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Genetic Identification of *ACC-RESISTANT2* Reveals Involvement of *LYSINE HISTIDINE TRANSPORTER1* in the Uptake of 1-Aminocyclopropane-1-Carboxylic Acid in *Arabidopsis thaliana*

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1-Aminocyclopropane-1-carboxylic acid (ACC) is a biosynthetic precursor of ethylene, a gaseous plant hormone which controls a myriad of aspects of development and stress adaptation in higher plants. Here, we identified a mutant in *Arabidopsis thaliana*, designated as *ACC-resistant2* (*are2*), displaying a dose-dependent resistance to exogenously applied ACC. Physiological analyses revealed that mutation of *are2* impaired various aspects of exogenous ACC-induced ethylene responses, while not affecting sensitivity to other plant hormones during seedling development. Interestingly, the *are2* mutant was normally sensitive to gaseous ethylene, compared with the wild type. Double mutant analysis showed that the ethylene-overproducing mutations, *eto1* or *eto3*, and the constitutive ethylene signaling mutation, *ctr1* were epistatic to the *are2* mutation. These results suggest that the *are2* mutant is not defective in ethylene biosynthesis or ethylene signaling per se. Map-based cloning of *ARE2* demonstrated that *LYSINE HISTIDINE TRANSPORTER1* (*LHT1*), encoding an amino acid transporter, is the gene responsible. An uptake experiment with radiolabeled ACC indicated that mutations of *LHT1* reduced, albeit not completely, uptake of ACC. Further, we performed an amino acid competition assay and found that two amino acids, alanine and glycine, known as substrates of LHT1, could suppress the ACC-induced triple response in a LHT1-dependent way. Taken together, these results provide the first molecular genetic evidence supporting that a class of amino acid transporters including LHT1 takes part in transport of ACC, thereby influencing exogenous ACC-induced ethylene responses in *A. thaliana*.

Keywords: ACC uptake • *Arabidopsis thaliana* • Ethylene • LHT1 • Triple response.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC oxidase; ACS, ACC synthase; ARE2, ACC-RESISTANT2; CTR1, CONSTITUTIVE TRIPLE RESPONSE1; EBF, EIN3 BINDING F-BOX; EBS, EIN3 BINDING SEQUENCE; EIL, EIN3-Like; EIN2, ETHYLENE-INSENSITIVE2; *eto*, *ethylene-overproducing*; LHT1, LYSINE HISTIDINE TRANSPORTER1; MS, Murashige and Skoog; PM, plasma membrane; PRE1, PACLOBUTRAZOL RESISTANCE1; RT–PCR, reverse transcription–PCR; SAM, *S*-adenosyl-L-methionine; suc, sucrose; TAIL-PCR, thermal asymmetric interlaced PCR; WT, wild type.

Introduction

In higher plants, ethylene plays essential roles in regulation of many developmental processes and stress adaptation throughout their life cycles, ranging from germination to senescence (Abeles et al. 1992, Bleecker and Kende 2000, Cho and Yoo 2009). Understanding of the mode of action of ethylene has been greatly advanced for the last two decades. By exploiting the triple response (apical hook exaggeration, shortened root and thickened hypocotyl) of dark-grown seedling as a representative ethylene response, a molecular genetic approach revealed core signaling components as well as multiple layers of regulation of ethylene signaling and biosynthesis (Guzman and Ecker 1990, Larsen and Chang 2001, Alonso et al. 2003a).

The level of ethylene in plants is tightly controlled mainly by ethylene biosynthesis, impacted by developmental cues as well as environmental stimuli (Yang and Hoffman 1984, Zarembinski and Theologis, 1994, De Paepe and Van Der Straeten 2005). Ethylene is synthesized from the amino acid methionine via *S*-adenosyl-L-methionine (SAM, also called AdoMet) by the enzyme methionine adenosyltransferase. SAM is then converted to the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC). Conversion of SAM to ACC is a rate-limiting step of ethylene biosynthesis, catalyzed

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by a family of ACC synthases (ACSs). ACC is a non-protein amino acid in which a three-membered cyclopropane ring is fused to the C(α)-atom of the amino acid. Afterwards, ACC is converted to ethylene by a family of ACC oxidases (ACOs). Molecular genetics and biochemical analyses have revealed multiple layers of sophisticated transcriptional and post-transcriptional control over both the ACS and ACO gene families, involving calcium signaling, phosphorylation and proteasome-mediated degradation (Chae and Kieber 2005, Argueso et al. 2007).

Upon perception of ethylene, the signal is transduced to inactivate multiple endoplasmic reticulum-localized receptors and a receptor-interacting Raf-like protein kinase, CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) (Chang et al. 1993, Kieber et al. 1993, Hua and Meyerowitz 1998). Then, the reduced CTR1 activity induces cleavage of the C-terminal ETHYLENE-INSENSITIVE2 (EIN2) fragment (EIN2-C) from the endoplasmic reticulum by unidentified protease(s), followed by nuclear translocation of EIN2-C (Ju et al. 2012, Qiao et al. 2012, Wen et al. 2012). Nuclear signaling, as yet unidentified, induces destabilization of F-box proteins EIN3 BINDING F-BOX1 (EBF1)/EIN3 BINDING F-BOX2 (EBF2) to relieve transcription factors ETHYLENE-INSENSITIVE3 (EIN3)/EIN3-Like (EIL) (Chao et al, 1997, Guo and Ecker 2003, Potuschak et al. 2003, Yanagisawa et al. 2003). The activated EIN3/EIL regulates transcriptional expression by binding to the EIN3 BINDING SEQUENCE (EBS) element in the promoter region of various target genes, which mediate various developmental phenotypes (Solano et al. 1998, An et al. 2010). Their distinct transcriptional outputs include several families of transcription factors, e.g. NAM, ATAF and CUC (NAC), ethylene response factor (ERF) and ethylene response DNA-binding factor (EDF) families, that underpin various ethylene-dependent responses as well as integration into a developmental network (Merchante et al. 2013). In addition to canonical ethylene signaling, the complex web of cross-talk between ethylene and other plant hormones has been revealed by genetic mutants with tissue-specific ethylene insensitivity (Larsen and Chang 2001, Alonso et al. 2003a). However, as exemplified by very recent identification of mutants impaired in the ethylene-induced triple response (Kim et al. 2013, Shin et al. 2013), genetic screening has not been saturated and would reveal additional components of ethylene signaling/responses.

Being an immediate biosynthetic precursor of ethylene, ACC has long been proposed to act as a mobile signal, relaying intercellular communication. In addition to ethylene biosynthesis/signaling, transport of its precursor, ACC, is thought to contribute to differential ethylene biosynthesis/responses within certain environmental or developmental contexts (Jackson 2002). For example, in terms of long-distance transport of the signaling molecule, ACC was detected in the xylem sap of plants exposed to flooding or salinity (Bradford and Yang 1980). In *Zinnia elegans*, the accumulation of ACC in the extracellular medium implicated its paracrine mode of action during differentiation into tracheary elements (Pesquet and Tuominen 2011). Although previous physiological experiments have implied that ACC transporter(s) might have the characteristics of an amino acid transporter (Lürssen 1981), little is known about the transport system for ACC at the molecular level.

Here, we identified a genetic mutant of *Arabidopsis thaliana*, originally named *ACC-resistant2* (*are2*), that exhibits various ACC resistance phenotypes. Hormonal sensitivity experiments revealed that *are2* causes resistance to ACC, but not to ethylene or other plant hormones. Molecular cloning demonstrated that *LYSINE HISTIDINE TRANSPORTER1* (*LHT1*), encoding an amino acid transporter with broad substrate specificity (Chen and Bush 1997, Hirner et al. 2006, Svennerstam et al. 2007), is the gene responsible for this mutation. Together with findings from our genetic analysis, the results of physiological and biochemical analyses suggest that LHT1 is involved in the uptake of ACC, influencing ACC-induced responses in *A. thaliana*.

Results

Identification of an ACC-resistant mutant

During the generation of transgenic plants using a pBI-GR-based vector for steroid-inducible expression of *PACLOBUTRAZOL RESISTANCE1* (*PRE1*) (Lloyd et al. 1994, Lee et al. 2006), we identified a mutant with early onset of the leaf senescence syndrome (**Supplementary Fig. S1**). Based on involvement of plant hormones in leaf senescence, we analyzed the hormonal sensitivity of the mutant. In the course of our physiological analyses, the mutant was found to be significantly resistant to exogenously applied ACC during seedling development, and was thus designated as *ACC resistant2* (*are2*) (**Fig. 1A, B**). Under light conditions, the wild type (WT) showed an elongated hypocotyl and shortened root growth in the presence of exogenous ACC (1 μM) as reported (Smalle et al. 1997). Compared with the WT, the *are2* mutant displayed ACC resistance phenotypes including a shorter hypocotyl and more elongated roots (**Fig. 1C**). The ACC-resistant phenotype of *are2* was dependent on the concentration of ACC (**Fig. 1A, B**). At higher concentrations of ACC (>10 μM), the ACC resistance of the *are2* mutant was attenuated with regard to hypocotyl and root growth. From our genetic analysis based on the F_1 heterozygote (*ARE2*/*are2*) exhibiting a phenotype of slightly longer roots in the presence of exogenous ACC (**Fig. 1C, D**), the ACC resistance of the *are2* mutation appeared to be partially dominant. Subsequent F_2 analysis with 1 μM ACC showed that this resistance followed a typical Mendelian segregation ratio (short root : long root, 301 : 95), indicative of monogenic inheritance of the *are2* mutation. After back-crossing to the WT at least four times, we subjected *are2* to further molecular and physiological analyses.

Various aspects of ACC-induced ethylene responses are impaired in the *are2* mutant

Ethylene exerts various developmental effects on seedling development. To assess further the roles of *ARE2* in the ethylene-dependent responses, we characterized various ethylene-induced responses in *are2* in detail. The triple

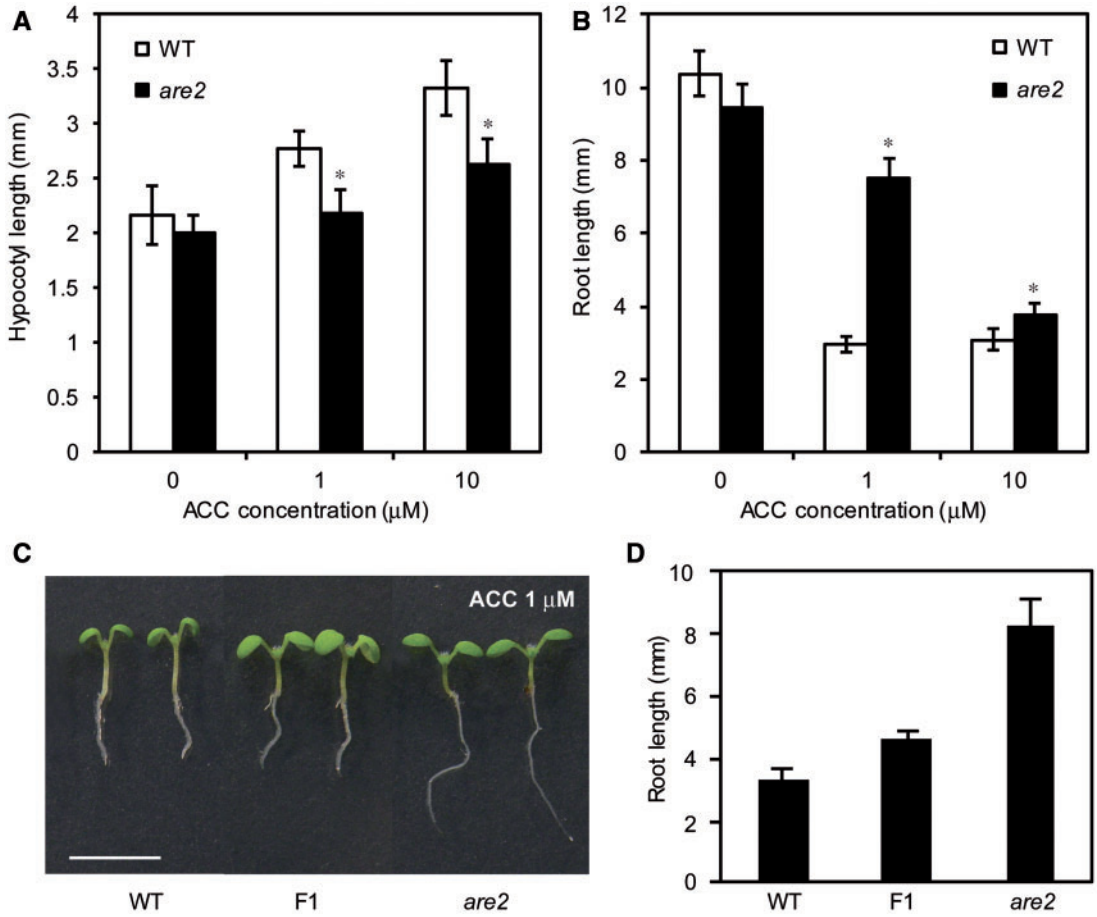

Fig. 1 Identification of the ACC resistant mutant, *are2*. (A and B) Hypocotyl and root growth in the presence of various concentrations of ACC. Plants were grown on MS-suc medium containing 0, 1 or 10 μM ACC under continuous white light for 6 d. Values for hypocotyl length and root length were averaged from at least 15 seedlings. Error bars indicate the SD. An asterisk represents statistical significance ($P < 0.05$) from the ACC-treated wild-type (WT) control, as revealed by Student's *t*-test. (C) Representative morphology of 7-day-old seedlings. Plants were grown on MS-suc medium containing 1 μM ACC under continuous white light. Scale bar = 5 mm. (D) Values for root length were averaged from at least 10 seedlings grown as in (C). Error bars indicate the SD.

response is a well-characterized ethylene-responsive developmental phenotype: inhibition of root elongation, apical hook exaggeration and thickening of hypocotyl (Guzman and Ecker 1990). To evaluate the triple response, we grew WT and mutant seedlings on Murashige and Skoog (MS) medium containing various concentrations of ACC in darkness. A typical triple response was observed in the WT when exposed to 1 μM ACC (**Fig. 2A–D**). In contrast, growth of the *are2* hypocotyls/roots was not significantly inhibited at that concentration. In addition, apical hook exaggeration was not observed in the presence of 1 μM ACC (**Fig. 2B**). Notably, an increased concentration of ACC could significantly restore the defective triple response of the *are2* mutant. Thus, these results indicated that the *are2* mutation abrogated the ACC-induced triple response in a dose-dependent manner.

Ethylene is known to promote the production of root hairs (Tanimoto et al. 1995). To examine whether ACC-induced root hair development is altered in the *are2* mutant, we grew seedlings vertically on MS-sucrose (suc) medium containing different concentrations of ACC. Fewer root hairs were formed in the mutant at 1 μM ACC, whereas this development was vigorous in both *are2* and the WT at 10 μM ACC (**Fig. 2E**).

We also observed that expression of *ETHYLENE RESPONSE FACTOR1* (*ERF1*), a primary target gene of EIN3 (Chao et al. 1997), was less responsive to ACC treatment in *are2* in a dose-dependent manner compared with the WT (**Fig. 2F**). Collectively, these results indicated that the *are2* mutation impaired multiple ACC-induced ethylene responses, implying its role in controlling ethylene-responsive growth and development.

To test whether the *are2* mutant showed an abnormal seedling growth phenotype in response to other plant hormones, we grew the WT and *are2* mutant in various concentration of hormones, including methyl jasmonate (MeJA), a synthetic auxin (2,4-D), a synthetic cytokinin (6-benzyl aminopurine, BA), epi-brassinolide (BL) and ABA. In contrast to ACC resistance, the *are2* mutant was normally sensitive to other plant hormones, compared with the WT (**Supplementary Fig. S2**). The ACC-specific resistance led us to focus on characterizing the *are2* mutation with regard to any ethylene-dependent responses.

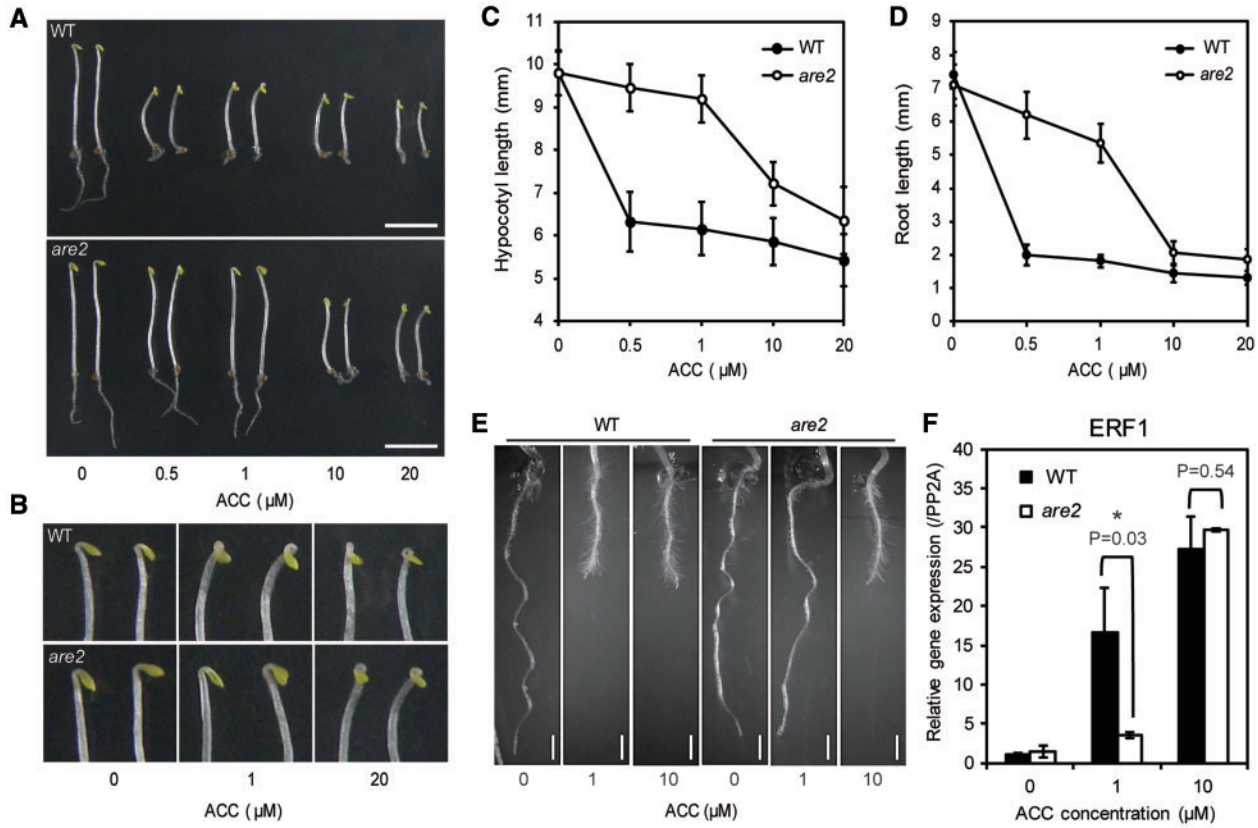

Fig. 2 *are2* affects various aspects of ACC-induced growth responses. (A) Representative phenotypes for the wild type (WT) and *are2* mutant. Seedlings were grown on MS-suc medium containing 0, 0.5, 1, 10 or 20 μM ACC in darkness for 4 d. Scale bar = 5 mm. (B) Close-up of the apical hook region from seedlings grown as in (A). (C and D) Hypocotyl lengths and root lengths of seedlings grown as in (A). The values for the hypocotyl and root length were averaged from at least 15 seedlings. Error bars represent the SD. (E) Reduction in hairy root formation of the *are2* mutant in the presence of exogenous ACC. Representative root phenotypes of the WT and *are2* mutant. Seedlings were grown vertically on MS-suc medium containing different concentrations of ACC under light for 4 d. Scale bar = 1 mm. Similar results were obtained from independent, replicated experiments. (F) Real-time RT–PCR analysis of *ERF1* expression. Total RNA was extracted from whole seedlings of the WT and *are2* mutant. Plants were grown on MS-suc medium containing 0, 1 or 10 μM ACC under continuous light for 4 d. A 1 μg aliquot of total RNA was subject to RT–PCR analysis. Relative levels of expression by *ERF1* from the WT and *are2* mutant are presented after normalization against that of *PP2A*. Data are means of triplicates from three independent experiments. Error bars represent the SD. Brackets superimposed on adjacent bars represent statistical comparisons (*P*-value by Student's *t*-test) between means of the WT and *are2* mutant grown with 10 μM ACC. An asterisk represents statistical significance ($P < 0.05$).

Normal sensitivity of *are2* to gaseous ethylene

To dissect further the defect of the *are2* mutant in the context of ethylene biosynthesis and signaling, we performed further physiological and genetic analysis. In order to verify the involvement of the *are2* mutation in ethylene signaling, we applied gaseous ethylene and examined the triple response. To our surprise, the mutant and the WT exhibited essentially the same dose–responses in regard to the ethylene-induced triple response (**Fig. 3A, B**). Over the concentrations of exogenous gaseous ethylene tested, the *are2* mutant and WT exhibited a similar dose–response relationship in regard to hypocotyl/root growth inhibition and apical hook exaggeration. This implied that the *are2* mutation may not affect ethylene perception/signaling per se.

Genetic epistasis analysis

To assess the possibility that the *are2* mutation affects ACC-responsive growth and development via canonical ethylene signaling, we constructed a double mutant of *are2* and *ctr1-1*, an upstream ethylene signaling mutant (Kieber et al. 1993), displaying the constitutive ethylene response in the absence of ACC. Then, we examined the triple response of the *are2ctr1-1* double mutant. The phenotype of the *are2ctr1-1* double mutant was similar to that of *ctr1-1*, implying that ARE2 and CTR1 function in the same pathway to control the ACC-induced triple response, via canonical ethylene signaling and that ARE2 functions upstream of CTR1 for the ACC-induced ethylene response (**Supplementary Fig. S3**).

In order to assess functional involvement of ARE2 in ethylene biosynthesis from ACC, we performed double mutant analysis with ACC-overproducing mutations, including *ethylene-overproducing1-1* (*eto1-1*) and *ethylene-overproducing3-1* (*eto3-1*) (Chae et al. 2003, Wang et al. 2004). The *eto1-1* mutant was found to have elevated ethylene production due to the increased function of ACS5 by stabilizing this protein, and mutation in *eto3-1* disrupts the protein degradation of ACS5 and ACS9, resulting in elevated ACS activity and subsequent ethylene overproduction.

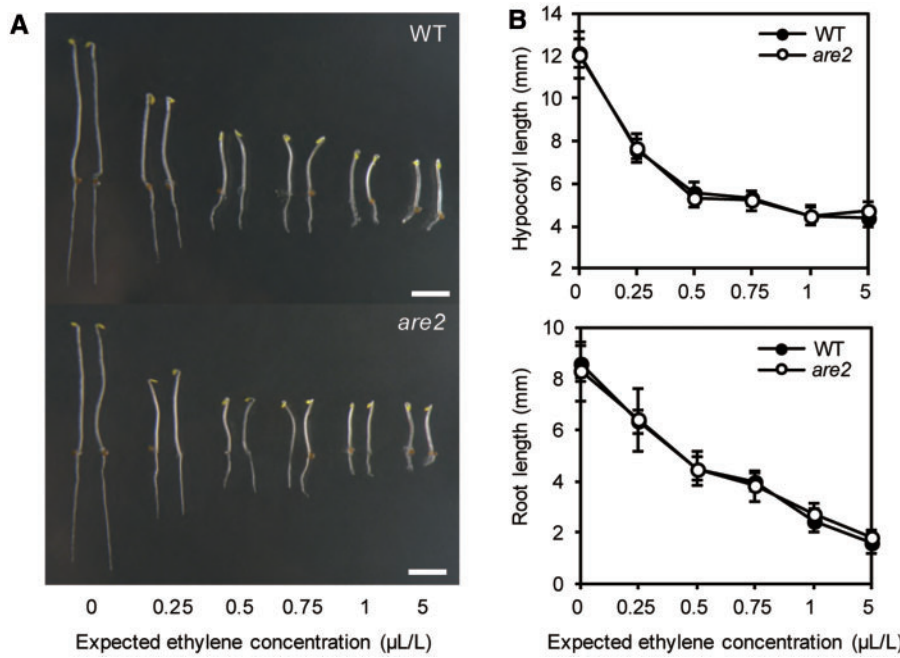

Fig. 3 Dose–responses of the *are2* mutant to gaseous ethylene. (A) Representative morphology of etiolated seedlings grown for 4 d in chambers containing various concentrations of ethephon. Scale bar = 5 mm. (B) Hypocotyl lengths (upper) and root lengths (lower) of seedling grown as in (A). Values for the hypocotyl and root length were averaged from at least 15 seedlings. Error bars indicate the SD ($n > 15$).

In the absence of exogenous ACC, the double mutant seedlings, *are2eto1-1* or *are2eto3-1*, exhibited constitutive ethylene response, like *eto1-1* or *eto3-1* single mutants did (**Fig. 4A, B**). These results suggested that the *are2* mutation may not affect ethylene responses induced by endogenously overproduced ACC or the conversion of ACC into ethylene for ethylene biosynthesis. In accordance with this, measurement of ethylene evolution showed that *are2* mutant seedlings produced levels of gaseous ethylene comparable with those measured from the WT (**Supplementary Fig. S5**).

Molecular nature of *are2*, a loss-of-function allele of *LHT1*

To gain insight into the molecular lesion of the *are2* mutation, we performed positional cloning based on the exogenous ACC resistance phenotypes of the mutant. Chromosomal mapping located the *ARE2* locus at the lower arm of chromosome 5, between *AT5G40220* and *AT5G41010* (**Fig. 5A**). We performed candidate gene sequencing and reverse transcription–PCR (RT–PCR) analyses of genes in the mapped region. As a result, the absence of its transcript in the mutant suggested *LHT1* (AT5G40780) as the gene responsible for *are2* mutation and implicated that *are2* is a null allele of *LHT1* (**Fig. 5B**). Further sequencing analysis of the genomic structure of *LHT1* in the mutant revealed a complicated insertion of T-DNA in the first intron (**Fig. 5C**).

Next, we performed an allelism test with *lht1-5*, a T-DNA insertional knock-out allele of *LHT1* (Svennerstam et al. 2007). This mutant exhibited the same ACC resistance phenotype as the *are2* mutant (**Fig. 5D**). Moreover, the double heterozygote

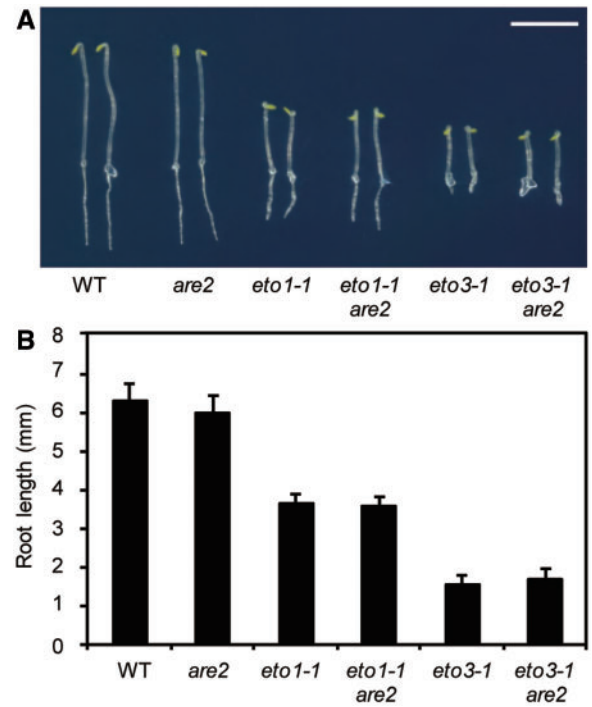

Fig. 4 Genetic epistasis analysis between *are2* and ACC-overproducing mutants. (A and B) Double-mutant analysis of *eto1-1are2* and *eto3-1are2*. (A) Representative morphology of 4-day-old seedlings grown on MS-suc medium under darkness. Scale bar = 5 mm. (B) Root lengths from seedlings grown as in (A). The value for root length was averaged from at least 15 seedlings. Error bars indicate the SD ($n > 15$).

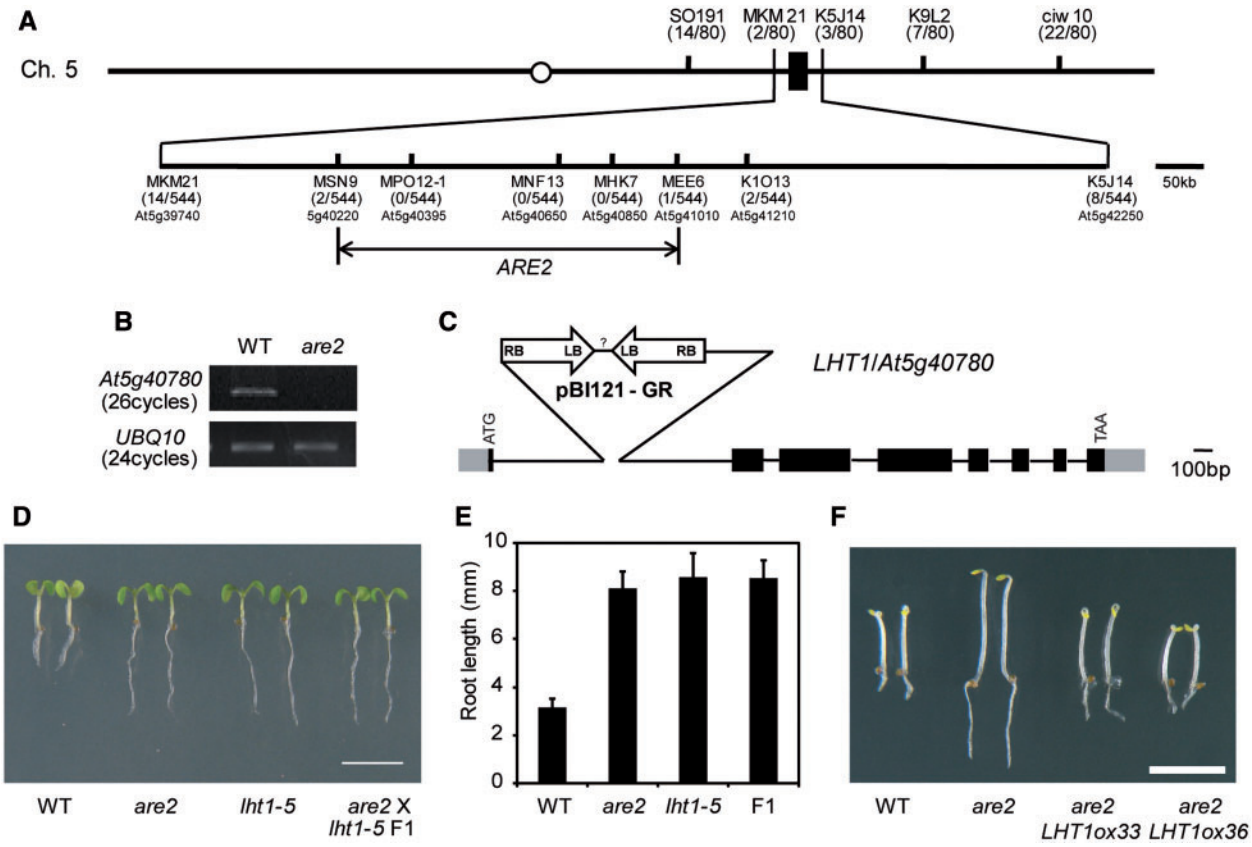

Fig. 5 Map-based cloning of *ARE2*. (A) Schematic illustration of genetic mapping. The *ARE2* locus was initially mapped between MKM21 and K5J14 markers on chromosome 5. Fine mapping narrowed the location to between MSN9 and MEE6 markers, corresponding to *At5g40220* and *At5g41010*, respectively. (B) RT–PCR analysis of the candidate gene *are2*. Total RNAs were extracted from 5-day-old light-grown seedlings and subject to RT–PCR analysis. *UBQ10* served as the loading control. (C) Genomic structure of *LHT1* in the *are2* mutant. Black rectangles indicate exons; triangle, insertion of T-DNA; gray boxes, 5′ and 3′ untranslated regions; black lines, introns. (D and E) Complementation test between *are2* and *lht1-5* mutants. (D) Representative morphology of 5-day-old seedlings, grown on MS-suc medium containing 1 μM ACC. Scale bar = 5 mm. (E) Root lengths from seedlings grown as in (D). Error bars represent the SD (n = 10). (F) Transgenic complementation of the *are2* mutant by the *LHT1* transgene. Two independent transgenic plants, *LHT1ox33* and *LHT1ox36*, in the *are2* mutant background were grown for 4 d under darkness along with the WT and *are2* mutant in the presence of 1 μM ACC. Scale bar = 5 mm.

derived from crossing *are2* with the *lht1-5* mutant revealed the same ACC resistant phenotypes as for the single mutants (**Fig. 5E**), indicating that the *are2* mutation is allelic to *lht1-5*. We also found that both *are2* and *lht1-5* exhibited resistance to the toxic amino acid, D-alanine, as well as an early leaf senescence phenotype (**Supplementary Fig. S5**), as reported (Hirner et al. 2006, Svennerstam et al. 2007).

To verify further that *LHT1* is the gene responsible for this *are2* mutation, we introduced full-length *LHT1* cDNA, expression of which is driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter, into the *are2* mutant. The resulting transgenic lines, with a single-copy T-DNA insertion and overexpression verified by RT–PCR, were examined for ACC sensitivity. Among the 15 tested lines, 13 showed a normal triple response, unlike the *are2* mutant (**Fig. 5F** and other data not presented here). Collectively, the results demonstrated that loss of function of the *LHT1* gene caused exogenous ACC-resistant phenotypes in the *are2* mutant.

It is often observed that expression of a transporter is inducible by its cognate substrate as a feed-forward control (Kang et al. 2010). To test whether *LHT1* expression is regulated by ACC treatment, we performed semi-quantitative RT–PCR analysis. Exogenous applications of ACC elevated the transcript level of *LHT1* within 2 h and sustained that level for up to 12 h (**Supplementary Fig. S6**), indicating that *LHT1* expression was ACC inducible.

LHT1 belongs to a small gene family of amino acid transporters which can mediate the uptake of a set of amino acids in planta (Tegeder and Ward 2012). To gain more insight into the function of the *LHT* family in ACC uptake, we examined whether other *lht* knock-out mutants show insensitivity to ACC. The results implicated that other close homologs of *LHT1* are not necessary for the ACC-induced triple response during seedling development (**Supplementary Fig. S7**).

Uptake of radiolabeled ACC is reduced in *lht1* mutants

Previous functional characterization of ARE2/LHT1 as a plasma membrane (PM)-localized amino acid transporter (Hirner et al. 2006) suggested that the ACC-resistant phenotypes of the *are2* mutant may be due to the reduced uptake of ACC. To assess

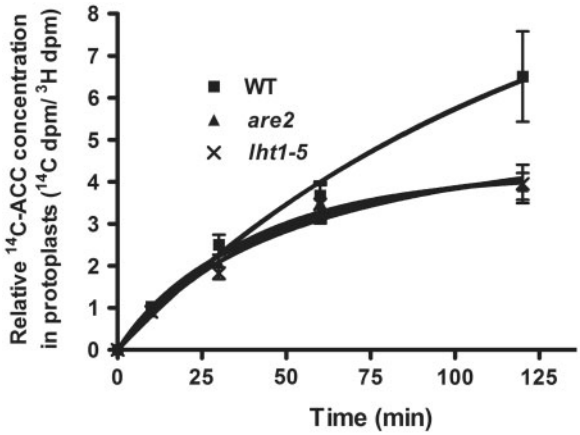

Fig. 6 ACC uptake assay. Time-dependent [^{14}C]ACC uptake in protoplasts of the wild type, and *lht1-5* and *are2* mutants. Protoplasts were suspended in loading buffer containing 1.85 kBq of [^{14}C]ACC per 0.1 ml and 1.85 kBq $^{3}H_2O$ per 0.1 ml, and were incubated for the indicated periods. Intact protoplasts were collected by centrifugation, and radiation from ^{14}C and ^{3}H was determined with a liquid scintillation counter. Values are [^{14}C]ACC counts normalized against $^{3}H_2O$ counts for protoplasts. Means ± SE are shown, obtained from four independent experimental repeats ($n = 4$, $N = 4$).

this possibility, we performed an uptake experiment using radiolabeled [^{14}C]ACC and protoplasts from WT or *are2*/*lht1* mutant leaf mesophyll cells. As shown in **Fig. 6**, although uptake of [^{14}C]ACC was gradually increased over time in all lines, concentrations in the two *lht1* mutant lines, *are2* and *lht1-5*, were approximately 60% of that measured in the WT protoplasts. These results indicated that uptake of ACC was reduced, albeit not completely, in *lht1* mutants.

Analyses of amino acid competition on the ACC-induced triple response

Based on previous findings that excess amino acids can interfere with uptake of ACC (Lürssen 1981), we hypothesized that excess amino acid may compete with exogenously treated ACC to reduce the ACC-induced triple response. To test this possibility, we performed an amino acid competition assay in regard to the ACC-induced triple response. We chose two amino acids, alanine and glycine, as substrates of LHT1, and glutamate as a non-substrate of LHT1 (Hirner et al. 2006). WT seedlings were grown on MS-suc media containing 1 μM ACC plus a high dose of amino acid (100 μM). The results showed that exogenously applied alanine or glycine could effectively, if not completely, suppress the triple response of etiolated WT seedlings in the presence of 1 μM of ACC, whereas the effect of glutamate was marginal. Notably, an increased concentration of ACC (10 μM) could restore the triple response of the WT even in the presence of exogenous alanine or glycine (**Fig. 7**). The competition effect of alanine or glycine was marginal in the *are2* mutant background, although alanine can partially suppress the amino acid competition, indicative of LHT1 dependence of amino acid competition. However, it is notable that alanine can suppress the triple response of the *are2* mutant at a high concentration of ACC (10 μM), suggestive of the presence of an alternative target of amino acid competition in the ACC-induced triple response (see the Discussion).

Discussion

Our results presented molecular genetic evidence for the hypothesis that ACC is transported through the amino acid transporter system. LHT1, a member of the amino acid transporter family, is necessary for the exogenous ACC-induced triple response. Loss-of-function mutations of *LHT1* caused dose-dependent resistance to exogenous ACC in regard to various aspects of ethylene-responsive growth and gene expression, irrespective of light conditions (**Figs. 1**, **2**, **5**). The normal response of *lht1* to gaseous ethylene and genetic analysis of double mutants of *lht1* with *ctr1*, *eto1* or *eto3* mutants excluded the possibility that the ACC-resistant phenotype of the *lht1* mutant was due to a defect in ethylene signaling or ethylene biosynthesis from ACC (**Figs. 3**, **4**). Instead, the reduced uptake rate of radiolabeled ACC in *lht1* mutant protoplasts suggested that LHT1 is involved in uptake of exogenous ACC. In accordance with the proposed role of LHT1 for ACC uptake, it was shown to be inducible by exogenous ACC (**Supplementary Fig. S6**). In line with previous findings that excess amino acids can compete with ACC for its uptake (Lürssen 1981), our results support the hypothesis that the amino acid transport system takes part in ACC transport in higher plants.

LHT1 is a PM-localized amino acid transporter, expressed at the rhizodermis and leaf mesophyll cells (Hirner et al. 2006). In consideration of structural analogy between amino acids and ACC, there has been physiological and biochemical evidence that ACC could move through the amino acid transporter system (Jackson 2002). Supporting this hypothesis, *lht1* mutation reduced movement of extracellular ACC into cells (**Fig. 6**). In accordance with this, we found that two amino acids, known as in planta substrates of LHT1, could effectively compete with ACC to reduce the ACC-induced triple response (**Fig. 7**). Although it is necessary to determine the biophysical characteristics of LHT1 as an ACC transporter, our data clearly indicate that LHT1 could function in transporting both amino acids and ACC. Further biochemical experiments using a heterologous expression system would provide more detailed characteristics of LHT1 in regard to ACC transporter activity, proving the function of LHT1 as a bona fide ACC transporter.

It is noteworthy that *are2*, a null mutant of *LHT1*, exhibited a nearly normal triple response at higher concentrations of ACC, and ACC uptake was not abolished completely in *lht1* mutant protoplast (**Figs. 2**, **6**). These results suggest that there would be additional ACC transporter(s). In *A. thaliana*, *LHT1* belongs to a small gene family of amino acid transporters, comprised of *LHT1–LHT10* (Tegeder and Ward 2012). Although we observed that loss-of-function mutants of other *LHT1* homologs were normal in regard to the ACC-induced triple response (**Supplementary Fig. S7**), it cannot be ruled out that other *LHT1* homologs may function in ACC uptake under specific

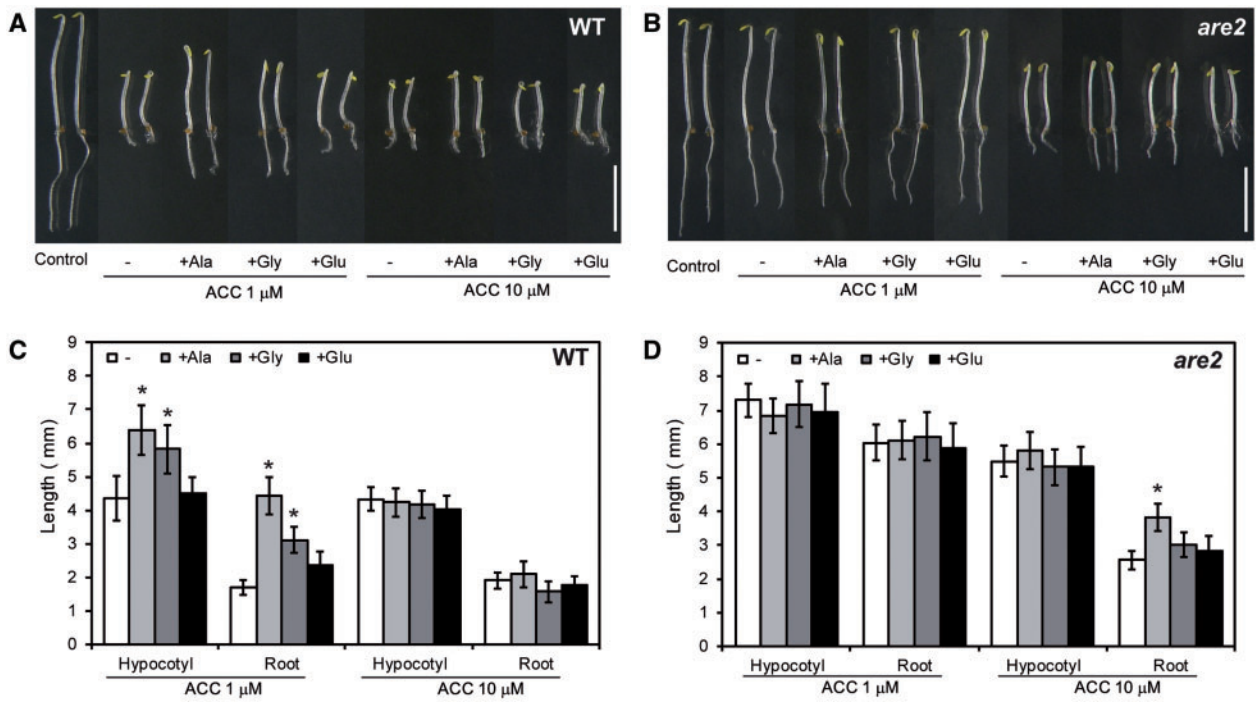

Fig. 7 Assay of amino acid competition based on the ACC-induced triple response. (A and B) Representative morphology of wild-type (WT) and *are2* seedlings grown for 4 d in darkness on MS-suc medium containing 1 or 10 μM ACC plus alanine, glycine or glutamate (100 μM). (C and D) Hypocotyl and root lengths were averaged from at least 15 wild-type and *are2* seedlings grown as in (A) and (B), respectively. Error bars indicate the SD ($n > 15$). An asterisk represents statistical significance ($P < 0.0001$) from the ACC-treated control, as revealed by Student's *t*-test.

environmental conditions or within a particular developmental context because *LHT1* homologous genes exhibit distinct and overlapping spatio-temporal expression patterns (Hirner et al. 2006, Foster et al. 2008). In support of this hypothesis, our ongoing transgenic complementation studies showed that at least one *LHT1* homolog could complement the *lht1* mutant, restoring the triple response in the presence of ACC (unpublished results). Moreover, there are several classes of amino acid transporters which exist in *A. thaliana*, comprised of >60 genes (Rentsch et al. 2007, Tegeder and Ward 2012). Thus, it will be of interest to investigate whether other amino acid transporters participate in ACC uptake/transport processes in *A. thaliana*. Combined with genetic resources including T-DNA insertional mutant lines (Alonso et al. 2003b), further transgenic complementation analysis as well as *lht1* enhancer screening would uncover additional transporter proteins for the ACC transport system.

In regard to the early senescence phenotype of the *lht1* mutant (Hirner et al. 2006, Liu et al. 2010; **Supplementary Fig. S1**), it is intriguing that LHT1 was proposed to modulate the redox status during senescence as well as defense responses (Liu et al. 2010). In agreement with the regulatory function of *LHT1*, its expression was reported to be early responsive to pathogenic challenges as well as developmental aging (van der Graff et al. 2006, Liu et al. 2010). However, it is not yet clear how LHT1 controls redox status during cell death responses during senescence or defense. Liu et al. (2010) suggested that altered amino acid homeostasis in the *lht1* mutant, particularly depletion of glutamine, may lead to accelerated cell death/senescence. In consideration of the promotive role of ethylene during leaf senescence (Lim et al. 2007), our present results raise an alternative, not mutually exclusive, hypothesis that differential apoplastic/cytoplasmic distribution of ACC might contribute to senescence/cell death responses, along with glutamine-responsive signaling. However, the ethylene signaling mutants, *etr1-1* and *ein2-1*, could not alter the accelerated cell death phenotype of the *lht1* mutant (Liu et al. 2010, unpublished results), excluding the possibility that ethylene signaling is necessary for the accelerated senescence/cell death responses of the *lht1* mutant. Recently, several studies have begun to reveal regulatory function(s) of amino acids and non-protein amino acids including ACC, independently of their metabolic roles (Yoon and Kieber 2013, Zeier 2013, Van de Poel and Van Der Straeten, 2014). Thus, it is conceivable that differential distribution of ACC as well as other substrate amino acids via LHT1 may provide multiple signal inputs into developmental senescence/cell death responses. Although the physiological significance of the ACC uptake activity of LHT1 remains speculative at this stage, our results showing that both amino acids and ACC can be transported through LHT1 in a competitive manner may provide a molecular link between metabolic cues (driven by amino acid transport) and ACC/ethylene signaling. In this context, it is noteworthy that several nitrate/peptide transporter family members have been shown to transport plant hormones (Krouk et al. 2010, Kanno et al. 2012). Along with these recent findings, our results add further evidence on

how nutritional and hormonal cues can elegantly be integrated through nutrient/hormone dual substrate transporters.

As our present results provide novel opportunities to explore the ACC transport system in higher plants, which may entail previously characterized amino acid transporters including influx/efflux carriers and intracellular transporters, undoubtedly further molecular identification and biochemical characterizations of LHT1 and other ACC transporter proteins would provide further insight into our understanding of how a plant co-ordinates its development and also adapts to environmental stresses via ACC, a key intermediate metabolite of ethylene.

Materials and Methods

Plant materials and growth conditions

All of the *A. thaliana* plant materials used in this study had the 'Col-0' ecotype background. Mutant seeds for *eto1-1*, *eto3-1*, *ein2-1*, *lht1-5* (SALK_115555C), *lht2ko* (SAIL_222_C2), *lht3ko* (GABI KAT_769F01), *lht5ko* (GABI KAT_036F08), *lht6ko* (SALK_049092C), *lht8ko* (SALK_059360), *lht9ko* (SALK_11416) and *lht10ko* (SAIL_009662C) were obtained from the Arabidopsis Biological Resource Center (ABRC). To identify mutants homozygous for the T-DNA insertion of *lht* mutants, we performed genotyping PCR with the primers listed in **Supplementary Table S1**. Unless specified otherwise, seedlings were grown on half-strength MS medium containing 1% (w/v) sucrose and 0.8% (w/v) phytoagar (Duchefa). After 7 d, they were transferred to soil (Sunshine Professional Growing Mix #5) for further growth under long days (16 h light/8 h dark) at 21–23°C. To observe seedling development, we held sterilized seeds for 3 d at 4°C to synchronize their germination. Afterward, the seeds were sown on MS medium and irradiated with white light for 12 h before being transferred to experimental lighting conditions. For growing plants vertically, the seeds were first sown on MS-suc medium supplemented with 1% (w/v) phytoagar.

Assays for hormonal responses

To test hormonal sensitivity, we sowed stratified seeds on plates of MS medium containing various concentrations of ACC (Sigma), 2,4-D (Sigma), BA (Duchefa), epi-BL (Sigma), MeJA (Sigma) or D-alanine (Sigma). The seedlings were then further grown under continuous light or darkness. For the application of gaseous ethylene, the plates were kept in gas-tight chambers containing ethephon (Sigma) in disodium hydrogen phosphate buffer (Na_2HPO_3, 5 mM), as previously described (Zhang and Wen 2010, Shin et al. 2013). The hypocotyls and roots were measured from at least 15 seedlings, using ImageJ software (http://rsbweb.nih.gov/ij/), with root lengths being normalized to those of the mock-treated control. Seedling images were captured with a digital camera (Nikon Coolpix P5000). To assay the sensitivity of germination to ABA (Sigma), we sowed seeds on MS plates containing (+/−)-*cis*, *trans*-ABA. After incubation for 5 d under white light, germination rates were scored based on radicle emergence. At least 70 seeds were used for each experimental set. Average values were obtained from three independent experiments.

Double mutant construction

To construct the double mutant lines *are2eto1-1* and *are2eto3-1*, we crossed the *are2* mutant with the *eto1-1* or *eto3-1* mutant. The resulting F_1 progeny were self-pollinated to produce F_2 seeds. Seedlings with constitutive ethylene responses (i.e. a shortened root phenotype on the MS medium) were first selected among the F_2 population and then subjected to genotyping with PCR-based molecular markers (**Supplementary Table S1**) for the *are2* mutation. The selected putative double mutants were tested for homozygosity in the F_3 generation and used for phenotypic analysis. For *are2ein2-1* double mutant construction, the F_2 seeds of self-pollinated F_1 plants that were derived from crossing *are2* with *ein2-1* were screened for ethylene insensitivity in the presence of 10 μM ACC (phenotype of *ein2*). The selected *ein2-1* mutants were tested for homozygosity of *are2* by genotyping with molecular markers for the *are2* mutation. The putative double mutants were confirmed in the F_3 generation and used for phenotypic comparison.

Expression analysis

Quantitative real-time PCR (qRT-PCR) analysis and semi-quantitative RT–PCR were performed with total RNA extracted from 5-day-old light-grown seedlings of the WT and *are2* mutant. After treatment with DNase I, 1 μg of total RNA was used for cDNA synthesis with Superscript II reverse transcriptase and an oligo$(dT)_{18}$ primer (Invitrogen). Ten-fold-diluted cDNA was used for semi-quantitative RT–PCR analysis or qRT-PCR analysis. qRT-PCR was conducted in an Eco™ Real-Time PCR System (Illumina), using QuantiMix SYBR Kits (JMC R&D) in a 10 μl volume. All primers are listed in **Supplementary Table S1**. The reactions were performed in triplicate for each run, with experimental duplicates. The comparative ΔΔCt method was used to evaluate the relative quantities of each amplified product in the samples, as described (Lee et al. 2013). Relative expression levels were normalized according to Ct values for *PP2A*.

Chromosomal mapping and genomic structure analysis of the *are2* mutant

The chromosomal location of the *are2* mutation was determined through recombination-based genetic mapping with molecular markers, as described previously (Lukowitz et al. 2000, Shin et al. 2013). The F_2 seedlings, derived from crossing *are2* with the Ler WT, were examined for their root phenotype in the presence of 1 μM ACC. Putative *are2* mutants with long roots were selected for genomic DNA extractions. Additional markers were designed based on Col/Ler polymorphism (TAIR). Primers for mapping are listed in **Supplementary Table S1**.

To determine the genomic structure of *LHT1* in the *are2* mutant, we performed genomic PCR analysis with various primer sets. Thermal asymmetric interlaced (TAIL)-PCR analysis revealed an insertion of T-DNA in the second intron of *LHT1*. We then investigated the flanking sequences for the insertional T-DNA fragment in *are2*, using TAIL-PCR with primers designed for the right border region of the pBI-GR vector (Liu and Whittier 1995). Sequencing of the amplified PCR products revealed that those pBI-GR T-DNAs were inserted in the first intron of the *LHT1* genomic DNA at 744 bp downstream from the transcriptional start site.

Transgenic complementation tests

To examine transgenic complementation of the *are2* mutant, we amplified the LHT1 full-length cDNA via RT–PCR, using the primers listed in **Supplementary Table S1**. The sequence-verified *LHT1* cDNA was subcloned into pENTR1A (Invitrogen). The resulting entry clone was recombined into a Gateway-compatible plant expression vector, pMDC32 (Curtis and Grossniklaus 2003), using LR clonase according to the manufacturer's instructions (Invitrogen). This pMDC32/LHT1 clone was introduced by the floral dip method (Clough and Bent 1998) into *Agrobacterium tumefaciens* GV3101 for transformation into the *are2* mutant plants. Putative transgenic plants were selected based on their hygromycin resistance. For phenotypic analysis, homozygous lines harboring a single T-DNA insertion were selected in the T_2 and T_3 generations.

Measurement of ethylene evolution

Seedlings (approximately 30 per vial) were grown for 4 d in 20 ml gas chromatography vials containing 3 ml of an agar (0.8%)-solidified half-strength MS-suc (1%) medium. Conditions included continuous light and 23°C. The accumulation of ethylene was monitored by a gas chromatograph–flame ionization detector (GC-FID) system (Varian 450-GC; Varian Inc.) equipped with a PLOT fused silica column and thermal desorber (Unity, Markes). A 5 ml sample of headspace was injected onto the column. The ethylene peaks were quantified with a Galaxie chromatography data system, based on comparisons with ethylene standards of 1 ml l^{-1} and 0.2 ml l^{-1}. Ethylene production was normalized for the number of seedlings in each vial. All observations were made from at least three replicates.

[^{14}C]ACC uptake assay

To evaluate ACC uptake, we purified mesophyll protoplasts from the leaves of 7-week-old WT, *are2* and *lht1-5* plants grown under short days

(8 h light/16 h dark). A 33% Percoll gradient was used as described by Martinoia et al. (1993). The protoplasts were re-suspended in a loading buffer [0.5 M sorbitol, 1 mM $CaCl_2$, 10 mM MES-KOH, 5 mM $KHCO_3$, 0.1% bovine serum albunin (BSA) and 1.85 kBq ^3H_2O (American Radiolabelled Chemicals, Inc.) per 0.1 ml; pH 5.6] to achieve a density of 1×10^7 protoplasts ml^{-1}. Samples were gently shaken at room temperature (25°C) for 30 min. For the [^{14}C]ACC uptake assays, 18 nmol of radiolabeled ACC [carboxyl ^{14}C] with 37 kBq (American Radiolabelled Chemicals, Inc.) was added to 2 ml of this protoplast suspension and gently shaken at room temperature for the indicated time. After incubation, 100 µl of the sample was loaded on silicon oil and 33% Percoll (pH 6.0) gradients. Intact protoplasts were collected by centrifugation at 10,000×*g* for 20 s. Their ^{14}C and ^{3}H contents were measured with a liquid scintillation counter as described by Kang et al. (2010).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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