated χ^2 value was based on the expected ratio of 3:1 for individuals responsive vs. unresponsive to acsinone7303, assuming that *cesa6*^{ret8} or *det2*^{ret41} is a single recessive mutation ($P > 0.05$).
[c] *ret8* (*cesa6*^{ret8};*eto1-4*) was crossed with *cesa6*$^{prc1\text{-}1}$ for allelic analysis. The mutations are indicated in Fig. 1A.
[d] *ret41* (*det2*^{ret41};*eto1-5*) was crossed with *det2-1* for allelic analysis. The mutations are indicated in Fig. 1B.

restoration of short hypocotyls (partial phenotype) or a complete triple response phenotype in the presence of acsinones. We named these mutants *revert to eto1* (*ret*), as likely being due to a reduced response or sensitivity to acsinone7303. We identified 19 *ret* mutants, 12 and 7 generated by EMS and T-DNA insertion of pSKI015, respectively, and confirmed their phenotype in the subsequent generation. Unlike the elongated hypocotyls of *eto1*, those of *ret* mutants remained short when germinated in the dark on medium containing acsinone7303. Reciprocal crosses were performed to show that all of the 19 *ret* mutants were in different complementation groups. We characterized 2 of the mutants, *ret8* and *ret41*, in this study. The 2 *ret* mutants were backcrossed with *eto1*, and the F_2 offspring showed a segregation in phenotype, with a ratio of 3 (*eto1*) to 1 (*ret*) (Table 1), which indicates that both *ret* mutants were caused by a recessive mutation in a single gene.

3.2. ret8 has a mutation in CESA6

To identify the genes mutated in *ret8* and *ret41* mutants, we used a map-based cloning approach. The mapping population was generated from a genetic cross of *ret* mutants (Col-0 background) to L*er*, and F2 seeds were obtained from self-pollinated F1 plants. We genotyped 1,264 F2 progeny from crossing *ret8* with L*er* and displayed the *ret* phenotype by using PCR with simple sequence length polymorphism (SSLP) and cleaved amplified polymorphic sequence (CAPS) markers (information available from the Monsanto Arabidopsis Polymorphism collection, http://www.arabidopsis.org/browse/Cereon/index.jsp) and mapped the *ret8* mutation to the bottom of chromosome 5, between markers Cer440906 and Cer441365 (Fig. 1A). By sequencing the candidate genes in this narrow region (BAC clone MVP7), we identified a mutation in G to A at nucleotide 3036 of the coding region of At5g64740 (Fig. 1A). The locus contains a gene that encodes CESA6, a member of the cellulose synthase catalytic subunit family, which contains 10 *CESA* genes [54]. Loss-of-function mutations in *CESA6/PROCUSTE1 (PRC1)/ISOXABEN RESISTANT2 (IXR2)* cause reduced elongation in roots and dark-grown hypocotyls [16], a phenotype consistent with that of *ret8*. To confirm that *ret8* bears a mutation in *CESA6* to exhibit the *ret* phenotype, we performed an allelic test by generating a genetic cross between *ret8* with *cesa6*$^{prc1\text{-}1}$, which is a null allele of *CESA6* [16]. Seedlings grown in the dark with acsinone7303 for all F1 progeny showed short hypocotyls and roots (Table 1). The results from the allelic test strongly indicate that the phenotype of *ret8* is caused by loss of function of *CESA6*. We named this new allele of *cesa6* in the *ret8* mutant as *cesa6*^{ret8}. For complementation analysis, we introduced *CESA6pro::GFP-CESA6* into the *cesa6*^{ret8} mutant and scored the phenotype of etiolated seedlings. *CESA6pro::GFP-CESA6* complemented both *cesa6*^{ret8} and *cesa6*$^{prc1\text{-}1}$ (Fig. 2A); the latter was reported previously [51]. This result confirms that *cesa6*^{ret8} is the mutation in the *ret8* mutant and is a new allele in the *CESA6* locus.

CESA6 has 8 putative membrane-spanning helices (UniProt ID: Q94JQ6) [16]. Tryptophan 1012 of CESA6 in *ret8* is mutated to create a stop codon (TGG to TGA) (Fig. S1), which results in a truncated protein with deletion of 73 amino acid residues at the C terminus, thus removing the last 2 predicted transmembrane domains.

3.3. ret41 has a mutation in DET2

Although *ret41* was identified from the activation-tagged T-DNA insertional mutant population, 4 of the insertion sites identified by thermal asymmetric interlaced PCR (TAIL-PCR) and plasmid rescue approaches were not co-segregated with the phenotype (data not shown). Therefore, we used a map-based cloning method to find the mutation in *ret41* mutant. With the same procedure used to map *ret8*, we genotyped 231 F2 plants from the cross of *ret41* and L*er* by using SSLP and CAPS markers (information available from the Arabidopsis Mapping Platform, http://amp.genomics.org.cn/) and mapped *ret41* to the lower arm of chromosome 2 between markers 8036 and 8117 (Fig. 1B). Because the adult plants of *ret41* show an extreme dwarf phenotype, with greatly reduced stature, and are nearly sterile, with tiny siliques containing mostly unfertilized ovules, we deliberately looked for candidate genes in the mapped genomic region that would produce a similar phenotype when mutated. *DE-ETIOLATED2* (*DET2*, At2g38050) is among the genes. Mutations in *DET2* cause severe dwarfism in rosette plants similar to that of *ret41* [55]. Sequencing the *DET2* gene from the genomic DNA of *ret41* revealed a 10-bp deletion (422–431 nt) proximal to the beginning of the second exon (Fig. 1B). *DET2* encodes a steroid 5α-reductase in the brassinolide biosynthetic pathway [49]. The dark-grown seedlings of brassinolide-deficient plants have short hypocotyls, a phenotype similar to that of *ret41* [49,55]. To verify whether *ret41* was allelic to *det2* mutation, we crossed *ret41* and *det2-1* and scored the phenotype of F1 plants. All 3-day-old dark-grown seedlings of the F1 progeny had short hypocotyls and were unresponsive to acsinone7303 (Table 1), which strongly indicated that *ret41* was allelic to *det2-1*. We further confirmed this result by functional complementation of *ret41* with the genomic sequence of *DET2*. Both the short hypocotyl phenotype in etiolated seedlings and the dwarf phenotype of *ret41* rosette plants were completely recovered by introducing a *DET2* gene (Fig. 2B and C). Therefore, the

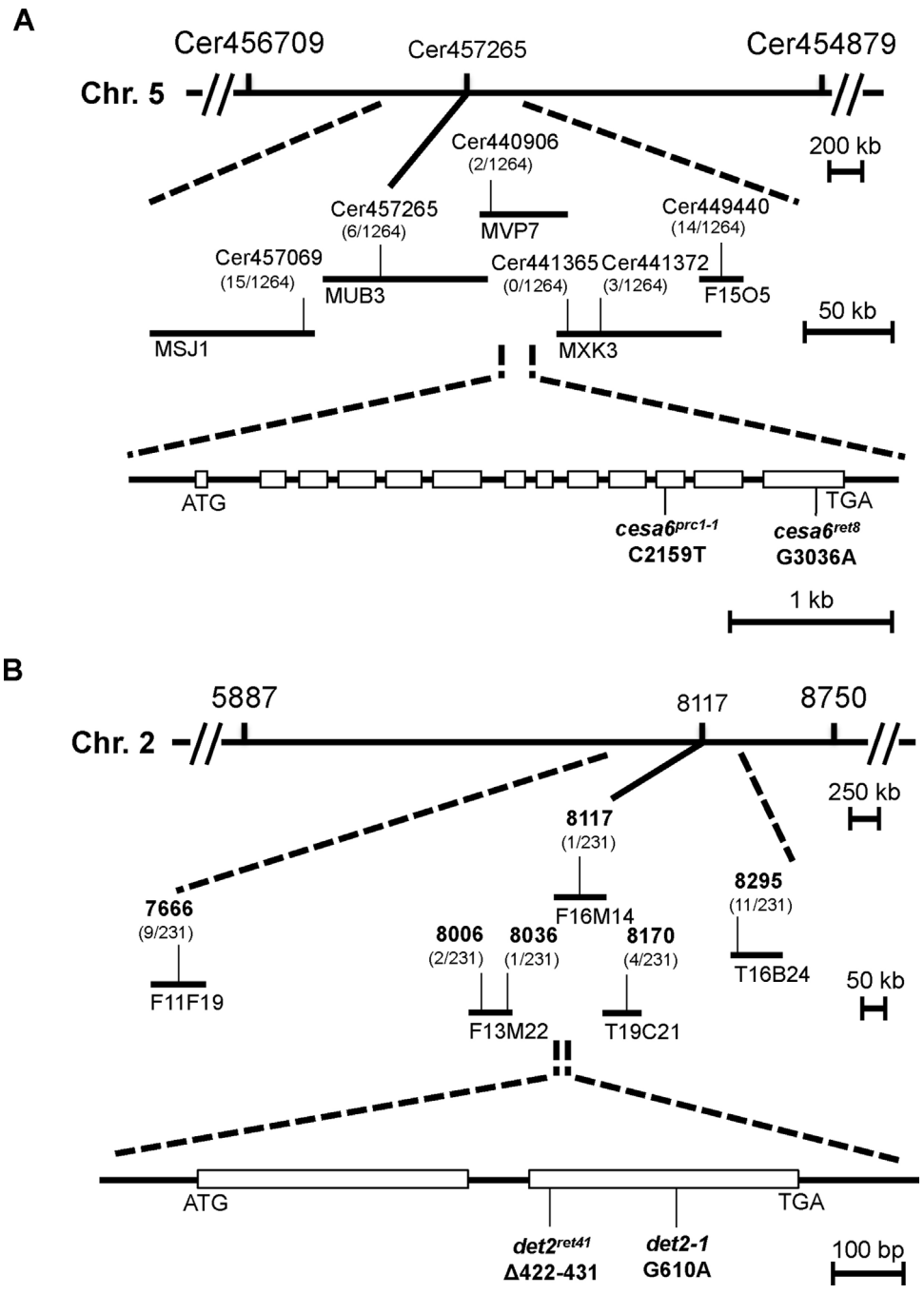

Fig. 1. Schematic diagrams of map-based cloning of *RET8/CESA6* and *RET41/DET2*. (A) Genetic and physical maps of the region between Cer456709 and Cer454879 in Arabidopsis chromosome 5 (Chr. 5). The gene structure of *RET8/CESA6* (At5g64740) is shown. The *cesa6*^{ret8} (G3036A), which generates W1012stop, and *cesa6*$^{prc1-1}$ (C2159T, Q720stop) mutations are indicated. (B) Genetic and physical maps of the region between simple sequence length polymorphism markers 5887 and 8750 in Arabidopsis chromosome 2 (Chr. 2). The gene structure of *RET41/DET2* (At2g38050) is shown. The positions and mutations of *det2-1* (G610A, E204K) and *det2*^{ret41} (deletion of 422-431 nt., V158stop) are indicated. BAC and TAC clones are depicted by filled bars. The last 4 digits of the marker ID are shown on the maps. Numbers above the bars indicate recombination events/chromosomal crosses. Open boxes represent protein-coding regions, and lines are introns and untranslated regions.

mutation with a 10-bp deletion in *DET2* is indeed responsible for the mutant phenotype of *ret41*. We designated the new *det2* allele in the *ret41* mutant as *det2*^{ret41}.

DET2 encodes a small protein of 262 amino acids with 6 potential transmembrane helices (UniProt ID: Q38944). The 10-bp deletion of *ret41* was predicted to change amino acid residues 141 to 157 and generated a premature stop codon at position 158 (Fig. S2). The *det2*^{ret41} mutation disrupts the fifth putative transmembrane helix to generate a truncated protein, if expressed, and is expected to be a null allele.

3.4. Ethylene enhances the short hypocotyl phenotype in dark-grown ret8 seedlings

Because the genetic background of *ret8* is *eto1-4*, the phenotype of *ret8* (*cesa6*^{ret8};*eto1-4*) may represent a composite consequence of mutations in both *CESA6* and *ETO1*. Etiolated seedlings of *cesa6*^{ret8};*eto1-4* exhibited an enhanced triple response phenotype, with much shorter hypocotyls than for *eto1-4* (Fig. 3A). In contrast to the hypocotyl phenotype of *eto1-4*, that of *cesa6*^{ret8};*eto1-4* remained unchanged with acsinone7303 treatment (Fig. 3A). The

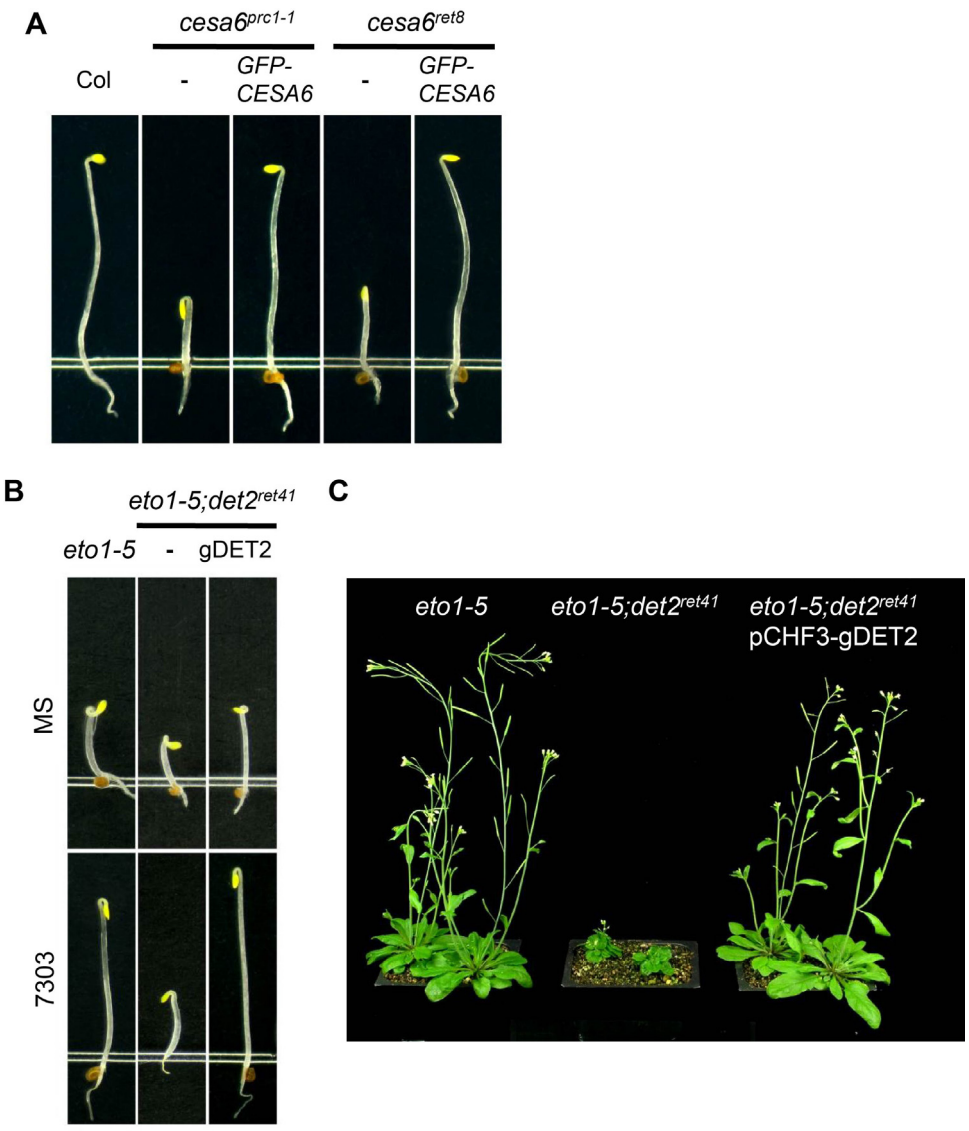

Fig. 2. Complementation of *ret* mutants. (A) Complementation of *cesa6*^{ret8} by *CESA6pro::GFP-CESA6*. The phenotype of short hypocotyls in 3-day-old etiolated *cesa6*^{ret8} and *cesa6*$^{prc1-1}$ seedlings was rescued by *CESA6pro::GFP-CESA6*, which was introduced into *cesa6*^{ret8} by a cross with *cesa6*$^{prc1-1}$ plants harboring the transgene. (B and C) Complementation of *eto1-5;det2*^{ret41} by the wild-type *DET2* genomic DNA (*gDET2*). Because homozygous *eto1-5;det2*^{ret41} plants barely produce seeds, a genomic construct containing the locus At2g38050 was introduced into *eto1-5;det2*^{ret41}/+ heterozygous plants by Agrobacterium-mediated transformation. T1 and T2 plants were subsequently genotyped to confirm the homozygous *det2*^{ret41} allele. The phenotype of *eto1-5;det2*^{ret41} was completely recovered in the presence of pCHF3-gDET2 in etiolated seedlings treated without (MS) or with acsinone7303 (10 μM) (B) and 6-week-old rosette plants (C).

roots of *cesa6*^{ret8};*eto1-4* treated with acsinone7303 were slightly longer and less swollen than those of untreated seedlings. To examine whether the phenotype of etiolated *cesa6*^{ret8};*eto1-4* depends on *eto1-4*, we used the isogenic segregants, wild type (Col-0), *cesa6*^{ret8}, *eto1-4*, and *cesa6*^{ret8};*eto1-4*, which were generated from progeny of a backcross between Col-0 and *cesa6*^{ret8};*eto1-4*, for phenotypic analysis (Fig. 3A) and quantitation of hypocotyl length (Fig. 4A). The hypocotyl length of *cesa6*^{ret8} was slightly longer than that of *cesa6*^{ret8};*eto1-4* and the same as that of *cesa6*$^{prc1-1}$. The hypocotyls of etiolated *cesa6*^{ret8} and *cesa6*$^{prc1-1}$ were not elongated with acsinone7303 treatment, which suggests that mutations in *CESA6* would sufficiently cause the short hypocotyl that was not affected by acsinone7303 (Fig. 3A). In combination with *eto1-4*, both *cesa6*^{ret8} (in *cesa6*^{ret8};*eto1-4*) and *cesa6*$^{prc1-1}$ (in *cesa6*$^{prc1-1}$;*eto1-4*) showed an extremely shortened hypocotyl phenotype, which was likely caused by elevated ethylene level in etiolated *eto1* seedlings (Fig. 3A). The adult plants of *cesa6*^{ret8};*eto1-4* had a rosette phenotype and height comparable to that of the wild type and *eto1-4*, although *cesa6*^{ret8};*eto1-4* bolted earlier and had slightly smaller rosette leaves similar to *cesa6*$^{prc1-1}$;*eto1-4* plants (Fig. 3B).

We wondered whether ethylene biosynthesis or response was involved in the shortened hypocotyl phenotype in *cesa6*^{ret8};*eto1-4*. We measured hypocotyls in etiolated seedlings with or without chemical treatments to suppress the ethylene response by an antagonist to ethylene receptors (STS) or inhibitors of ethylene biosynthesis (acsinone7303 and AVG). The hypocotyl length of *cesa6*^{ret8} and *cesa6*^{ret8};*eto1-4* dark-grown seedlings remained short with AVG and STS treatment, whereas that of *eto1-4* was elongated (Fig. 4A). The ethylene level of *cesa6*$^{prc1-1}$ was nearly the same as that of the wild type with or without acsinone7303, whereas that of *cesa6*^{ret8} was approximately two-fold higher than that of the wild type without acsinone7303 (Fig. 4B). The ethylene level of *cesa6*^{ret8};*eto1-4* was nearly six-fold higher than that of the wild type. Acsinone7303 reduced the amount of ethylene emission of *cesa6*^{ret8};*eto1-4*, similar to *eto1-4*. We next asked whether the ethylene response in *cesa6*^{ret8};*eto1-4* was affected by acsinone7303 by

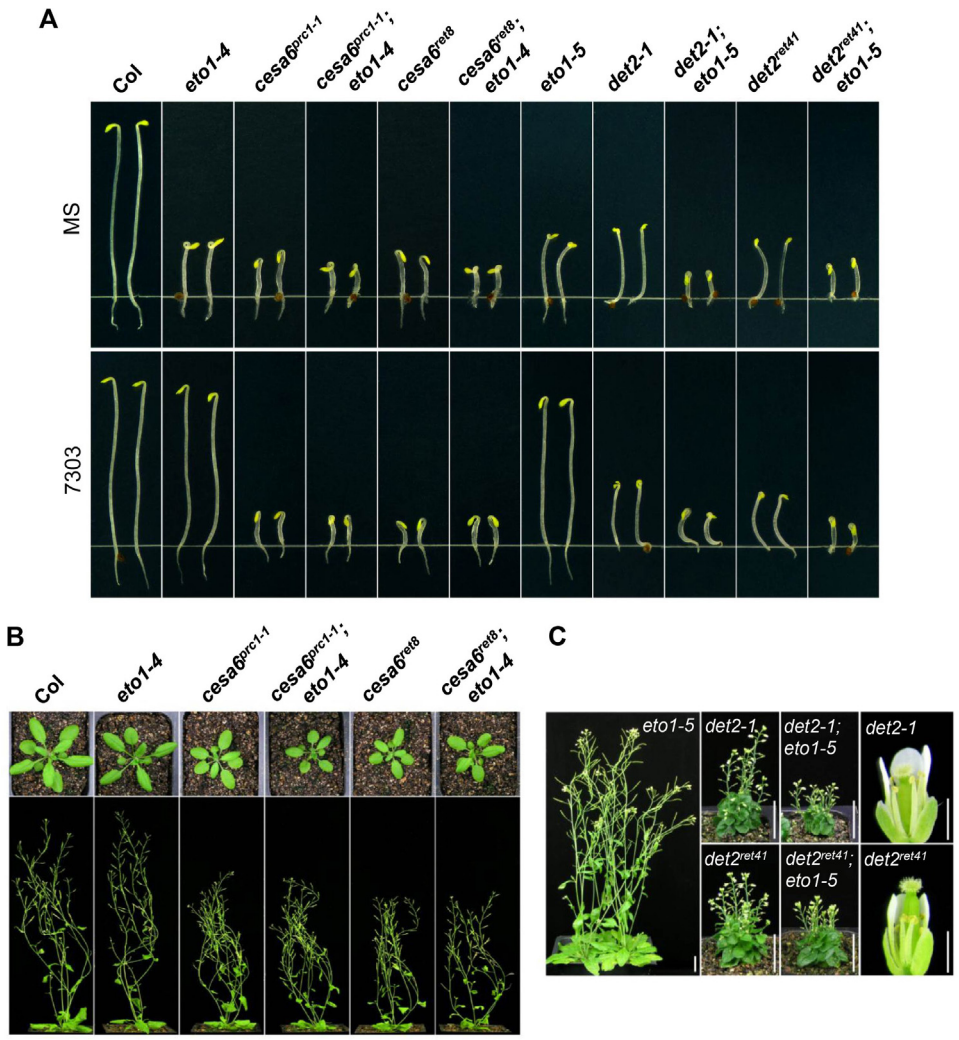

Fig. 3. Phenotypes of etiolated seedlings and rosette plants of *cesa6*^{ret8} and *det2*^{ret41}. (A) Phenotypes of 3-day-old etiolated seedlings of various mutants as indicated treated without (MS) or with acsinone7303 (10 μM). (B) Rosette plants of *cesa6*^{ret8}. Photos were taken when plants were 5 (top) or 7 (bottom) weeks old. (C) Rosette plants and flowers of *det2* mutants at anthesis. Photos were 7-week-old plants of *eto1-5* and 9-week-old plants of *det2* and *det2*;*eto1-5* mutants. Bars for plants = 2 cm; for flowers = 1 mm.

using the *5xEBS:LUC* reporter gene present in the *cesa6*^{ret8};*eto1-4* mutant, in which the luciferase activity was responsive to ethylene level. The luminescence emission (Fig. 5A) and luciferase activity (Fig. 5B) in etiolated *eto1-4* seedlings were completely inhibited by acsinone7303, STS and AVG treatment. Acsinone7303, AVG, and STS all reduced luciferase activity in etiolated *cesa6*^{ret8};*eto1-4* seedlings comparably (Fig. 5). Therefore, the ethylene response was suppressed by the chemical treatments. The short hypocotyl phenotype of *cesa6*^{ret8} and *cesa6*^{ret8};*eto1-4* is likely regulated by the defective cellulose synthesis instead of ethylene response or ethylene level.

3.5. Combination of det2 and eto1 mutations results in an exaggerated apical hook in etiolated seedlings

Etiolated seedlings of *ret41* (*det2*^{ret41};*eto1-5*) showed a strong triple response phenotype, including short roots, short and swelling hypocotyls and exaggerated apical hooks (Fig. 3A). With acsinone7303 treatment, the hypocotyls of *det2*^{ret41};*eto1-5* remained short. We removed the *eto1* mutation from *det2*^{ret41};*eto1-5* genetic background by outcrossing with the wild type. The *det2*^{ret41} showed short hypocotyls and roots in dark-grown seedlings, which are similar to those in *det2-1* (Fig. 3A). Apical hooks were more prominent in the presence of *eto1-5* (Fig. 3A, compare *det2-1* vs. *det2-1*;*eto1-5* and *det2*^{ret41} vs. *det2*^{ret41};*eto1-5*). Thus, ethylene level appears to modulate apical hook curvature in etiolated seedlings. The hypocotyls were shorter for both *det2*^{ret41};*eto1*-5 and *det2-1*; *eto1-5* than *det2*^{ret41} and *det2-1*, respectively (Fig. 3A). The increased amount of endogenous ethylene caused by *eto1* likely enhanced the short-hypocotyl phenotype in the *det2*;*eto1* double mutants.

Surprisingly, the hypocotyl length of *det2-1* and *det2*^{ret41} was not increased as in *eto1-5* with AVG and STS treatments (Fig. 4A). The hypocotyls of *det2*^{ret41};*eto1-5* were elongated with AVG and STS treatments, not to the same degree as in *eto1-5* but to the final lengths as in the *det2*^{ret41} and *det2-1* (Fig. 4A). Thus, in the dark, both ethylene and *det2* contribute to an additive effect on hypocotyl shortening in *det2*^{ret41};*eto1-5* seedlings. This notion was confirmed by analyzing the ethylene levels in etiolated seedlings of two mutants defective in brassinolide biosynthesis, *det2* and *cbb1*/*dwf1* [49,56], which both produced a comparable levels of ethylene as the wild type with or without acsinone7303 treatment (Fig. 4B). Therefore, the short hypocotyls of etiolated *det2-1* are not a consequence of overproduced ethylene.

3.6. det2^{ret41} is a strong allele in DET2 that causes infertility

The rosette plants of *det2*^{ret41};*eto1-5* showed a marked phenotype of dwarfism, with reduced apical dominance (Fig. 3C).

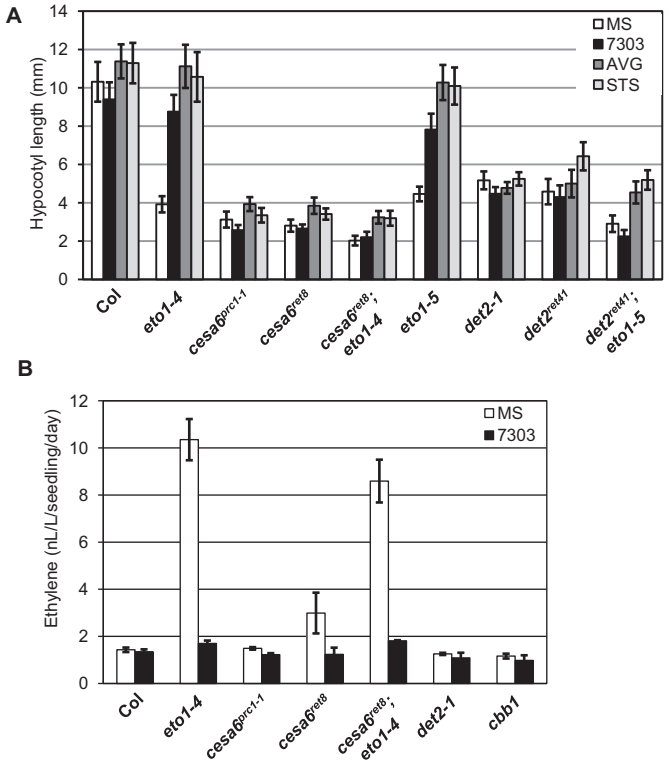

Fig. 4. Quantitative analyses of hypocotyl length and ethylene levels in etiolated *cesa6*^{ret8} and *det2*^{ret41} seedlings. (A) Hypocotyl length of 3-day-old etiolated seedlings of mutants without (MS) or with acsinone7303 (10 μM), AVG (5 μM), and STS (10 μM). In total, 20 to 30 individuals were measured for each line/treatment. (B) Ethylene levels of 3-day-old dark-grown seedlings of mutants without (MS) or with acsinone7303. Data are mean ± standard error from at least 3 replicates.

The leaves are dark green and small. The *det2*^{ret41};*eto1-5* plants grew slowly and had delayed flowering time and extended longevity. These phenotypes are characteristics of plants with loss-of-function mutations in *DET2* [49]. The phenotype of adult plants of *det2*^{ret41} was the same as for *det2*^{ret41};*eto1-5* and both produced mostly small empty siliques (unfertilized carpels) (Fig. 3C). Although the overall morphologic features of *det2-1* were similar to those of *det2*^{ret41}, homozygous *det2-1* produced small siliques with viable seeds.

Compared with *det2*^{ret41}, *det2-1* is apparently a weak allele in terms of fertility. We examined the floral organs of *det2* mutants and noted that filaments of *det2*^{ret41};*eto1-5* and *det2*^{ret41} remained short at anthesis, and the well-developed stigma was not pollinated by pollen grains (Fig. 3C). Outcrossing *det2*^{ret41};*eto1-5* and *det2*^{ret41} as pollen donors or recipients produced viable seeds, which indicates that the carpels and pollen grains are fertile. Apparently, because of the short filaments, mature pollen grains of *det2*^{ret41};*eto1-5* and *det2*^{ret41} cannot reach the stigma, and therefore fertilization fails. This phenotype was previously documented by studies of another sterile *det2* allele, *transient defective exine1* (*tde1*) [57]. In contrast, *det2-1* is fertile because filaments of *det2-1* were long enough to reach the stigma (Fig. 3C).

The *det2*^{ret41} is an allele with a 10-bp deletion that results in a frameshift mutation with an alteration of 17 terminal amino acids to cause a premature termination with a truncation of 105 residues at the C terminus of DET2, if translated (Fig. S2). The *tde1* mutant had 26 amino acids altered at the C terminus of DET2 [57]. The *det2-1* allele was predicted to have a substitution of a positively charged lysine for a negatively charged glutamate at position 204. Currently available sequence data showed this glutamate to be conserved in land plants [NCBI blastp; 58] and it is absolutely required for the activity of human steroid reductase [49]. The differential severity in defective enzyme activity resulting from different alleles may affect the elongation of filaments in *det2* mutants to various degrees. Under the same growth conditions, the rosette

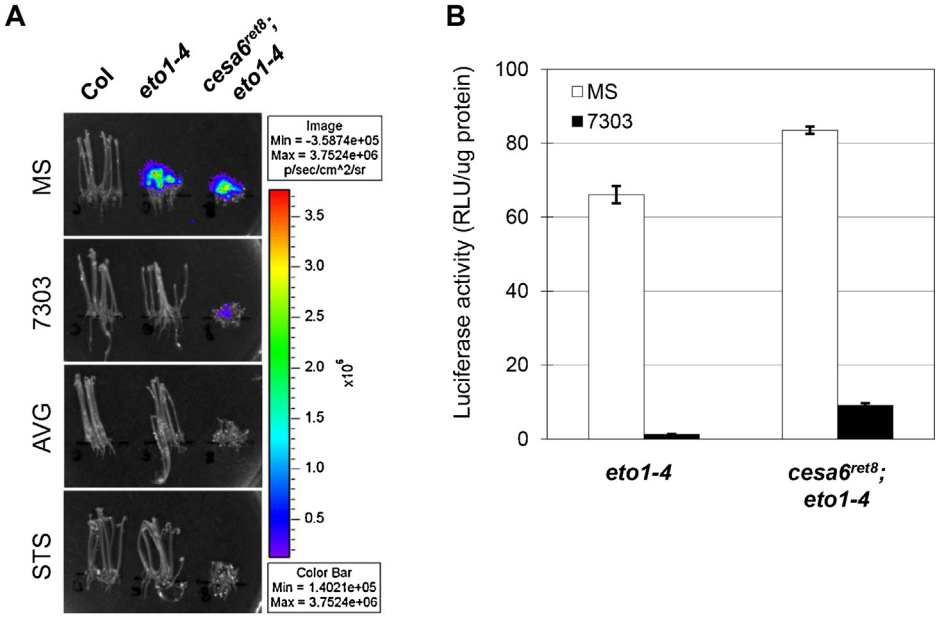

Fig. 5. Ethylene response in etiolated *cesa6*^{ret8};*eto1-4* seedlings is suppressed by acsinone7303. (A) Images of 3-day-old etiolated seedlings for luciferase activity in *eto1-4* and *cesa6*^{ret8};*eto1-4*, both containing *5xEBS::LUC*, treated without (MS) or with acsinone7303 (10 μM), AVG (5 μM), and STS (10 μM). The superimposed pseudocolor represents the photons emitted by the live cells after luciferin treatment (2 mM); the color scale bar on the right shows the photon counts (photon/s/cm^2/sr). (B) Quantitative analysis of luciferase activity without (MS) or with acsinone7303 (10 μM). Luciferase activity is measured as relative light unit (RLU)/μg protein. Data are mean ± standard error of 3 replicates.

plants of both *det2*^{ret41};*eto1-5* and *det2-1*;*eto1-5* double mutants were smaller than plants of *det2-1* and *det2*^{ret41} single mutants (Fig. 3B). Thus, the *eto1* mutation may also exacerbate the degree of dwarfism in *det2* plants.

3.7. The seedling phenotypes of det2 mutants are recovered by brassinolide treatment

The phenotype of BR deficiency can be rescued by treating plants with BRs [49,59]. To confirm whether the phenotype of *det2*^{ret41};*eto1-5* was caused by brassinolide deficiency, we treated mutants with 0.1 μM EBL to determine whether the seedling phenotype of *det2*^{ret41};*eto1-5* and *det2*^{ret41} could be rescued. As a positive control, *det2-1* exhibited elongated hypocotyls after the treatment under both dark- (Fig. 6A) and light-grown (Fig. 6B) conditions. Hypocotyl elongation of wild-type seedlings was inhibited in the dark and was promoted in the light with the same concentration of EBL (Fig. 6A), which is consistent with previous observations [12]. Interestingly, EBL did not induce hypocotyl growth of *eto1-5* in the dark (Fig. 6A) but greatly promoted hypocotyl elongation in the light (Fig. 6B). The etiolated hypocotyls of *eto1* mutant was previously found insensitive to EBL treatment [60]. Because EBL promotes ethylene production in the dark, the EBL-induced shortening of etiolated wild-type hypocotyls is likely ethylene dependent [12]. Whether EBL can further increase the already elevated level of ethylene production in *eto1* is unclear, but EBL does not significantly affect hypocotyl elongation of etiolated *eto1* (Fig. 6A).

Smalle et al. showed that ethylene treatment promoted hypocotyl elongation of seedlings under light [19]. The elevated level of ethylene in *eto1-5* seedlings might have enhanced BR-induced hypocotyl elongation in the light as compared with the wild type (Fig. 6B). The elongation of hypocotyls in both *det2*^{ret41};*eto1-5* and *det2*^{ret41} was promoted by EBL in the dark or light (Fig. 6). With EBL treatment, hypocotyls of *det2*^{ret41} were elongated to a level similar to that of *det2-1* in the dark (Fig. 6A). Nevertheless, under the light, the effect of EBL on hypocotyl growth of *det2*^{ret41} was not as prominent as that of *det2-1* (Fig. 6B). In addition to filament elongation, the 2 alleles of *det2* differ in hypocotyl elongation in the light. EBL treatment caused the hypocotyls of *det2*^{ret41};*eto1-5* to elongate in the dark to a length similar to that of *eto1-5*, *det2-1*, or *det2*^{ret41} without EBL treatment (Fig. 6A), which suggests that ethylene suppressed hypocotyl elongation even with exogenous EBL. In the light, *eto1* had an additive effect on EBL-induced hypocotyl elongation because hypocotyls of *det2*^{ret41};*eto1-5* were longer than those of *det2*^{ret41} with EBL treatment (Fig. 6B). Thus, both ethylene and BRs regulate hypocotyl growth, which is enhanced by light.

4. Discussion

In this study, we report the results of a genetic screen for mutants with an altered response to acsinone7303, which suppresses ACS activity and the triple response phenotype of the *eto1* mutant. Acsinones are a group of chemical compounds with a qulinazolinone backbone and are novel uncompetitive inhibitors of ACS [46]. Acsinone treatment inhibits ACS activity and reduces ethylene level in etiolated Arabidopsis seedlings. We identified several *ret* mutants by scoring the reduced response of *eto1* mutants to ascinone7303 and further characterized 2 of them. Map-based cloning revealed that *ret8* carried a mutation in *CESA6* (*cesa6*^{ret8}) and *ret41* a mutation in *DET2* (*det2*^{ret41}). When the *eto1* mutation was removed from *ret8* and *ret41*, the hypocotyls in dark-grown seedlings remained shorter than those in wild type, which indicates that the hypocotyl phenotype did not entirely depend on elevated ethylene level. Inhibitors of ACS (ascinone7303 and AVG) and an antagonist of ethylene perception (STS) effectively suppressed the triple response in *eto1* mutants to elongate hypocotyls but not in *cesa6*^{ret8} and *det2*^{ret41} seedlings. Thus, the short hypocotyls in etiolated *cesa6*^{ret8} and *det2*^{ret41} are caused by loss-of-function mutations of *CESA6* and *DET2*, respectively, which play an independent role in regulating seedling development. Excessive ethylene in *eto1* enhances the hypocotyl phenotype and exaggerates the curvature of apical hooks in the *cesa6* and *det2* mutants.

The aim of the genetic screen for *ret* mutants was to uncover the genes and cellular functions affected by the newly identified small molecule acsinone7303. We were also interested in disclosing the unknown properties of acsinone7303 other than as an ACS inhibitor. Because the main phenotype affected by acsinone7303 was the triple response of dark-grown seedlings of *eto1*, the screening strategy was designed to screen mutants with altered sensitivity to acsinone7303. We expected to uncover mutations in genes encoding components in the ethylene signaling pathway, such as *CTR1* and *RAN1*, among the *ret* mutants, as well as mutations in genes involved in seedling development contributing to a partial or complete triple response phenotype. *ret8* and *ret41* indeed represented the latter case.

The *cesa6*^{ret8} in *ret8* mutant represents a novel mutant allele in *CESA6*, a gene crucially involved in formation of the plant cell wall. Plant cell walls are complex structures composed of polysaccharides, proteins, and lignins. In most cell types, the bulk of walls are made up of the polysaccharide cellulose. Higher plants have 2 major types of cell wall, primary and secondary, that are structurally distinct. During cell growth, the primary walls are initiated and form outside the plasma membrane. The secondary walls are deposited inside the primary walls after the cessation of cell growth in certain cell types. Experimental data support that cellulose is synthesized by membrane-bound complexes that can be visualized by freeze etching. The 20–30-nm hexameric rosette-like structures are proposed to comprise 6 globular complexes, and each of the globular complexes is in turn proposed to hold 6 CESA proteins. Glucan chains are synthesized by the CESA complexes and extrude into the extracellular space [61].

The Arabidopsis genome contains 10 *CESA* and 29 *cellulose synthase-like* (*CSL*) genes [54]. Among the *CESA* genes, *CESA1*, *CESA3*, and *CESA6* are parts of the primary wall CESA complexes [51]. Null mutations of *CESA1* and *CESA3* are gametophytic lethal, whereas mutations in *CESA6* produce a relatively mild phenotype. Functional redundancy of CESA2 and CESA5 with CESA6 is likely to result in the weak phenotype of *cesa6* mutants [51]. This finding is consistent with our observation that phenotypes of *cesa6*^{ret8} and wild type (Col-0) in rosette plants do not differ much.

In general, deficiency in primary wall cellulose synthesis is characterized by short and swollen etiolated hypocotyls, reduced root elongation, ectopic accumulation of lignin, dwarfism, and sterility [61]. Mutations in CESA1 (*rsw1*) [62], CESA3 (*cev1*) [63], and CESA6 (*prc1*) [16] typically result in short and swollen dark-grown hypocotyls and short roots. The dark-grown phenotype of *cesa6* mutants can be phenocopied by treating wild-type seedlings with inhibitors perturbing cellulose biosynthesis, such as 2, 6-dichlorobenzonitrile (DCB) or isoxaben [16]. Cross sections of drug-treated seedlings showed the cells radically expanded in the short and swollen hypocotyls. Cellulose synthesis is reduced in the *cesa* mutants (*rsw1, cev1, and prc1*) and in seedlings treated with DCB or isoxaben. The reduction in cellulose synthesis is apparently associated with the phenotype of short and swollen dark-grown hypocotyls.

The phenotype of dark-grown seedlings of the above-mentioned *cesa* mutants resembles that of the triple response, particularly

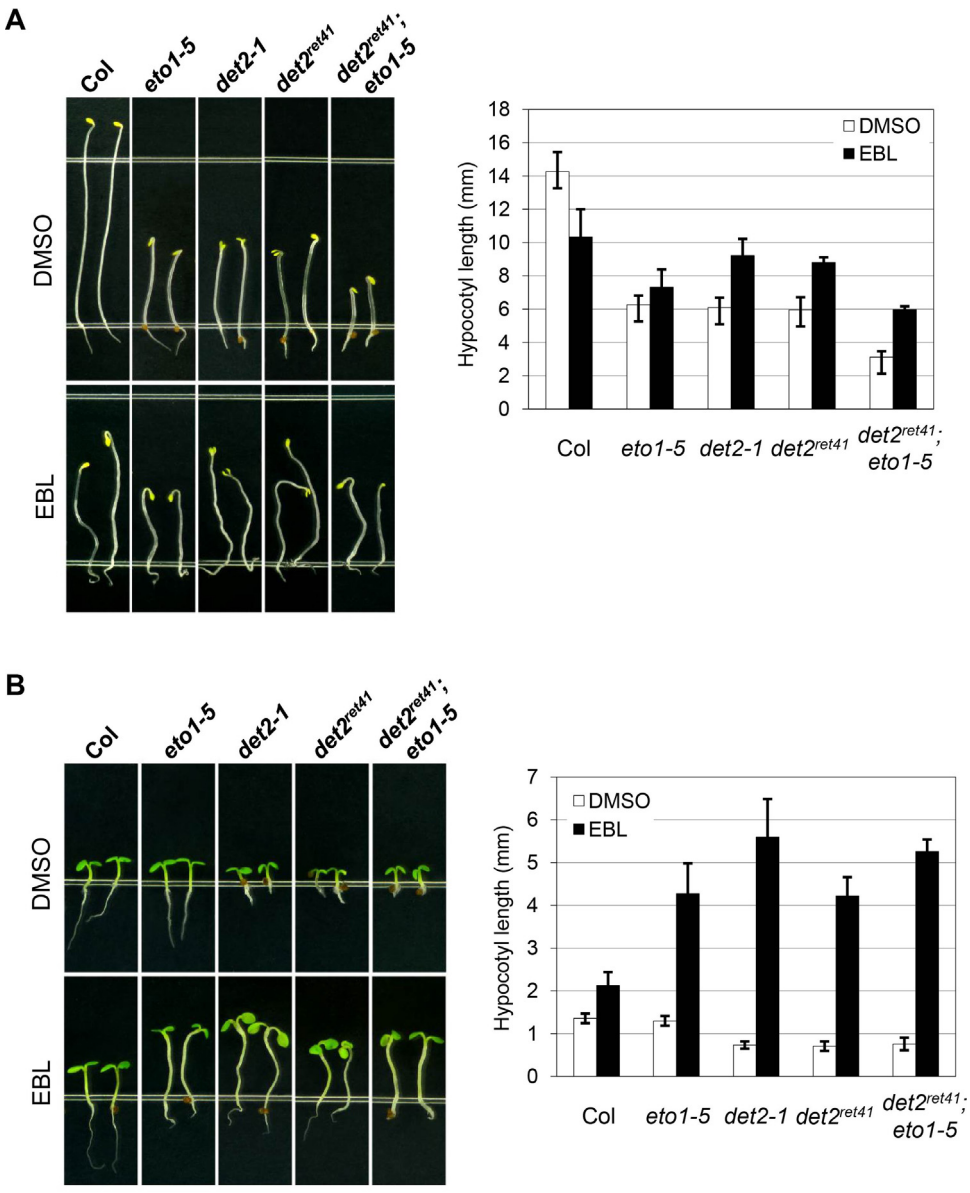

Fig. 6. Effect of brassinolide treatment on hypocotyl elongation in light- and dark-grown seedlings of *det2*^{ret41}. Four-day-old etiolated (A) or light-grown (B) seedlings were treated without (DMSO) or with 2,4-epibrassinolide (EBL; 0.1 μM). The short hypocotyls of *det2* mutants were elongated with EBL treatment. Data are mean ± standard error of 20–30 individuals for each line/treatment.

in the short hypocotyls. Nevertheless, the apical hooks of *cesa* mutants are not in an exaggerated angle as those induced by excessive ethylene. Our data also support this notion that the angle of apical hooks in *cesa6* mutants is less prominent than in *cesa6;eto1* double mutants (*cesa6*^{ret8}*;eto1-4* and *cesa6*$^{prc1-1}$*;eto1-4*, Fig. 3A). Acsinone7303 treatment did not substantially change the phenotype of *cesa6;eto1*, which consequently led to identification of *cesa6*^{ret8}*;eto1-4* from the genetic screening. We found that the formation of the *cesa6* mutant phenotype did not depend on ethylene. Interestingly, the presence of short and swollen hypocotyls of dark-grown *cev1*, a mutant allele of *CESA3*, requires a functional *ETR1* gene [63]. *cev1* plants show increased production of ethylene and jasmonate (JA) and they constitutively express the JA-and ethylene-induced genes, *THI2.1* and *PDF1.2*. We found that in the dark, the ethylene level was slightly higher for *cesa6*^{ret8} than the wild type. Nevertheless, the ethylene level of another *cesa6* mutant, *cesa6*$^{prc1-1}$, was similar to that of the wild type (Fig. 4B). Changes in cell wall structure or composition may trigger a stress signal to induce ethylene and JA responses [63]. Exactly what kind of cell wall changes cause the stress signal and whether ethylene emission is part of the stress response remain to be clarified.

De-etiolation is used to describe a photomorphogenesis process stimulated by light after seed germination, which results in inhibition of hypocotyl elongation, opening of apical hooks, cotyledon expansion, accumulation of anthocyanins, and induction of greening [60,64]. *De-etiolated* mutants, with characteristics of light-grown plants even under dark-grown conditions, were first isolated for study of light-dependent development [55]. *det2* seedlings were found to have short hypocotyls when grown in the dark for less than 5 days, with significant cotyledon expansion and opening occurring after more than 1 week of dark growth [64]. This description is

consistent with our observation of the *det2*^{ret41} mutant phenotype (Fig. 3A).

DET2 encodes steroid 5α-reductase, an enzyme known to have an important role in brassinolide biosynthesis. More than 50 natural occurring BRs exist, and brassinolide is the most biologically active BR. The biosynthesis of brassinolides involves many oxidation steps, with only 2 steps of reduction [65]. DET2 catalyzes the reduction of campesterol to campestanol, which occurs early in the pathway. Because of no similar sequence in the Arabidopsis genome [49], mutants with loss-of-function *DET2* show typical phenotypes of brassinolide deficiency, including dwarfism, small green leaves, reduced male fertility, and de-etiolated seedlings. Other mutants involved in BR biosynthesis, such as *cbb1* [50,56] and *cpd* [60], or the BR signaling pathway, such as *bri1* [66], typically have similar phenotypes.

BRs are well known to promote cell elongation, and the direct result of cell elongation is a long hypocotyl [67]. The mechanistic role of the BR action in cell elongation is not completely clear. Modification of cell wall structure is likely involved in the process, which was suggested by microarray data [68] and by findings showing the regulation of Arabidopsis *CESA* genes by BRs [69]. Besides the cell-wall–related genes, 3 related receptor-like kinases, which functionally interact with the BR pathway, were found required for cell elongation during vegetative growth [70]. Although in general, BRs promote hypocotyl growth under light, the application of high concentrations of BRs (> 0.1 μM) to dark-grown wild-type seedlings does not significantly change hypocotyl length or even reduces hypocotyl length [data from 12,49]. Nevertheless, BR treatment can promote hypocotyl elongation of certain mutants impaired in hypocotyl growth in darkness [60].

Ethylene is known to reduce cell elongation in hypocotyls of dark-grown seedlings but stimulate hypocotyl elongation in the light [19]. The synergetic effect of BR and ethylene on cell elongation in the light was demonstrated with EBL in *eto1-5* as compared with the wild type (Fig. 6B). BRs have been shown to induce ethylene production, possibly by stabilizing ACS [71]. Because BR and ethylene mutually promote the biosynthesis of each other [72], elongation of hypocotyls under light is possibly regulated by the interaction of the 2 hormones. In line with our observation, ethylene and BRs were proposed to be in balance to regulate hypocotyl elongation [12].

The *ret* mutants identified from our genetic screens may be defective in displaying the phenotype we aimed to score, whether dependent on ethylene or not. Additional criteria must be used in screening *ret* mutants to identify genes involved in the ethylene response network. Introducing an ethylene-responsive reporter construct, such as *5xEBS::LUC*, during the initial phase of mutant screening, can be used to score the *ret* phenotype and monitor the ethylene response simultaneously. The *ret* mutants that maintain high luciferase activity with acsinone7303 treatment are more likely to be involved directly in the ethylene pathway. One such mutant, *ret12*, which maintains high luciferase activity in the presence of acsinone7303 and inhibitors of ethylene response, was identified (unpublished results). Genetic analysis showed that *ret12* was a recessive mutation and was not allelic to *ctr1* or *ran1*. RET12 may represent a new component downstream of ethylene receptors in the pathway. We did not find *ctr1*, which is the most likely *ret* mutant candidate, which suggests that the mutant screen was not exhaustive. We can continue to uncover novel elements in the ethylene biosynthetic and response pathways by using this genetic screen with a modified strategy. Further characterization of the remaining *ret* mutants will reveal additional information about seedling development regulated by ethylene and other modulators.

Acknowledgements

We thank ABRC and the Salk Institute for seeds and T-DNA lines and Dr. Samantha Vernhettes for seeds of *CESA6p::GFP-CESA6*. This work was supported by grants from Development Program of Industrialization for Agricultural Biotechnology (100S0030016, to LCW), National Science Council (101-2324-B-008-001, to SJW; 101-2311-B-001-011 to LCW) and Institute of Plant and Microbial Biology, Academia Sinica, Taiwan (to LCW and WSL). We appreciate Ms. Laura Smales for editing the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2013.04.005.

References

[1] E.M. Josse, K.J. Halliday, Skotomorphogenesis: the dark side of light signalling, Curr. Biol. 18 (2008) R1144–R1146.
[2] M. Chen, J. Chory, Phytochrome signaling mechanisms and the control of plant development, Trends Cell Biol. 21 (2011) 664–671.
[3] A.A. Arsovski, A. Galstyan, J.M. Guseman, J.L. Nemhauser, Photomorphogenesis, Arabidopsis Book 10 (2012) e0147.
[4] L. De Grauwe, F. Vandenbussche, O. Tietz, K. Palme, D. Van Der Straeten, Auxin, ethylene and brassinosteroids: tripartite control of growth in the *Arabidopsis* hypocotyl, Plant Cell Physiol. 46 (2005) 827–836.
[5] A. Lehman, R. Black, J.R. Ecker, *HOOKLESS1*, an ethylene response gene, is required for differential cell elongation in the *Arabidopsis* hypocotyl, Cell 85 (1996) 183–194.
[6] F. Vandenbussche, J. Petrasek, P. Zadnikova, K. Hoyerova, B. Pesek, V. Raz, R. Swarup, M. Bennett, E. Zazimalova, E. Benkova, D. Van Der Straeten, The auxin influx carriers AUX1 and LAX3 are involved in auxin-ethylene interactions during apical hook development in *Arabidopsis thaliana* seedlings, Development 137 (2010) 597–606.
[7] F. An, X. Zhang, Z. Zhu, Y. Ji, W. He, Z. Jiang, M. Li, H. Guo, Coordinated regulation of apical hook development by gibberellins and ethylene in etiolated *Arabidopsis* seedlings, Cell Res. (2012) 1–13.
[8] W.H. Vriezen, P. Achard, N.P. Harberd, D. Van Der Straeten, Ethylene-mediated enhancement of apical hook formation in etiolated *Arabidopsis thaliana* seedlings is gibberellin dependent, Plant J. 37 (2004) 505–516.
[9] J. Gallego-Bartolome, M.V. Arana, F. Vandenbussche, P. Zadnikova, E.G. Minguet, V. Guardiola, D. Van Der Straeten, E. Benkova, D. Alabadi, M.A. Blazquez, Hierarchy of hormone action controlling apical hook development in *Arabidopsis*, Plant J. 67 (2011) 622–634.
[10] F. Vandenbussche, J. Smalle, J. Le, N.J. Saibo, A. De Paepe, L. Chaerle, O. Tietz, R. Smets, L.J. Laarhoven, F.J. Harren, H. Van Onckelen, K. Palme, J.P. Verbelen, D. Van Der Straeten, The *Arabidopsis* mutant *alh1* illustrates a cross talk between ethylene and auxin, Plant Physiol. 131 (2003) 1228–1238.
[11] F. Vandenbussche, B. Vancompernolle, I. Rieu, M. Ahmad, A. Phillips, T. Moritz, P. Hedden, D. Van Der Straeten, Ethylene-induced *Arabidopsis* hypocotyl elongation is dependent on but not mediated by gibberellins, J. Exp. Bot. 58 (2007) 4269–4281.
[12] S.D. Deslauriers, P.B. Larsen, FERONIA is a key modulator of brassinosteroid and ethylene responsiveness in *Arabidopsis* hypocotyls, Mol. Plant 3 (2010) 626–640.
[13] A.N. Stepanova, J. Yun, A.V. Likhacheva, J.M. Alonso, Multilevel interactions between ethylene and auxin in *Arabidopsis* roots, Plant Cell 19 (2007) 2169–2185.
[14] K. Ruzicka, K. Ljung, S. Vanneste, R. Podhorska, T. Beeckman, J. Friml, E. Benkova, Ethylene regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution, Plant Cell 19 (2007) 2197–2212.
[15] P. Achard, W.H. Vriezen, D. Van Der Straeten, N.P. Harberd, Ethylene regulates arabidopsis development via the modulation of DELLA protein growth repressor function, Plant Cell 15 (2003) 2816–2825.
[16] M. Fagard, T. Desnos, T. Desprez, F. Goubet, G. Refregier, G. Mouille, M. McCann, C. Rayon, S. Vernhettes, H. Höfte, PROCUSTE1 encodes a cellulose synthase required for normal cell elongation specifically in roots and dark-grown hypocotyls of *Arabidopsis*, Plant Cell 12 (2000) 2409–2424.
[17] J. Dugardeyn, D. Van Der Straeten, Ethylene Fine-tuning plant growth and development by stimulation and inhibition of elongation, Plant Sci. 175 (2008) 59–70.
[18] P. Guzman, J.R. Ecker, Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants, Plant Cell 2 (1990) 513–523.
[19] J. Smalle, M. Haegman, J. Kurepa, M. Van Montagu, D.V. Straeten, Ethylene can stimulate *Arabidopsis* hypocotyl elongation in the light, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 2756–2761.

[20] S. Zhong, M. Zhao, T. Shi, H. Shi, F. An, Q. Zhao, H. Guo, EIN3/EIL1 cooperate with PIF1 to prevent photo-oxidation and to promote greening of Arabidopsis seedlings, Proc. Natl. Acad. Sci. U.S.A. 106 (2009) 21431–21436.
[21] A.B. Bleecker, H. Kende, Ethylene: a gaseous signal molecule in plants, Annu. Rev. Cell Dev. Biol. 16 (2000) 1–18.
[22] G. Roman, B. Lubarsky, J.J. Kieber, M. Rothenberg, J.R. Ecker, Genetic analysis of ethylene signal transduction in *Arabidopsis thaliana*: five novel mutant loci integrated into a stress response pathway, Genetics 139 (1995) 1393–1409.
[23] A.B. Bleecker, J.J. Esch, A.E. Hall, F.I. Rodriguez, B.M. Binder, The ethylene-receptor family from *Arabidopsis*: structure and function, Philos. Trans. R. Soc. Lond. B: Biol. Sci. 353 (1998) 1405–1412.
[24] Y.-F. Chen, M.D. Randlett, J.L. Findell, G.E. Schaller, Localization of the Ethylene Receptor ETR1 to the Endoplasmic Reticulum of *Arabidopsis*, J. Biol. Chem. 277 (2002) 19861–19866.
[25] J.M. Alonso, T. Hirayama, G. Roman, S. Nourizadeh, J.R. Ecker, EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*, Science 284 (1999) 2148–2152.
[26] J.J. Kieber, M. Rothenberg, G. Roman, K.A. Feldmann, J.R. Ecker, *CTR1*, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the raf family of protein kinases, Cell 72 (1993) 427–441.
[27] R. Solano, A. Stepanova, Q. Chao, J.R. Ecker, Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1, Genes Dev. 12 (1998) 3703–3714.
[28] Q. Chao, M. Rothenberg, R. Solano, G. Roman, W. Terzaghi, J.R. Ecker, Activation of the ethylene gas response pathway in *Arabidopsis* by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins, Cell 89 (1997) 1133–1144.
[29] A.B. Bleecker, M.A. Estelle, C. Somerville, H. Kende, Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*, Science 241 (1988) 1086–1089.
[30] A.N. Stepanova, J.M. Alonso, Ethylene signaling and response: where different regulatory modules meet, Curr. Opin. Plant Biol. 12 (2009) 548–555.
[31] J.M. Alonso, A.N. Stepanova, R. Solano, E. Wisman, S. Ferrari, F.M. Ausubel, J.R. Ecker, Five components of the ethylene-response pathway identified in a screen for *weak ethylene-insensitive* mutants in *Arabidopsis*, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 2992–2997.
[32] A.N. Stepanova, J. Robertson-Hoyt, J. Yun, L.M. Benavente, D.Y. Xie, K. Dolezal, A. Schlereth, G. Jurgens, J.M. Alonso, TAA1-mediated auxin biosynthesis is essential for hormone crosstalk and plant development, Cell 133 (2008) 177–191.
[33] A.N. Stepanova, J.M. Hoyt, A.A. Hamilton, J.M. Alonso, A link between ethylene and auxin uncovered by the characterization of two root-specific ethylene-Insensitive mutants in *Arabidopsis*, Plant Cell 17 (2005) 2230–2242.
[34] M.J. Christians, P.B. Larsen, Mutational loss of the prohibitin AtPHB3 results in an extreme constitutive ethylene response phenotype coupled with partial loss of ethylene-inducible gene expression in *Arabidopsis* seedlings, J. Exp. Bot. 58 (2007) 2237–2248.
[35] P.B. Larsen, J.D. Cancel, Enhanced ethylene responsiveness in the *Arabidopsis eer1* mutant results from a loss-of-function mutation in the protein phosphatase 2A A regulatory subunit, *RCN1*, Plant J. 34 (2003) 709–718.
[36] L.M. Robles, J.S. Wampole, M.J. Christians, P.B. Larsen, Arabidopsis *enhanced ethylene response 4* encodes an EIN3-interacting TFIID transcription factor required for proper ethylene response, including *ERF1* induction, J. Exp. Bot. 58 (2007) 2627–2639.
[37] M.J. Christians, L.M. Robles, S.M. Zeller, P.B. Larsen, The *eer5* mutation, which affects a novel proteasome-related subunit, indicates a prominent role for the COP9 signalosome in resetting the ethylene-signaling pathway in *Arabidopsis*, Plant J. 55 (2008) 467–477.
[38] H.E. Blackwell, Y. Zhao, Chemical genetic approaches to plant biology, Plant Physiol. 133 (2003) 448–455.
[39] C.J. O'Connor, L. Laraia, D.R. Spring, Chemical genetics, Chem. Soc. Rev. 40 (2011) 4332–4345.
[40] R. Toth, R.A. van der Hoorn, Emerging principles in plant chemical genetics, Trends Plant Sci. 15 (2010) 81–88.
[41] S.Y. Park, P. Fung, N. Nishimura, D.R. Jensen, H. Fujii, Y. Zhao, S. Lumba, J. Santiago, A. Rodrigues, T.F. Chow, S.E. Alfred, D. Bonetta, R. Finkelstein, N.J. Provart, D. Desveaux, P.L. Rodriguez, P. McCourt, J.K. Zhu, J.I. Schroeder, B.F. Volkman, S.R. Cutler, Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins, Science 324 (2009) 1068–1071.
[42] J.I. Armstrong, S. Yuan, J.M. Dale, V.N. Tanner, A. Theologis, Identification of inhibitors of auxin transcriptional activation by means of chemical genetics in *Arabidopsis*, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 14978–14983.
[43] B. De Rybel, D. Audenaert, G. Vert, W. Rozhon, J. Mayerhofer, F. Peelman, S. Coutuer, T. Denayer, L. Jansen, L. Nguyen, I. Vanhoutte, G.T. Beemster, K. Vleminckx, C. Jonak, J. Chory, D. Inze, E. Russinova, T. Beeckman, Chemical inhibition of a subset of *Arabidopsis thaliana* GSK3-like kinases activates brassinosteroid signaling, Chem. Biol. 16 (2009) 594–604.
[44] J.M. Gendron, A. Haque, N. Gendron, T. Chang, T. Asami, Z.-Y. Wang, Chemical genetic dissection of brassinosteroid–ethylene interaction, Mol. Plant 1 (2008) 368–379.
[45] W. He, J. Brumos, H. Li, Y. Ji, M. Ke, X. Gong, Q. Zeng, W. Li, X. Zhang, F. An, X. Wen, P. Li, J. Chu, X. Sun, C. Yan, N. Yan, D.Y. Xie, N. Raikhel, Z. Yang, A.N. Stepanova, J.M. Alonso, H. Guo, A small-molecule screen identifies L-kynurenine as a competitive inhibitor of TAA1/TAR activity in ethylene-directed auxin biosynthesis and root growth in *Arabidopsis*, Plant Cell 23 (2011) 3944–3960.
[46] L.-C. Lin, J.-H. Hsu, L.-C. Wang, Identification of novel inhibitors of 1-aminocyclopropane-1-carboxylic acid synthase by chemical screening in *Arabidopsis thaliana*, J. Biol. Chem. 285 (2010) 33445–33456.
[47] S. Savaldi-Goldstein, T.J. Baiga, F. Pojer, T. Dabi, C. Butterfield, G. Parry, A. Santner, N. Dharmasiri, Y. Tao, M. Estelle, J.P. Noel, J. Chory, New auxin analogs with growth-promoting effects in intact plants reveal a chemical strategy to improve hormone delivery, Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 15190–15195.
[48] H. Kende, Ethylene Biosynthesis, Annu. Rev. Plant Physiol. Plant Mol. Biol. 44 (1993) 283–307.
[49] J. Li, P. Nagpal, V. Vitart, T.C. McMorris, J. Chory, A role for brassinosteroids in light-dependent development of *Arabidopsis*, Science 272 (1996) 398–401.
[50] A. Kauschmann, A. Jessop, C. Koncz, M. Szekeres, L. Willmitzer, T. Altmann, Genetic evidence for an essential role of brassinosteroids in plant development, Plant J. 9 (1996) 701–713.
[51] T. Desprez, M. Juraniec, E.F. Crowell, H. Jouy, Z. Pochylova, F. Parcy, H. Höfte, M. Gonneau, S. Vernhettes, Organization of cellulose synthase complexes involved in primary cell wall synthesis in *Arabidopsis thaliana*, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 15572–15577.
[52] D. Weigel, J.H. Ahn, M.A. Blazquez, J.O. Borevitz, S.K. Christensen, C. Fankhauser, C. Ferrandiz, I. Kardailsky, E.J. Malancharuvil, M.M. Neff, J.T. Nguyen, S. Sato, Z.Y. Wang, Y. Xia, R.A. Dixon, M.J. Harrison, C.J. Lamb, M.F. Yanofsky, J. Chory, Activation tagging in *Arabidopsis*, Plant Physiol. 122 (2000) 1003–1013.
[53] K.L.C. Wang, H. Yoshida, C. Lurin, J.R. Ecker, Regulation of ethylene gas biosynthesis by the *Arabidopsis* ETO1 protein, Nature 428 (2004) 945–950.
[54] T.A. Richmond, C.R. Somerville, The cellulose synthase superfamily, Plant Physiol. 124 (2000) 495–498.
[55] J. Chory, P. Nagpal, C. Peto, Phenotypic, genetic analysis of *det2*, a new mutant that affects light-regulated seedling development in *Arabidopsis*, Plant Cell 3 (1991) 445–459.
[56] U. Klahre, T. Noguchi, S. Fujioka, S. Takatsuto, T. Yokota, T. Nomura, S. Yoshida, N.H. Chua, The Arabidopsis DIMINUTO/DWARF1 gene encodes a protein involved in steroid synthesis, Plant Cell 10 (1998) 1677–1690.
[57] T. Ariizumi, T. Kawanabe, K. Hatakeyama, S. Sato, T. Kato, S. Tabata, K. Toriyama, Ultrastructural characterization of exine development of the *transient defective exine 1* mutant suggests the existence of a factor involved in constructing reticulate exine architecture from sporopollenin aggregates, Plant Cell Physiol. 49 (2008) 58–67.
[58] S.F. Altschul, T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.
[59] S. Choe, B.P. Dilkes, B.D. Gregory, A.S. Ross, H. Yuan, T. Noguchi, S. Fujioka, S. Takatsuto, A. Tanaka, S. Yoshida, F.E. Tax, K.A. Feldmann, The *Arabidopsis dwarf1* mutant is defective in the conversion of 24-methylenecholesterol to campesterol in brassinosteroid biosynthesis, Plant Physiol. 119 (1999) 897–908.
[60] M. Szekeres, K. Németh, Z. Koncz-Kálmán, J. Mathur, A. Kauschmann, T. Altmann, G.P. Rédei, F. Nagy, J. Schell, C. Koncz, Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and deetiolation in *Arabidopsis*, Cell 85 (1996) 171–182.
[61] A. Endler, S. Persson, Cellulose synthases and synthesis in *Arabidopsis*, Mol. Plant 4 (2011) 199–211.
[62] T. Arioli, L. Peng, A.S. Betzner, J. Burn, W. Wittke, W. Herth, C. Camilleri, H. Höfte, J. Plazinski, R. Birch, A. Cork, J. Glover, J. Redmond, R.E. Williamson, Molecular analysis of cellulose biosynthesis in *Arabidopsis*, Science 279 (1998) 717–720.
[63] C. Ellis, I. Karafyllidis, C. Wasternack, J.G. Turner, The *Arabidopsis* mutant *cev1* links cell wall signaling to jasmonate and ethylene responses, Plant Cell 14 (2002) 1557–1566.
[64] N. Wei, X.W. Deng, The role of the *COP/DET/FUS* genes in light control of *Arabidopsis* seedling development, Plant Physiol. 112 (1996) 871–878.
[65] S. Fujioka, T. Yokota, Biosynthesis and metabolism of brassinosteroids, Annu. Rev. Plant Biol. 54 (2003) 137–164.
[66] S.D. Clouse, M. Langford, T.C. McMorris, A brassinosteroid-insensitive mutant in *Arabidopsis thaliana* exhibits multiple defects in growth and development, Plant Physiol. 111 (1996) 671–678.
[67] T.W. Wang, D.J. Cosgrove, R.N. Arteca, Brassinosteroid stimulation of hypocotyl elongation and wall relaxation in Pakchoi (*Brassica chinensis* cv Lei-Choi), Plant Physiol. 101 (1993) 965–968.
[68] H. Goda, Y. Shimada, T. Asami, S. Fujioka, S. Yoshida, Microarray analysis of brassinosteroid-regulated genes in *Arabidopsis*, Plant Physiol. 130 (2002) 1319–1334.
[69] L. Xie, C. Yang, X. Wang, Brassinosteroids can regulate cellulose biosynthesis by controlling the expression of *CESA* genes in *Arabidopsis*, J. Exp. Bot. 62 (2011) 4495–4506.
[70] H. Guo, L. Li, H. Ye, X. Yu, A. Algreen, Y. Yin, Three related receptor-like kinases are required for optimal cell elongation in *Arabidopsis thaliana*, Proc. Natl. Acad. Sci. U.S.A. 106 (2009) 7648–7653.
[71] M. Hansen, H.S. Chae, J.J. Kieber, Regulation of ACS protein stability by cytokinin and brassinosteroid, Plant J. 57 (2009) 606–614.
[72] Y.H. Shi, S.W. Zhu, X.Z. Mao, J.X. Feng, Y.M. Qin, L. Zhang, J. Cheng, L.P. Wei, Z.Y. Wang, Y.X. Zhu, Transcriptome profiling, molecular biological, and physiological studies reveal a major role for ethylene in cotton fiber cell elongation, Plant Cell 18 (2006) 651–664.