Original research article

Increased expression of ethylene receptor genes during low pH-induced root hair formation in lettuce (*Lactuca sativa* L.) seedlings: direct and indirect induction by ethylene and auxin, respectively

Hidenori Takahashi[1], Asuka Nakamura[1], Wakana Harigaya[1], Ryouhei Fujigasaki[1], Takahiro Iwasa[2] and Yasunori Inoue[2,3]

[1]Department of Biology, Faculty of Science, Toho University, 2-2-1 Miyama, Funabashi, Chiba, 274-8510 Japan
[2]Department of Applied Biological Sciences, Faculty of Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba, 278-8510 Japan
[3]Division of Plant Biotechnology, Tissue Engineering Research Center, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba, 278-8510 Japan
Corresponding author: H. Takahashi, E-mail: takahide@bio.sci.toho-u.ac.jp, Phone: +81-47-472-5362, Fax: +81-47-472-1188

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Abstract: The plant hormones ethylene and auxin mediate the formation of root hairs on lettuce seedlings that are transferred from pH 6.0 to pH 4.0 medium. To investigate the regulatory mechanism of ethylene, we isolated ethylene receptor genes from lettuce. Three putative transmembrane domains were found in Ls-ERS1 and Ls-ETR1 and four in Ls-ETR2 and Ls-ETR3. Five bacterial histidine kinase motifs were highly conserved in Ls-ERS1 and Ls-ETR1, but not Ls-ETR2 or Ls-ETR3. Phylogenetic analysis supported these similarities among family members. Genomic Southern hybridization revealed that each gene existed as a single copy in the genome. mRNAs of these genes were detected in seedling roots after pre-culture at pH 6.0. After transfer to pH 4.0 medium, *Ls-ERS1* and *Ls-ETR2* expression and ethylene production increased and were maintained at higher levels than those found at pH 6.0. The addition of 1-aminocyclopropane-1-carboxylic acid (ACC) to the pH 6.0 medium noticeably induced both ethylene production and *Ls-ERS1* and *Ls-ETR2* expression. A marked increase in the mRNA level of *Ls-ERS1*, with a slight increase in *Ls-ETR2* mRNA level, was noted with the addition of indole-3-acetic acid (IAA); however, ethylene production was also induced. Simultaneous treatment with an ethylene biosynthesis inhibitor and IAA markedly inhibited ethylene production and ethylene receptor gene expression. These results suggest that ethylene receptor gene expression is differentially regulated among the family members during low pH-induced root hair formation in lettuce seedlings, and that the increased expression of *Ls-ERS1* and *Ls-ETR2* during this process is induced by ethylene rather than by auxin.

Keywords: ethylene, ethylene receptor, lettuce (*Lactuca sativa* L.), low pH, root hair

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACS, ACC synthase; AVG, aminoethoxyvinylglycine; bp, base pairs; IAA, indole-3-acetic acid; ORF, open reading frame; PCIB, 2-(*p*-chlorophenoxy)-2-methylpropionic acid; RT-PCR, reverse transcriptase-polymerase chain reaction; SAM, *S*-adenosyl-*L*-methionine; UTR, untranslated region

Note: The nucleotide sequences reported in this paper have been submitted to GenBank under accession numbers AF350320 (*Ls-ERS1*), AF350321 (*Ls-ETR1*), AF350322 (*Ls-ETR2*), and AF350323 (*Ls-ETR3*).

Introduction

Root hairs are tubular projections originating from a specialized subset of root epidermal cells that increase the surface area of roots, thereby increasing

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their absorptive capacity for water and nutrients, helping to anchor plants in soil, and providing sites of interaction for symbiotic micro-organisms (Clarkson 1985, Dolan et al. 1994, Ridge 1995, 1996, Hofer 1996, Peterson and Farquhar 1996). Root hair morphogenesis involves hair initiation, bulge formation, tip growth, and growth cessation (Schiefelbein 2000). Because root hairs originate from single root epidermal cells, they are easily observed and follow a precise morphogenetic pathway; thus, they have been used as a model to study the mechanisms underlying cell patterning, differentiation, and growth in higher plants.

Genetic and physiological studies have demonstrated that the plant hormone ethylene is a positive regulator of root hair formation. In *Arabidopsis*, application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) induces root hair formation at ectopic positions. In contrast, aminoethoxyvinylglycine (AVG), an ethylene biosynthesis inhibitor, or Ag^{+}, an ethylene action inhibitor, prevents root hair development (Masucci and Schiefelbein 1994, Tanimoto et al. 1995). Phenotypic analyses of the ethylene-signaling mutants *ein2* and *etr1* (Pitts et al. 1998) and *ctr1* (Dolan et al. 1994) support the positive effect of ethylene on root hair formation.

In higher plants, ethylene is synthesized via a biosynthetic pathway that involves the conversion of *S*-adenosyl-*L*-methionine (SAM) to ACC, and ACC to ethylene (Adams and Yang 1979). Ethylene receptors capture the synthesized ethylene, initiating signal transduction pathways that elicit various responses. Since the cloning of the *Arabidopsis ETR1* gene (Chang et al. 1993), the isolation of ethylene receptor genes from various plants, including tomato (Wilkinson et al. 1995, Zhou et al. 1996, Lashbrook et al. 1998, Tieman and Klee 1999, Imanishi et al. 2001), tobacco (Knoester et al. 1997, Zhang et al. 1999, 2001a, 2001b, Terajima et al. 2001), *Rumex* (Vriezen et al. 1997), carnation (Shibuya et al. 2002), and melon (Sato-Nara et al. 1999), has advanced our understanding of ethylene perception.

Arabidopsis has five ethylene receptor genes, *ETR1*, *ETR2*, *ERS1*, *ERS2*, and *EIN4* (Chang et al. 1993, Hua et al. 1995, 1998, Sakai et al. 1998). Each receptor contains putative hydrophobic transmembrane domains at its amino-terminus and a region that is similar to the bacterial histidine kinase domain. In addition to these domains, ETR1, ETR2, and EIN4 have a receiver domain at the carboxyl-terminus, which is lacking in ERS1 and ERS2. Ethylene receptors have been recognized as negative regulators of ethylene responses since loss-of-function mutations in *ETR1*, *ETR2*, *ERS2*, and *EIN4* result in strong constitutive ethylene responses (Hua and Meyerowitz 1998).

Previously, we found that the transfer of lettuce seedlings from medium at pH 6.0 to medium at pH 4.0 induced root hair growth within 4 h, whereas no root hairs were initiated on seedlings transferred to pH 6.0 medium (Inoue et al. 2000, Inoue and Hirota 2000). The application of ACC or indole-3-acetic acid (IAA) to pH 6.0 medium also induced root hair formation. In contrast, the addition of ethylene or auxin inhibitors to pH 4.0 medium inhibited root hair formation (Takahashi et al. 2003c). These results suggest that ethylene and auxin positively regulate root hair formation in lettuce seedlings. After the transfer to pH 4.0 medium, the expression of two ACC synthase (ACS) genes increased, indicating that ethylene production plays a key role in regulation (Takahashi et al. 2003b). To fully understand how ethylene regulates root hair formation, the steps involved in ethylene production and ethylene reception must be elucidated, including the expression level of ethylene receptors during root hair formation. However, whether ethylene receptor expression plays a regulatory role in root hair formation, as do ACS genes, is unclear. Furthermore, information about the expression of ethylene receptor genes in plant roots, in contrast to that in aboveground organs, is lacking.

In this study, we isolated four ethylene receptor genes from lettuce and examined the effect of a low pH on their expression. We also investigated their expression in ACC- and IAA-treated seedlings and measured their ethylene production to clarify the role of ethylene receptors during low pH-induced root hair formation in lettuce.

Materials and Methods

Plant materials

Virus-free lettuce (*Lactuca sativa* L. cv. Grand Rapids) seeds were purchased from South Pacific Seeds (Griffith, NSW, Australia) and kept under dry conditions at 4°C until use.

Culture conditions

Seeds were immersed in tap water for 3 h in white light (7.5 W m^{-2}) to induce germination then kept in the dark at 4°C for 24 h to synchronize the timing of germination. They were then sown on a nylon mesh mounted on a polystyrene frame and pre-cultured hydroponically in pH 6.0 medium for 24 h at 25°C under white light to induce main-root formation. Following the pre-culture period, the medium was exchanged for fresh medium with a pH of 4.0 or 6.0, with or without 1 μM ACC, 0.1 μM IAA or 1 μM aminoethoxyvinylglycine (AVG), and the seedlings

were further cultured at 25°C under white light. The culture period was 3 h for gene isolation, 0-4 or 3 h for Northern hybridization analysis, 24 h for binocular microscopic observation, and 0-6 h for the measurement of ethylene production.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was performed using a High-Fidelity RNA PCR Kit (Takara, Kyoto, Japan). cDNA was synthesized from total RNA extracted from seedling roots cultured at pH 4.0 for 3 h. The primer pairs used were F1 and R1 and F2 and R1 (Table 1). Amplification was carried out at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 38°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 2 min. The amplified fragments were TA-cloned into pT7 Blue (Novagen, Madison, WI, USA), and the nucleotide sequences of the fragments were determined using an automated DNA sequencer (model 4000L; LI-COR, Lincoln, NE, USA).

cDNA library screening and DNA sequence analysis

DNA fragments of three ethylene receptor homologs obtained via RT-PCR were labeled using the ECL Direct Nucleic Acid Labeling and Detection System (GE Healthcare, Buckinghamshire, UK) and used as probes to screen a cDNA library (Takahashi et al. 2003b). Approximately 600,000 plaques were screened with each probe using the ECL system. After primary and secondary screenings, all candidate fragments were subjected to the ExAssist/SOLR *In Vivo* Excision System (Stratagene, La Jolla, CA, USA). pBluescript SK(–) phagemids containing cDNAs were cloned into *Escherichia coli* XL-1 Blue MRF′ cells, and the nucleotide sequences of both cDNA strands were determined. Phylogenetic analysis was performed on the deduced amino acid sequences using ClustalW (Thompson et al. 1994).

Table 1. PCR primers used in this study

Primer name	Primer sequence (5′→ 3′)
F1	GCITTYATIRTICTITGYGG
R1	GGBRTCCASAIIGMRCABTY
F2	GCIVTIGCITAYTTYTCRATICC
Ls-ERS1-F1	ACGTACGGATCCGTAGCAATCTT CTAGCAACA
Ls-ETR1-F1	AATTAACCCTCACTAAAGGATCA GGCCTGTTGCATCAG
Ls-ETR2-F1	AATTAACCCTCACTAAAGAAAGA AGCTGCACATCTTGC
Ls-ETR3-F1	ACGTACGAATTCATTGGAACCGG TGATGGTGG
M13(-20)	GTAAAACGACGGCCAGT

DNA extraction and Southern hybridization

Genomic DNA was extracted from young leaves using an ISOPLANT II Kit (Nippon Gene, Tokyo, Japan). Isolated DNA (10 μg of each) was digested with the appropriate restriction enzymes, electrophoresed on a 1% (W/V) agarose gel in TAE buffer, and transferred to a Hybond-N^+ nylon membrane (GE Healthcare). The following sets of primers were used to prepare the templates for transcribing gene-specific RNA probes: Ls-ERS1-F1 and M13 (-20); Ls-ETR1-F1 and M13 (-20); Ls-ETR2-F1 and M13 (-20); and Ls-ETR3-F1 and M13 (-20) (Table 1). The following amplification program was used: 94°C for 3 min, followed by 25 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. After digestion of the *Ls-ERS1* and *Ls-ETR1* probes with *Bam*HI and of the *Ls-ETR2* and *Ls-ETR3* probes with *Eco*RI, the anti-sense RNA probes were transcribed with T7 RNA polymerase using a DIG RNA Labeling Kit (Roche, Basel, Switzerland). Hybridization was performed at 65°C. The products were washed twice with 2× SSC [1× SSC: 0.15 M NaCl, 15 mM Na-citrate (pH 7.0)] plus 0.1% (W/V) SDS at 25°C and twice with 0.1× SSC plus 0.1% SDS at 65°C. Hybridization signals were detected using a DIG Luminescent Detection Kit (Roche).

RNA extraction and Northern hybridization

Total RNA was isolated from roots using an ISOGEN RNA Extraction Kit (Nippon Gene) then electrophoresed (3 μg) on 1% agarose gel in 20 mM MOPS buffer (pH 7.0) containing 5 mM Na-acetate, 1 mM EDTA, and 2.2 M formaldehyde. The RNA was then transferred to a Hybond-N^+ nylon membrane (GE Healthcare). Hybridization was performed at 65°C with the same probes used for Southern hybridization. Washing was performed twice with 2× SSC plus 0.1% SDS at 25°C and twice with 0.1× SSC plus 0.1% SDS at 65°C. Hybridization signals were detected using a DIG Luminescent Detection Kit (Roche) and quantified by subtracting the background level of each lane on the X-ray film using ImageJ software from the U.S. National Institutes of Health (rsb.info.nih.gov/ij). RNA bands corresponding to rRNAs were used as internal controls to normalize the amount of total RNA loaded. The experiments were performed multiple times, confirming the reproducibility of our results.

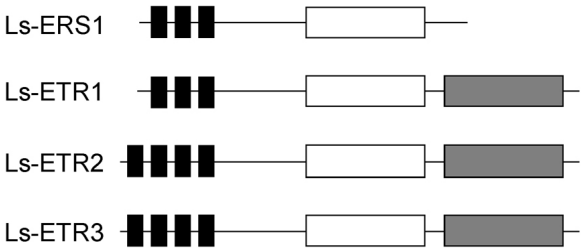

Fig. 1. Structures of the four lettuce ethylene receptors. Black, white, and gray boxes represent hydrophobic transmembrane regions, histidine kinase domains, and receiver domains, respectively.

Measurement of ethylene production

The amount of ethylene produced by the seedlings was measured by withdrawing 1 ml of the headspace gas from an airtight container in which the seedlings were cultured and injecting it into a gas chromatograph (model GC-17A; Shimadzu, Kyoto, Japan). A certified standard of 9.9 μL L^{-1} ethylene was used to calculate the ethylene concentration.

Results

Isolation of lettuce ethylene receptor genes

Degenerate PCR primers, which were designed based on the nucleotide sequences of the five *Arabidopsis* ethylene receptor genes, were used for RT-PCR of total RNA from seedling roots. Amplified fragments of the expected sizes were recovered from an agarose gel and TA-cloned into a plasmid vector. DNA sequencing of the clones revealed three types of DNA fragments, corresponding to three distinct putative ethylene receptor genes. To obtain full-length cDNA clones, a cDNA library from seedling roots was screened using the RT-PCR fragments as probes. DNA sequencing of the cDNA clones from a secondary screen revealed four ethylene receptor homologs (designated clones 1-4), with cDNAs 2797, 3114, 2357, and 2950 base pairs (bp) in length and open reading frames (ORFs) that putatively encoded proteins comprised of 737, 758, 639, and 763 amino acids, respectively.

Characterization of the deduced amino acid sequences of the ethylene receptors

Among the five *Arabidopsis* ethylene receptors, the deduced amino acid sequences of clones 1-4 showed the highest identities with ETR1 (78.7%), ETR2 (57.2%), ERS1 (72.7%), and EIN4 (59.0%), respectively. Clones 1 and 3 had three, while clones 2 and 4 had four putative hydrophobic transmembrane domains at their amino-termini. In addition to the predicted histidine kinase domains, clones 1, 2, and 4 had the predicted receiver domains at their carboxyl-termini, which clone 3 lacked. This suggests that clones 1, 2, and 4 are "ETR-type" ethylene receptors, while clone 3 is an "ERS-type" receptor. Based on these features and similarities with *Arabidopsis* ethylene receptors, we designated the genes for clones 1-4 as *Ls-ETR1* (GenBank Accession No. AF350321), *Ls-ETR2* (AF350322), *Ls-ERS1* (AF350320), and *Ls-ETR3* (AF350323), respectively (Fig. 1).

Comparisons of the deduced amino acid sequences revealed that the putative amino-terminal transmembrane domains were most highly conserved in lettuce ethylene receptors (Fig. 2A). Within the predicted histidine kinase domain, Ls-ERS1 and Ls-ETR1 had all five conserved motifs of bacterial histidine kinases (H, N, G1, F, and G2; Parkinson and Kofoid 1992), whereas the corresponding motifs in Ls-ETR2 and Ls-ETR3 diverged from the consensus sequences (Fig. 2B). Most strikingly, in Ls-ETR2, the conserved histidine residue in the H motif, which is postulated to serve as a phosphorylation site in bacterial two-component proteins, was replaced by a lysine residue. Within the receiver domains, three consensus residues found in bacterial receiver modules were conserved in all three ETR-type lettuce ethylene receptors (Fig. 2C).

Phylogenetic analysis of lettuce ethylene receptors

To analyze the lettuce ethylene receptor proteins using phylogenetic methods, we aligned protein sequences from various plant species and drew a phylogenetic tree (Fig. 3). In the tree, Ls-ERS1, Ls-ETR1, Ls-ETR2, and Ls-ETR3 were most closely related to *Arabidopsis* ERS1, ETR1, ETR2, and EIN4, respectively, which corroborates the aforementioned amino acid percentage-based similarity patterns. Members of subfamily I, comprising Ls-ERS1 and Ls-ETR1, appeared to be more closely related to one another, despite the presence or absence of a response-regulator domain. Subfamily II, comprising Ls-ETR2 and Ls-ETR3, appeared to be more divergent and contained numerous amino acid substitutions in the five sequence motifs that are characteristic of bacterial histidine kinase domains.

Genomic organization of the lettuce ethylene receptor genes

Southern hybridizations were performed to determine the genomic organization of the ethylene receptor

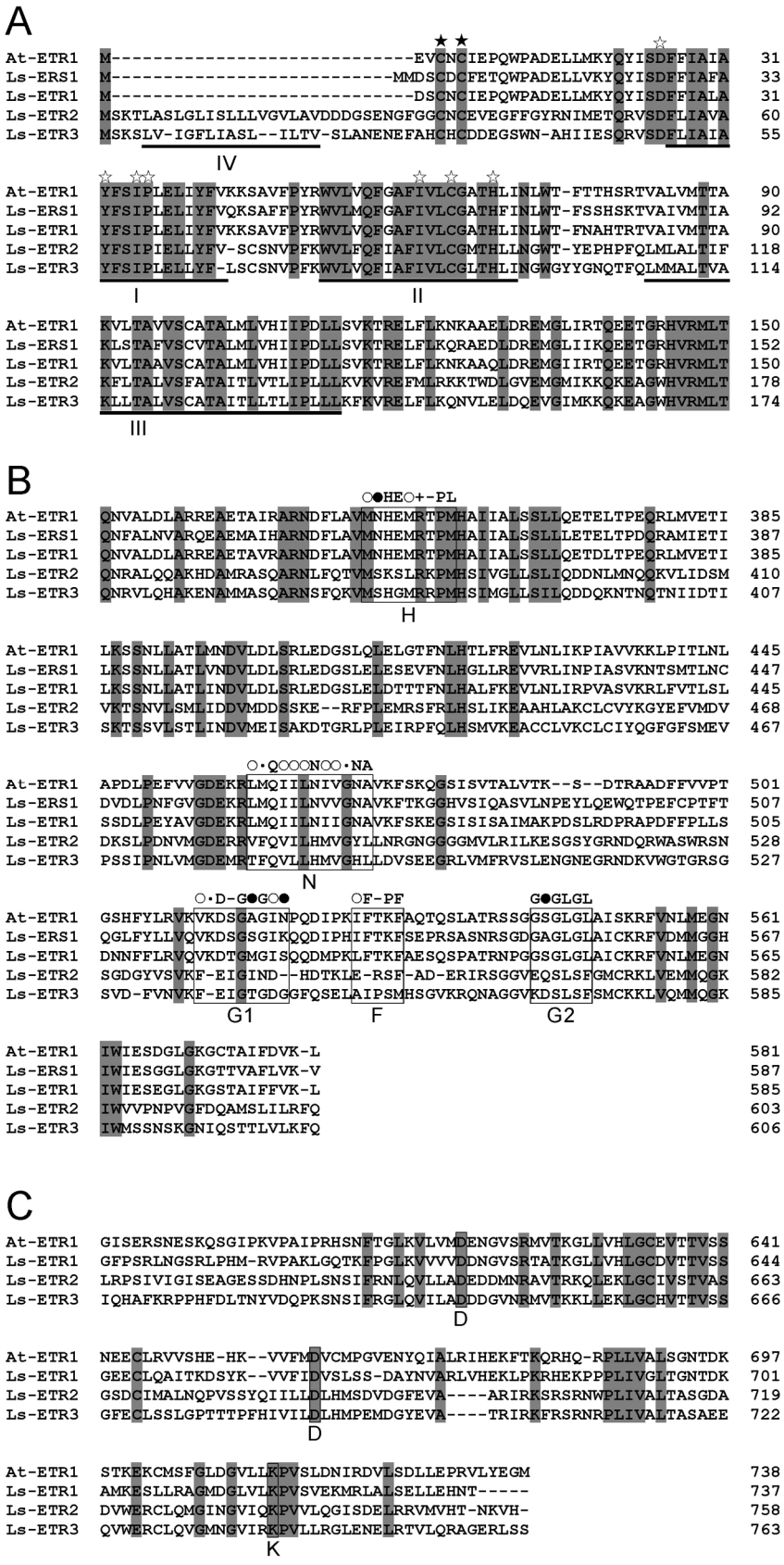

Fig. 2. Amino acid sequences of the putative lettuce ethylene receptors compared with that of *Arabidopsis* ETR1. (A) Alignment of the amino-terminal domains. The four putative hydrophobic transmembrane domains (I-IV) are underlined. Solid stars indicate the two cysteine residues that are thought to be involved in the formation of extracytoplasmic disulfide bridges during ETR1 dimerization (Schaller et al. 1995). Open stars indicate residues that are important for ethylene binding by ETR1 (Schaller et al. 1995, Hall et al. 1999, Rodriguez et al. 1999, Wang et al. 2006). (B) Alignment of the putative histidine protein kinase domains. The five consensus motifs (H, N, G1, F, and G2; Parkinson and Kofoid 1992) of bacterial histidine protein kinases are indicated below the sequences. Open and filled circles indicate nonpolar and polar residues, respectively. Plus signs indicate basic residues, while minus signs indicate acidic or amidic residues. Black dots indicate positions with <50% conservation in bacterial histidine kinases. These symbols are modified from those of Parkinson and Kofoid (1992) and Hua et al. (1998). (C) Alignment of the receiver domains. Conserved aspartate (D) and lysine (K) residues are indicated below the sequences. In (A-C), dashes indicate gaps that were added to facilitate alignment. Shaded amino acids are conserved among the five represented ethylene receptors.

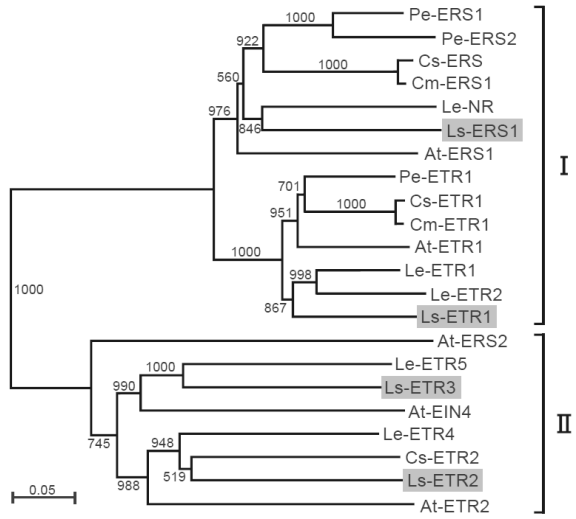

Fig. 3. Phylogenetic tree of ethylene receptors constructed from deduced amino acid sequences using the neighbor-joining method. The accession numbers of sequences in the GenBank database are shown in parentheses: *Arabidopsis* At-ERS1, At-ERS2, At-ETR1, At-ETR2, and At-EIN4 (U21952, AF047976, L24119, AF047975, and AF048982, respectively); cucumber Cs-ERS, Cs-ETR1, and Cs-ETR2 (AB026499, AB026498, and AB026500, respectively); melon Cm-ERS1 and Cm-ETR1 (AF037368 and AF054806, respectively); tomato Le-NR, Le-ETR1, Le-ETR2, Le-ETR4, and Le-ETR5 (U38666, AF043084, AF043085, AF118843, and AF118844, respectively); lettuce Ls-ERS1, Ls-ETR1, Ls-ETR2, and Ls-ETR3 (AF350320, AF350321, AF350322, and AF350323, respectively); and passion fruit Pe-ERS1, Pe-ERS2, and Pe-ETR1 (AB015497, AB070652, and AB015496, respectively). The numbers at the branch points show local bootstrap probabilities (out of 1000) of the various branches. The scale reflects the base-substitution rate.

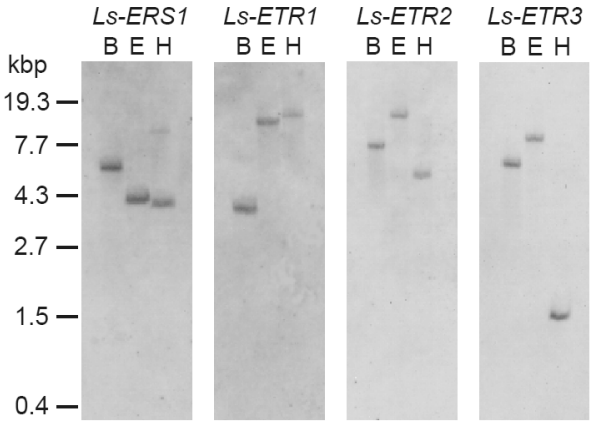

Fig. 4. Genomic Southern hybridization analysis of the ethylene receptor genes. Lettuce genomic DNA was digested with *Bgl*II (B), *Eco*RV (E), and *Hin*dIII (H). *Sty*I-digested λ-DNA was used as a molecular size marker.

genes (Fig. 4). For each gene, a fragment approximately 1 kbp from the 3′-end, which encompassed a partial ORF and the 3′-untranslated region (UTR), was used as a probe. One or two bands were detected in each lane, indicating that each ethylene receptor gene exists as a single copy in the lettuce genome. Moreover, the probes did not cross-react, as no common hybridization bands were observed. Therefore, we used these regions as gene-specific probes in a subsequent Northern hybridization gene expression analysis.

Expression of ethylene receptor genes during root hair initiation

Acidification of the medium induced root hair formation on lettuce seedlings, with root hair bulges beginning to appear 4 h after the pH was lowered (Inoue and Hirota 2000). Northern hybridization analyses were performed to investigate the expression of the ethylene receptor genes during this process (Fig. 5). Expression of each gene was detected at 0 h (i.e., the end of pre-culture at pH 6.0). After transfer of the seedlings to pH 4.0 medium, *Ls-ERS1* and *Ls-ETR2* expression increased, with the increase in *Ls-ETR2* exceeding that of *Ls-ERS1*. Their expression levels were higher than those in control seedlings transferred to fresh pH 6.0 medium. On the other hand, the expression of *Ls-ETR1* and *Ls-ETR3* was largely unchanged after the transfer to pH 4.0 medium and comparable to those in the seedlings transferred to pH 6.0 medium.

Effects of ACC and IAA on ethylene receptor gene expression and ethylene production

We next examined the effect of ACC and IAA on ethylene receptor gene expression in the roots. As expected, the addition of ACC induced root hair formation, even at pH 6.0 (Fig. 6). Seedlings treated with ACC produced far more ethylene than those grown on control (pH 6.0) medium; 10.7-fold more ethylene was produced compared with the pH 6.0 seedlings 6 h after the start of treatment (Fig. 7). Northern hybridization analysis revealed that ACC markedly induced *Ls-ERS1* and *Ls-ETR2* expression but had little effect on the mRNA levels of *Ls-ETR1* and *Ls-ETR3* (Fig. 8).

Addition of IAA also induced root hair formation (Fig. 6). During this process, IAA enhanced the accumulation of *Ls-ERS1* mRNA to the same level as in ACC-treated seedlings. mRNA accumulation was also observed for *Ls-ETR2*; however, the level was lower than that in the ACC-treated seedlings. Little or no induction was observed for *Ls-ETR1* and *Ls-ETR3*, respectively (Fig. 8).

Gas chromatography revealed that ethylene production was also induced by IAA treatment (Fig.

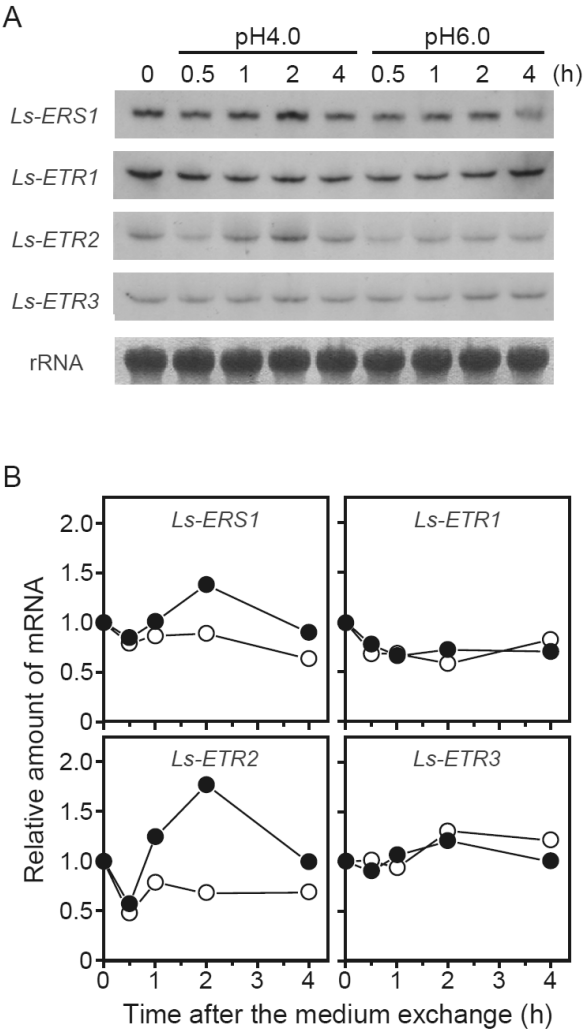

Fig. 5. Expression pattern of ethylene receptor genes in the roots of seedlings cultured at pH 4.0 or 6.0, following pre-culture at pH 6.0. Total RNA extracted after medium exchange at the indicated time was hybridized using gene-specific probes. (A) Hybridization profiles. The rRNA level was used as an internal control to normalize the amount of total RNA loaded. (B) Relative mRNA levels. Filled and open circles represent mRNA levels in the seedling roots at pH 4.0 and 6.0, respectively. For each gene, the mRNA level is shown relative to the baseline level at 0 h, which was assigned a value of 1.0.

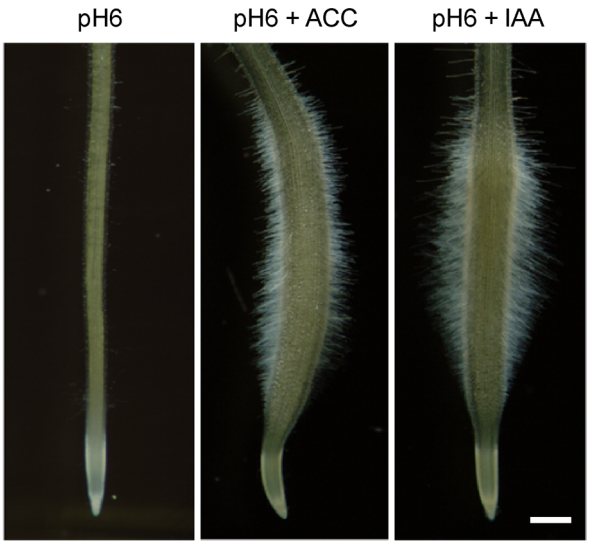

Fig. 6. Effects of ACC and IAA on root hair formation. After preculture at pH 6.0, seedlings were transferred to fresh pH 6.0 medium with or without ACC and IAA. Roots were observed using a binocular microscope 24 h after the start of treatment. Scale bar = 0.5 mm.

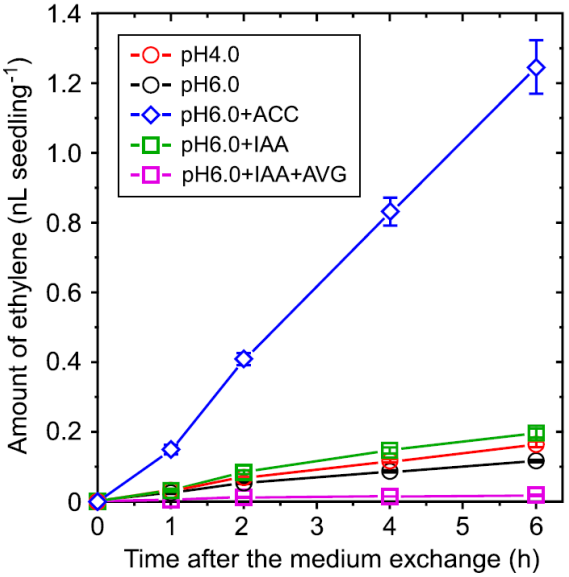

Fig. 7. Lettuce seedling ethylene production. The vertical axis indicates the accumulation of ethylene per seedling as the mean ± standard error. The amount of ethylene at the start of treatment (0 h) was set at 0 nL per seedling.

7). Although its level was 16% of that following ACC treatment, it peaked at 1.2- and 1.7-fold level in the pH 4.0 and pH 6.0 seedlings, respectively, 6 h after the start of treatment. To clarify whether IAA itself or IAA-induced ethylene was responsible for the mRNA levels detected in the IAA-treated seedlings, we used the ethylene biosynthesis inhibitor AVG together with IAA. Ethylene production was markedly inhibited under these conditions; it decreased to 15 and 9% of that in the pH 6.0 and IAA-treated seedlings, respectively, 6 h after the start of treatment (Fig. 7). In this condition, ethylene receptor gene expression was also decreased to a level that was lower than that in the pH 6.0 seedlings (Fig. 8).

Discussion

We isolated four ethylene receptor genes from lettuce, including one ERS-type (*Ls-ERS1*) and three ETR-type (*Ls-ETR1*, *2*, and *3*) genes (Fig. 1). All four lettuce ethylene receptors had domains that were similar to the bacterial histidine kinase domain. Five

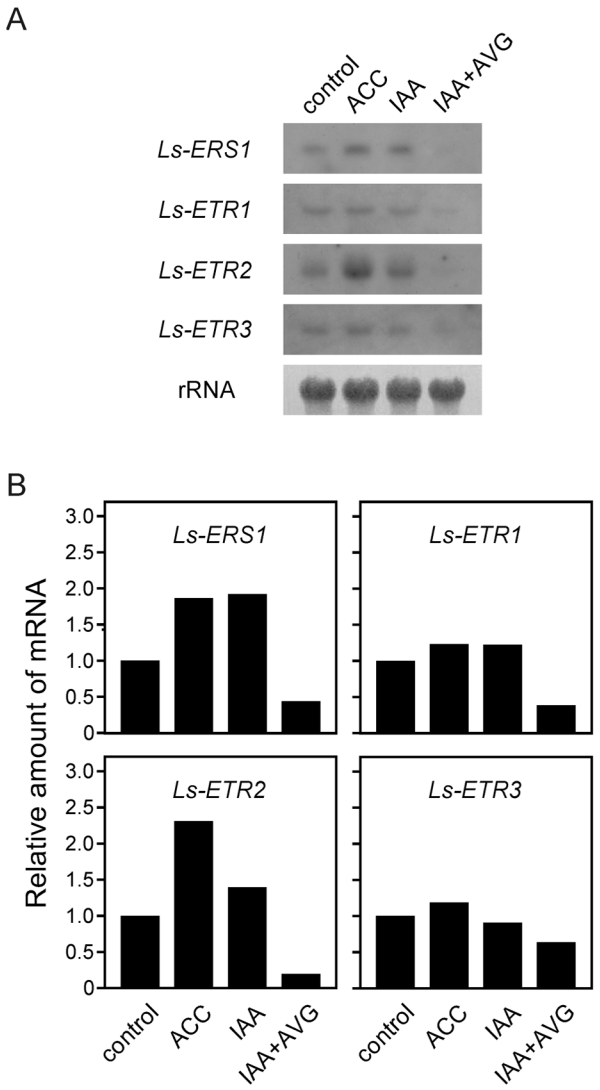

Fig. 8. Effects of ACC, IAA, and IAA plus AVG on ethylene receptor gene expression in the roots. (A) Hybridization profiles. Total RNA was extracted 3 h after the start of the treatments. The rRNA level was used as an internal control to normalize the amount of total RNA loaded. (B) Relative mRNA levels. For each gene, the mRNA level is shown relative to the level in seedling roots cultivated at pH 6.0, which was assigned a value of 1.0.

conserved motifs present in bacterial histidine protein kinases (H, N, G1, F, and G2; Parkinson and Kofoid 1992) were also well conserved in Ls-ERS1 and Ls-ETR1 (Fig. 2), which belong to subfamily I in the phylogenetic analysis (Fig. 3). A histidine residue (His-353 in *Arabidopsis* ETR1), which is postulated to serve as an auto-phosphorylation site in bacterial two-component proteins, was also conserved in both sequences. Conservation in histidine kinase domain has been found in other subfamily I members, including ERS1 (Hua et al. 1995) and ETR1 (Chang et al. 1993).

In contrast to subfamily I receptors, the H, N, G1, F, and G2 motifs in Ls-ETR2 and Ls-ETR3 diverged from the canonical bacterial histidine kinase sequences (Fig. 2). In particular, the probable autophosphorylatable histidine residue is replaced by a lysine residue in Ls-ETR2. The substitution of this histidine residue has also been reported in *Arabidopsis* ETR2 (Sakai et al. 1998) and ERS2 (Hua et al. 1998) and in tomato LeETR5 (Tieman and Klee 1999). These ethylene receptors formed subfamily II in the phylogenetic tree together with Ls-ETR2 and Ls-ETR3 (Fig. 3).

Despite the difference in histidine kinase domain conservation between subfamily I and II members, ethylene-binding activity of both subfamily members has been detected (O'Malley et al. 2005). In lettuce, all four ethylene receptors contain residues Ala-31, Ile-62, Cys-65, and Ala-102, which are substituted in the ethylene-insensitive *etr1* mutant (Bleecker and Schaller 1996), and Cys-4 and -6, which are thought to be involved in the formation of disulfide bridges during dimerization of ETR1 (Schaller et al. 1995), at the corresponding sites. Furthermore, seven residues that are important in the high-affinity binding of ethylene (Schaller and Bleecker 1995, Hall et al. 1999, Rodriguez et al. 1999, Wang et al. 2006) were also conserved (Fig. 2). These significant structural similarities suggest that lettuce ethylene receptors form dimers and have the ability to bind ethylene.

To reveal the expression pattern of the ethylene receptor genes during root hair formation, we examined their mRNA levels using Northern hybridization. Interestingly, the expression patterns differed from our expectations. Ethylene positively regulates root hair formation in lettuce (Takahashi et al. 2003c) as it does in *Arabidopsis* (Wilson et al. 1990, Dolan et al. 1994, Masucci and Schiefelbein 1994, Tanimoto et al. 1995). Since ethylene receptors are negative regulators of ethylene responses (Hua and Meyerowitz 1998, Ciardi et al. 2000, Hackett et al. 2000, Tieman et al. 2000), we had expected that ethylene receptor gene expression would decrease during root hair formation. However, the mRNA level of neither ethylene receptor gene decreased after acidification; in fact, *Ls-ERS1* and especially *Ls-ETR2* expression increased (Fig. 5).

Why, then, did ethylene receptor gene expression increase? First, consider the case in which the number of ethylene receptors increases in hair cells. Recent research by Plett et al. (2009) suggests that *Arabidopsis* ETR2 plays a unique role in mediating the involvement of ethylene in establishing cell shape by affecting microtubule stability. ETR2 shows the highest similarity to Ls-ETR2 among lettuce ethylene receptors. The importance of microtubule organization dynamics in root hair formation has been observed not only in *Arabidopsis* (Bao et al. 2001,

Van Bruaene et al. 2004) and *Zea mays* (Baluška et al. 2000), but also in lettuce (Takahashi et al. 2003a, c). Thus, we suspect that *Ls-ETR2*, with its dramatic increase in expression prior to root hair initiation, plays an important role in lettuce root hair formation by adjusting microtubule organization. Although inhibition of the ethylene response is intensified due to the increased ethylene receptor expression, the increased amount of ethylene produced at pH 4.0 (Fig. 7, Takahashi et al. 2003c) would saturate the receptor binding sites, thereby turning off their negative regulation and turning on the ethylene response (i.e., root hair formation). Even if the amount of ethylene was insufficient to promptly saturate the receptor binding sites, this would not pose a problem because ethylene-bound receptors are degraded in a ligand-induced manner (Chen et al. 2007, Kevany et al. 2007); thus, receptor levels would decrease. Because ethylene receptors are negative regulators, their degradation would allow the remaining receptors to be readily saturated by ethylene, resulting in increased ethylene sensitivity and root hair formation. Indeed, if sufficient numbers of ethylene receptors were removed by loss-of-function mutations, induction of the ethylene response was observed in the absence of ethylene (Hua and Meyerowitz 1998).

Alternatively, increased ethylene receptor expression could turn off the ethylene response in hair cells temporarily once root hair initiation has progressed to a certain stage. Ethylene is required not only for root hair initiation but also for root hair elongation (Takahashi and Inoue 2008). Once the ethylene response is turned off, the ratio of ethylene receptor members, especially those in subfamilies I and II, which have different enzymatic activities and different roles in signaling (Gamble et al. 1998, 2002, Wang et al. 2003, Mounssatche and Klee 2004, Qu and Schaller 2004), may be modified at the protein level as needed in the later stages of root hair formation. The proposed special role of hybrid ethylene receptors (O'Malley et al. 2005) highlights the importance of ethylene receptor composition.

Next, consider the second case in which increased ethylene receptor expression occurs in cells other than hair cells. Even in this case, a certain number of ethylene receptors must be present because root hair formation requires ethylene (Takahashi et al. 2003c) and, therefore, its receptors. The basal expression level of ethylene receptor genes before pH 4.0 treatment (at 0 h) observed in this study (Fig. 5) is not inconsistent with this. The ethylene receptors in the hair cells would readily capture the increased amount of ethylene induced by exposure to pH 4.0. The machinery needed for the induction of root hair formation after the binding of ethylene to the receptors is the same as described above.

These possibilities are based on the hypothesis that ethylene production occurs in hair cells. However, even if ethylene production occurs in cells other than hair cells, it would not affect our explanation because ethylene can diffuse through aqueous and lipid environments. Furthermore, seedling roots at 0 h can be as short as 6 mm and around 0.3 mm in thickness (data not shown); thus, ethylene produced somewhere in the root will easily reach the hair cells and induce root hair formation in proportion to its concentration. The idea that hair cells can respond to ethylene produced somewhere is supported by the observation that lettuce seedlings cultured on pH 6.0 medium formed root hairs when ethylene gas was injected into the culturing case (data not shown). If increased ethylene production occurred in hair cells, root hair formation would be induced all the more readily.

Many ethylene receptor genes are up-regulated by ethylene, including *Arabidopsis ERS1*, *ETR2*, and *ERS2* (Hua et al. 1998); tomato *NR*, *LeETR4*, and *LeETR5* (Ciardi et al. 2000); strawberry *FaEtr2* (Trainotti et al. 2005); Rumex *RP-ERS1* (Vriezen et al. 1997); cucumber *CS-ETR2* and *CS-ERS* (Yamasaki et al. 2000); and mung bean *VR-ERS1* (Kim et al. 1999). Lettuce *Ls-ERS1* and *Ls-ETR2* expression also increased in the pH 4.0 seedlings (Fig. 5), where ethylene production was enhanced (Fig. 7, Takahashi et al. 2003c). Furthermore, these gene expression was also up-regulated by the addition of ACC (Fig. 8). These results suggest that *Ls-ERS1* and *Ls-ETR2* expression is induced by ethylene as in many other ethylene receptor genes.

The addition of IAA also induced *Ls-ERS1* and *Ls-ETR2* expression (Fig. 8), suggesting that they are also up-regulated by auxin. However, gas chromatography revealed that IAA treatment, in fact, induced ethylene production at the same time (Fig. 7). When an ethylene biosynthesis inhibitor was used together with IAA, not only ethylene production but also their mRNA accumulation was markedly inhibited (Figs. 7 and 8). These results suggest that the IAA-induced expression of *Ls-ERS1* and *Ls-ETR2* was not due to IAA itself, but rather to IAA-induced ethylene.

In contrast to the effect of ethylene on the expression of its receptor, the effect of auxin has not been well characterized. Although a slight increase in ethylene receptor gene expression has been observed in auxin-treated soybean (Xie et al. 2007) and strawberry (Trainotti et al. 2005), it is unclear whether this was caused by auxin because the mRNA level was not examined in the presence of ethylene inhibitors.

Although Dong et al. (2008) detected ethylene receptors in *Arabidopsis* root hairs, this is the first report of how ethylene receptor gene expression

changes during root hair formation. In contrast to our expectations, the mRNA level of some ethylene receptor genes increased (Fig. 5). Regarding this increase, there are several plausible explanations (see above); however, we currently do not know which is most likely. Further studies, including investigations of ethylene receptor protein expression and whether ethylene-producing cells, ethylene receptor-increasing cells, and hair cells are identical, will provide insight into the role of ethylene receptors in low pH-induced root hair formation in lettuce.

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