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Original Paper

The *Lotus* intrinsic ethylene receptor regulates both symbiotic and non-symbiotic responses

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Abstract The phytohormone ethylene regulates plant growth, development, and responses to both biotic and abiotic stresses. Ethylene also negatively regulates rhizobial symbiosis in legumes, although the intrinsic ethylene signaling components in legumes are still largely unclear. We report a novel ethylene insensitive mutant named *Ljetr1* from the model legume *Lotus japonicus*. *Ljetr1* showed growth tolerance to high concentrations of 1-amino-cyclopropane-carboxylic acid, the biosynthetic precursor of ethylene. Petal senescence and abscission were delayed and number of nodules was slightly increased compared to wild-type. Mapping analysis and genome sequencing showed that *Ljetr1* bears a mutation in the ethylene-binding domain of the *Arabidopsis ETR1* homolog. Our results suggest that the *Lotus* intrinsic ethylene receptor LjETR1 regulates the ethylene signaling pathway in both non-symbiotic and legume-specific symbiotic responses.

Key words: Ethylene, ETR1, *Lotus japonicus*, nodulation, symbiosis.

Leguminous plants establish a mutualistic symbiosis with rhizobial bacteria and form specialized root organs, known as nodules. In this symbiosis, rhizobia convert atmospheric nitrogen to ammonia and provide it as nutrient to host plants in exchange for photosynthates. This symbiosis enables host legume plants to grow vigorously under nitrogen-nutrient deficient conditions. On the other hand, excessive nodulation impairs host plant growth, because establishment and maintenance of the symbiosis has a high cost in photosynthates. Therefore, leguminous host plants strictly regulate the number of nodules and maintain an appropriate level of symbiosis (Suzaki et al. 2015).

Application of the gaseous phytohormone ethylene, or its biosynthetic precursor 1-amino-cyclopropane-carboxylic acid (ACC), strongly inhibits nodulation in a wide variety of leguminous plants including soybeans (Schmidt et al. 1999), *Medicago truncatula* (Penmetsa and Cook 1997), *Macroptilium atropurpureum* and *Lotus japonicus* (Nukui et al. 2000). Conversely, a treatment with an ethylene biosynthesis inhibitor, L-α-(2-aminoethoxyvinyl)-glycine (AVG), slightly promotes the number of rhizobial infections and nodulations (Nukui et al. 2000, Oldroyd et al. 2001, Penmetsa and Cook 1997). These pharmacological studies suggest that the ethylene signaling pathway has a role in the negative regulation of nodulation.

The ethylene signaling pathway in plants has been extensively studied in the non-legume *Arabidopsis*. In *Arabidopsis*, ethylene is recognized at the endoplasmic reticulum (ER) membrane by a family of ethylene receptors, consisting of ETR1 (ETHYLENE RESPONSE1), ETR2, ERS1 (ETHYLENE RESPONSE SENSOR1), ERS2, and EIN4 (ETHYLENE INSENSITIVE4) that share similarities with bacterial two-component histidine kinases (reviewed in Guo and Ecker 2004). In the absence of ethylene, these ethylene receptors redundantly suppress ethylene responses. Recognition of ethylene inactivates the ethylene receptors and accordingly results in the cleavage of EIN2, an integral ER membrane protein consisting of an N-terminal transmembrane domain and a C-terminal domain containing an unknown motif (Alonso et al. 1999). The released C-terminal segment of EIN2 is translocated to the nucleus and activates the transcription of ethylene-responsive genes (Qiao et al. 2012). Consistent with this model, ethylene receptors bearing a mutation in the amino acid sequences required for ethylene binding (e.g., *etr1-1* and Cm-ERS1/H70A) are persistently active in the presence of ethylene and

Abbreviations: ACC, 1-amino-cyclopropane-carboxylic acid; AVG, L-α-(2-aminoethoxyvinyl)-glycine; EIN, ETHYLENE INSENSITIVE; ETR, ETHYLENE RESPONSE; ERS, ETHYLENE RESPONSE SENSOR; RT-PCR, reverse transcription PCR; GAF, cGMP-specfic phosphodiesterase, adenylyl cyclases and FhlA.

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confer a dominant ethylene-insensitive phenotype similar to the recessive *ein2* mutant.

Genetic signaling components for ethylene responses have also been characterized in legume plants. In *M. truncatula*, an ethylene insensitive mutant named *sickle* showed a strong insensitivity to ethylene or ACC, and formed a clustered and dramatically-increased number of nodules (Penmetsa and Cook 1997). The *SICKLE* gene encodes an orthologous protein to EIN2 in *Arabidopsis* (Penmetsa et al. 2008). Similarly, a simultaneous suppression of two *Lotus* EIN2 homologs, LjEIN2-1 and LjEIN2-2, confers a strong ethylene insensitivity and results in a hypernodulation phenotype comparable to the *Medicago sickle* mutant (Miyata et al. 2013). These results support the importance of ethylene in the negative regulation of nodulation, and indicate the role of EIN2 in the legume ethylene signaling pathway. On the other hand, the soybean ethylene receptor mutant, *etr1-1*, shows no significant difference in nodulation phenotype when compared with wild-type plants, whereas the *etr1-1* mutation is highly tolerant of ACC or ethylene (Schmidt et al. 1999). Nukui et al. conferred ethylene-insensitivity to *L. japonicus* by overexpressing the mutated melon ethylene receptor Cm-ERS1/H70A (Nukui et al. 2004). The transgenic plants showed a slightly increased nodulation phenotype. Similarly, Lohar et al. transformed *Lotus* plants with a mutated *Arabidopsis* ethylene receptor, *etr1-1*, under the control of CaMV-35S promoter and the transgenic plants were named *LjETR1-1* (Lohar et al. 2009). The roots of these transgenic *LjETR1-1* plants also showed insensitivity to ethylene and formed an increased number of nodules when compared to those of wild-type plants under ethylene accumulating condition. However, the degree of increased nodulation in transgenic plants bearing mutated *melon* or *Arabidposis* ethylene receptors is moderate and not comparable to the defects of *Lotus* EIN2 genes (Lohar et al. 2009; Miyata et al. 2013; Nukui et al. 2004). Results regarding ethylene receptors were thus not fully consistent with those regarding EIN2.

Because symbiotic nodulation is restricted in legumes, it is possible that ethylene receptors and/or downstream signaling pathways have specifically evolved for controlling the newly-acquired mechanism in these plants. However, the function of intrinsic ethylene receptors in model legumes (*L. japonicus* or *M. truncatula*) has not yet been characterized, although the effects of heterologous mutated ethylene receptors were reported for *L. japonicus* (Lohar et al. 2009; Nukui et al. 2004). In the present study, we screened for *Lotus* ethylene-insensitive mutants and isolated a novel mutant lacking ethylene responses. As the mutant bears a mutation in the *Lotus* ETR1 homolog, our results indicate that the intrinsic ethylene receptor engages in both symbiotic and non-symbiotic ethylene signaling pathways.

Materials and methods

Plant materials and growth conditions

Mutagenized M2 seeds of *L. japonicus* ecotype MG-20 for mutant screening were kindly provided from Prof. Norio Suganuma (Hakoyama et al. 2012). These M2 seeds were germinated and grown on half-strength Gamborg's B5 medium (Wako) including 10 μM ACC and grown in an artificially-lit growth cabinet at 24°C for 16 h (light) and 22°C for 8 h (dark). The method for nodulation analysis has been previously described (Nakagawa et al. 2011).

Map-based cloning

The causal gene of the *Ljetr1* mutant was identified by map-based cloning as described previously (Yano et al. 2009). A total of 87 F2 plants generated by crossing with *L. japonicus* B-129 Gifu were assessed in a co-segregation analysis with *Lotus* DNA markers provided by the *Lotus* Genome Sequencing Project (Kazusa DNA Research Institute). Genomic DNA of LjETR1 was amplified by the primers LjETR1-F (5′-ACC GTC TTC ACC AGC TAG CTA-3′) and LjETR1-R (5′-AAA CAT AAA TAA TCT TTT ATT CAC AAA AAT GG-3′) in both the *eti1* and *eti2* mutant lines, then sequenced and compared to wild-type LjETR1.

Expression analysis

Experimental methods for total RNA extraction and real-time PT-PCR analysis were described previously (Nakagawa et al. 2011). The gene specific primers used for LjETR1 were 5′-TTG CTC TTA CGG GAA ACA CC-3′ and 5′-CAC CCC TCA TTT TGT CAA CAG-3′.

Results

It is well known that dark-grown etiolated seedlings show shorter roots and hypocotyls in the presence of ethylene or ACC (Figure 1A). To obtain *Lotus* ethylene-insensitive mutants, we screened 1,500 EMS (Ethyl methanesulfonate)-mutagenized seeds (ecotype Miyakojima (MG-20)) by focusing on growth tolerance in the presence of 10 μM ACC. Two ethylene-insensitive mutants were found, tentatively named *eti1* and *eti2*. The *eti1* mutant was backcrossed to its non-parental ecotype Gifu (B-129) for mapping analysis. Interestingly, F1 progenies also showed weak tolerance to ACC similar to the *Arabidopsis etr1-1* mutant (Figure 1A) (Chang et al. 1993). In addition, mapping analysis using F2 progenies suggests that the mutation affecting ethylene insensitivity was mapped to a location near TM0436 (10.5 cM) on chromosome 3 (Figure 1C), where the *Lotus* ETR1 homolog (chr3.CM0634.490.nc) was placed by the *Lotus* Genome Sequencing Project. We cloned and sequenced the corresponding genomic region of

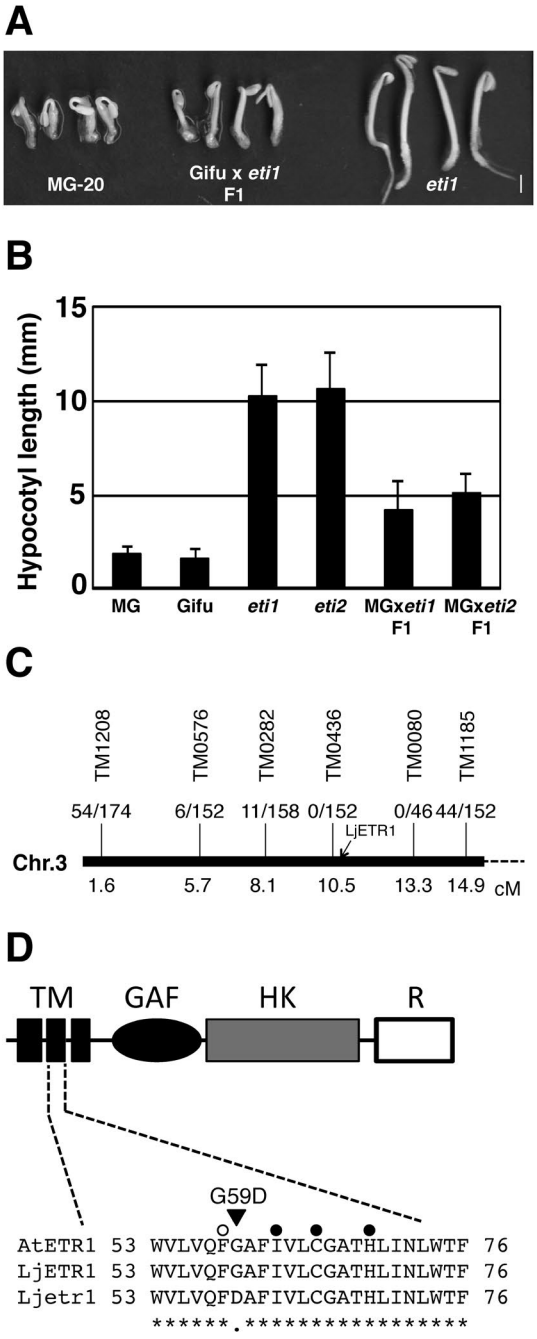

Figure 1. Ethylene-insensitive *eti1* and *eti2* mutants show tolerant growth under high concentrations of ACC and bear a mutation in LjETR1. (A) Dark-grown seedlings (5-day-old) of wild-type MG-20, F1 progenies of Gifu and *eti1*, and *eti1* homozygous F2 plants. Bar: 1 mm. (B) Hypocotyl length under 10 μM ACC. The data are expressed as means and SDs of more than 10 plants. (C) Genetic map of the LjETR1 region with markers and numbers of recombinant events above the line. The genetic distances of each marker from the terminal marker TM0793 of chromosome 3 are also shown. (D) Protein structure of LjETR1. TM: ethylene binding transmembrane domain containing three hydrophobic segments, GAF: GAF domain, HK: histidine kinase domain, R: receiver domain. The alignment of amino acid sequences in the second hydrophobic segment of ethylene binding domain is also shown. Open and closed circles represent essential residues for ethylene signaling or ethylene binding in AtETR1, respectively.

ETR1 in both *eti1* and *eti2* mutants and found the same G to A mutation in both mutants, which conferred an amino acid change from Gly to Asp at protein position 59 in the second hydrophobic segment of the ethylene binding segment (Figure 1D). The region around G59 in ETR1 is well conserved between *Lotus* and *Arabidopsis* (Figure 1D). Although the exact role of G59 has not yet been determined, previous studies of *Arabidopsis* ETR1 predicted the position of this amino acid residue to be in the vicinity of residues essential for both ethylene binding (I62) and ethylene signaling (F58) in the 3D conformation of ETR1 (Wang et al. 2006). We also backcrossed these *eti1* and *eti2* mutants to parental MG-20 and compared the ethylene insensitivity in their progenies. Both F1 and F2 progenies of *eti1* showed similar phenotypes to those of *eti2* (Figure 1B). As these lines may therefore be the siblings from the same M1 plant, we renamed these mutants as *Ljetr1*. The following analyses used the twice-backcrossed progenies of the *eti1* line as *Ljetr1*.

To evaluate the intensity of the ethylene-insensitive phenotype, homozygous *Ljetr1* and the F1 progenies of *Ljetr1* and MG-20 were subjected to different

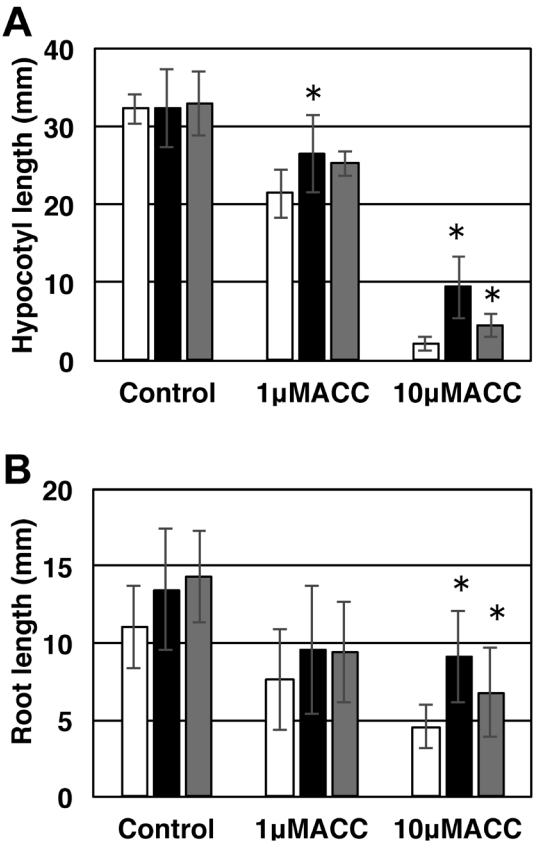

Figure 2. The phenotype of *Ljetr1* is more prominent in higher concentrations of ACC. Hypocotyl (A) or root (B) length of MG-20 (open bar), homozygous *Ljetr1* (black bar) and F1 progenies of MG-20 and *Ljetr1* (gray bar). The data are expressed as means and SDs of more than 7 plants. Black stars indicate significant differences from MG-20 in each condition by Student's *t*-test ($p<0.01$).

concentrations of ACC (Figure 2). In the absence of ACC, neither root growth nor shoot growth were significantly different from the parental wild-type plants. Lower concentrations of ACC (1 μM) decreased shoot and root growth in both wild-type plants and *Ljetr1* mutant. Tolerant growth of *Ljetr1* mutant or F1 progenies was prominent under high concentrations of ACC (10 μM). These results indicate that the *Ljetr1* mutant has a decreased response but does not completely lose ethylene responsiveness.

It is well known that ethylene is involved in floral petal abscission. In *L. japonicus*, pistils and stamens

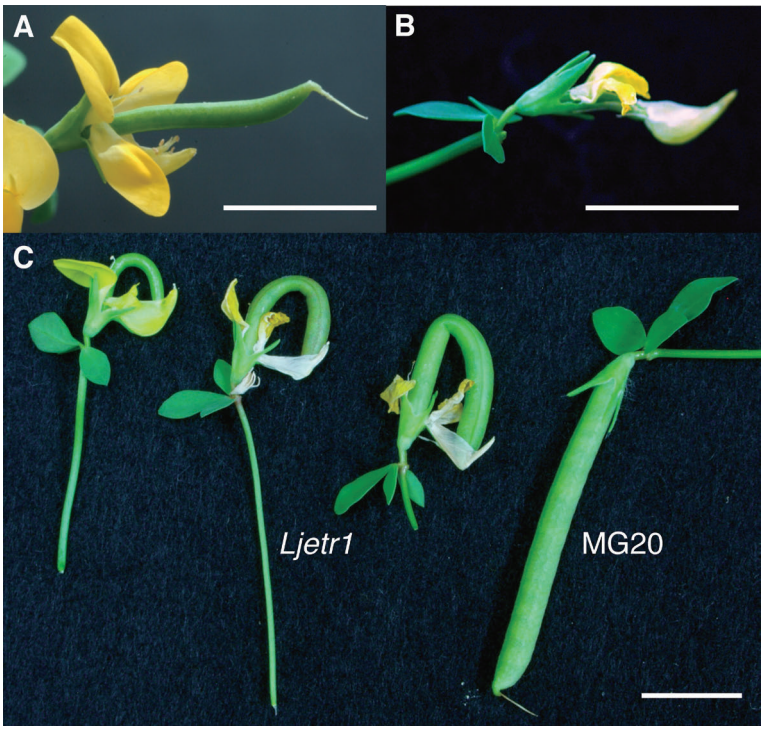

Figure 3. Flowering phenotype of *Ljetr1*. Petals were withered after the pollination in MG-20 (B) but still fresh in *Ljetr1* (A). Pod elongation of *Ljetr1* was often interfered with by the persistently-attached keel petal (C). Bars: 1 cm.

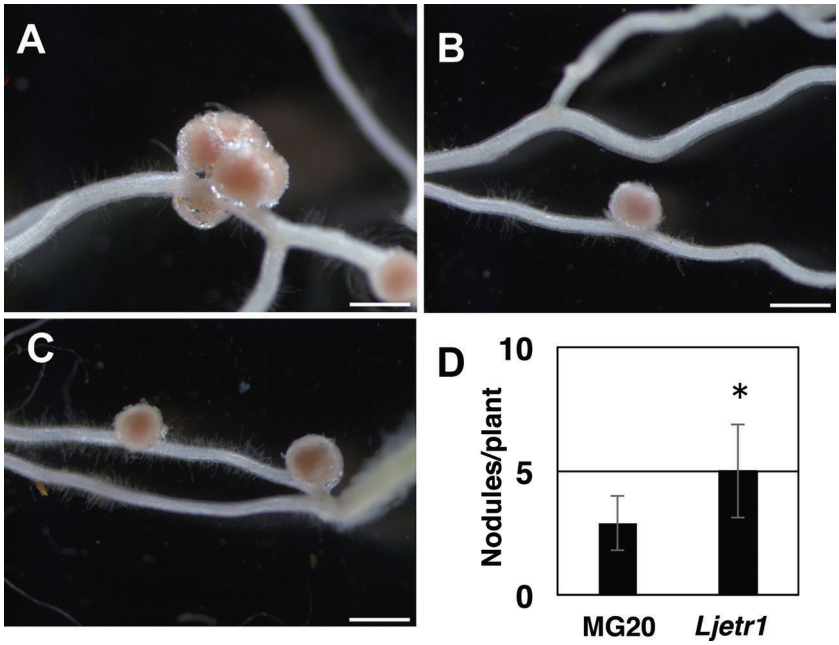

Figure 4. Nodulation phenotype of *Ljetr1*. Nodules formed on *Ljetr1* were often clustered (A) whereas others formed independently (B), similar to wild-type plants (C). D, Number of nodules in MG-20 and *Ljetr1* at 23 days after inoculation. The data are expressed as means and SDs of more than 10 plants. The numbers of nodules were statistically different between MG-20 and *Ljetr1* mutant ($p<0.01$).

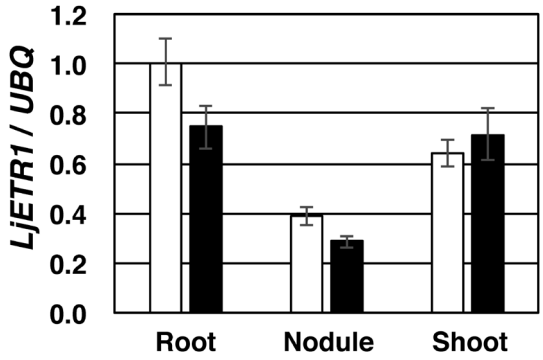

Figure 5. Real time RT-PCR analysis of LjETR1 in both wild-type MG-20 (open bar) and *Ljetr1* mutant (black bar). The data are expressed as means and SDs of three biological replications.

are covered by keel petals. After fertilization, the petals wither and detach from flowers before pod elongation (Figure 3B). In contrast, the petals of *Ljetr1* remained fresh and stayed in place during the pod elongation process (Figure 3A). As a result, the pod elongation of *Ljetr1* was often interfered with, and the pods were bent (Figure 3C).

To investigate the symbiotic phenotype of *Ljetr1*, the mutant was inoculated with *M. loti* MAFF303099 and its nodulation phenotype was compared with wild-type plants (Figure 4). *Ljetr1* mutant often formed clustered nodules (Figure 4A), and the number of nodules was slightly but significantly increased (Figure 4D). However, the degree of increased nodulation was not comparable to EIN2-suppressed plants (Miyata et al. 2013).

The expression pattern of *LjETR1* was investigated by semi-quantitative reverse transcription RT-PCR (Figure 5). *LjETR1* was expressed in both shoot and root, and also in nodules. A similar expression level of *LjETR1* was also observed in *Ljetr1* mutant. These results support the theory that mutated *LjETR1* proteins interfere with the intrinsic ethylene signaling pathway and result in the ethylene-insensitive phenotype in *Ljetr1* mutant.

Discussion

In the model legumes *L. japonicus* and *M. truncatula*, the function of intrinsic ethylene receptors has not been functionally characterized, although the effects of mutated heterologous ethylene receptors have been investigated. Here, we report that the ethylene-insensitive *Ljetr1* mutant bears a mutation in *LjETR1*. In *Arabidopsis*, mutations in I62, C65 or H69 of the second hydrophobic segment of the ethylene-binding domain completely abolish ethylene-binding activity. F58 is not required for ethylene binding, but is essential for ethylene signaling (Wang et al. 2006) (Figure 1D). Therefore, the G59D mutation in *Ljetr1* might disturb the conformation of the second hydrophobic segment of the ethylene-binding domain and result in a constitutively active LjETR1 in the presence of ethylene. On the other hand, the symbiotic phenotype of *Ljetr1* (Figure 4) is seemingly weaker than in previously reported LjEIN2-suppressed plants (Miyata et al. 2013). These results might imply the possibility that the G59D mutation is not sufficient to eliminate the ethylene-binding activity or to suppress the ethylene responses. However, note that overexpression of *Arabidopsis etr1-1* or mutated melon *CmERS1* also showed a phenotype with modestly increased nodulation (Lohar et al. 2009; Nukui et al. 2004). It is possible that ethylene receptor(s) other than LjETR1 also participate in the symbiotic ethylene-signaling pathway.

The phenotype of the *Ljetr1* mutant was more prominent at higher concentrations of ethylene (Figure 2). Ethylene receptors are constantly active to suppress the downstream signaling pathways in the absence of ethylene, and most might still be active under low ethylene concentration. Therefore, *Ljetr1* mutant might only rarely show a significant difference when compared to wild-type plants in low ethylene conditions (Figure 2). On the contrary, higher concentrations of ethylene inactivated most ethylene receptors and made the effects of the persistently active Ljetr1 protein more pronounced.

Ethylene is engaged in the regulation of plant growth, development, and responses to both biotic and abiotic stresses. Multiple copies of ethylene receptors might reflect such divergent roles of ethylene (Gallie 2015a). Detailed analyses of ethylene receptors in *Arabidopsis* indicate that some receptors also have specialized physiological functions in addition to a common and overlapping ethylene-signaling suppression activity (Liu et al. 2010; Liu and Wen 2012; Plett et al. 2009; Wilson et al. 2014). In addition, phylogenetic and structural analysis indicated that the composition and structure of ethylene receptors varies among land plants (Gallie 2015b). Land plants may thus have been coordinating ethylene-signaling pathways to adapt to environmental changes through the modification of ethylene receptors (Gallie 2015a). Legumes may also have adapted their ethylene-signaling pathways to control the newly acquired mechanism of symbiotic nodulation. Interestingly, the amino acid sequences of the N-terminal ethylene binding domain and GAF (cGMP-specfic phosphodiesterase, adenylyl cyclases and FhlA) domain are well conserved between LjETR1 and AtETR1 (identity: 90%, similarity 98%), whereas the similarity in the histidine kinase and receiver domain (by which ethylene receptors control the downstream signaling pathway) between these genes is rather low (identity: 71%, similarity 94%). It is intriguing to hypothesize that the modification of the histidine kinase and/or receiver domain enables LjETR1 to regulate the downstream symbiotic ethylene-signaling pathway more efficiently, although overexpression of the mutated *Arabidopsis etr1-1* or melon Cm-ERS1/H70A can also affect it. Future

functional analyses of other *Lotus* ethylene receptors and downstream symbiotic ethylene-signaling pathways can be expected to shed more light on this interpretation.

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