Title:

Evidence of the Functional Role of the Ethylene Receptor Genes *SlETR4* and *SlETR5* in Ethylene Signal Transduction in Tomato

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Abstract

Ethylene receptors are key factors for ethylene signal transduction. In tomato, six ethylene receptor genes (*SlETR1–SlETR6*) have been identified. Mutations in different ethylene receptor genes result in different phenotypes that are useful for elucidating the roles of each gene. In this study, we screened mutants of two ethylene receptor genes, *SLETR4* and *SLETR5,* from a Micro-Tom mutant library generated by TILLING. We identified two ethylene receptor mutants with altered phenotypes and named them *Sletr4-1* and *Sletr5-1*. *Sletr4-1* has a mutation between the transmembrane and GAF domains, while *Sletr5-1* has a mutation within the GAF domain. *Sletr4-1* showed increased hypocotyl and root lengths, compared to those of wild type plants, under ethylene exposure. Moreover, the fruit shelf life of this mutant was extended, titratable acidity was increased and total soluble solids was decreased, suggesting a reduced ethylene sensitivity. In contrast, in the absence of exogenous ethylene, the hypocotyl and root lengths of *Sletr5-1* were shorter than those of the wild type, and the fruit shelf life was shorter, suggesting that these mutants have increased ethylene sensitivity. Gene expression analysis showed that *SlNR* was up-regulated in the *Sletr5-1* mutant line, in contrast to the down-regulation observed in the *Sletr4-1* mutant line, while the down regulation of *SlCTR1*, *SlEIN2, SlEIL1, SlEIL3,* and *SlERF.E4* was observed in *Sletr4-1* mutant allele, suggesting that these two ethylene receptors have functional roles in ethylene signalling and demonstrating, for the first time, a function of the GAF domain of ethylene receptors. These results suggest that the *Sletr4-1* and *Sletr5-1* mutants are useful for elucidating the complex mechanisms of ethylene signalling through the analysis of ethylene receptors in tomato.

Keywords: ethylene receptor, gene expression, mutant, tomato

1. Introduction

Ethylene biosynthesis and signalling are modulated during the development of plant tissues and are responsible for inducing many biochemical processes, such as dormancy release, leaf abscission, stem and root elongation, root hair development, epinastic growth, flower senescence, pollination and wound response (Abeles et al. 1992). The ethylene biosynthesis pathway is regulated by both positive and negative feedback (Kende, 1993). Ripening fruits and senescing flowers exert positive feedback on the regulation of ethylene biosynthesis. Ethylene biosynthesis in higher plants has been well-characterized, and 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) have been recognized as the rate-limiting enzymes of ethylene biosynthesis (Yang and Haffman, 1984; Kende, 1993).

Tomato belongs to the group of climacteric fruits. It is mostly used as a plant model for studying fleshy fruit development, softening, ripening and metabolism (Brummell and Harpster, 2001; Carrari and Fernie 2006; Giovannoni 2004). The inhibition of either ethylene production or perception in climacteric fruits leads to improper ripening (Kevany et al. 2007). Therefore, ethylene plays an important role in the normal ripening process of climacteric fruits.

Ethylene receptors function as key factors in ethylene signal transduction. In tomato, at least six ethylene receptor genes (*LeETR1–6*) have been identified (Payton et al. 1996), but the separate roles of the ethylene receptor genes have not been well elucidated. Among the six ethylene receptors, *SlETR1* and *NR* have been extensively studied using the tomato mutant lines *Sletr1-1*, *Sletr1-2* and *Nr*. These studies showed that *SlETR1* and *NR* have essential functions in the tomato ripening process (Rick and Butler 1956; Okabe et al. 2011). However, no study has yet determined the functions of the other four ethylene receptor genes, *SlETR2, SlETR4, SlETR5* and *SlETR6*. Many studies have only shown data on their expression levels and patterns during tomato development. Alexander and Grierson (2002) stated that the expression of each tomato receptor varies temporally and spatially based on the developmental stage and external stimuli. *LeETR2* is expressed constitutively in all tissues throughout development; *LeETR4* is up-regulated during ripening, senescence, and abscission; and *LeETR5* is expressed in fruit and flowers and during pathogen infection (Tieman and Klee, 1999; Payton et al. 1996).

Ethylene receptor proteins can be structurally separated into three domains: the sensor domain, the kinase domain and the response regulator domain (Ciardi and Klee, 2001). The sensor domain is subdivided into an amino-terminal ethylene-binding subdomain and a GAF subdomain (Aravind and

Ponting 1997). The ethylene binding subdomain is an important region, as it acts as the ethylene binding site. Three established ethylene receptor mutants, *Nr, Sletr1-1* and *Sletr1-2,* have been used to clearly demonstrate the function of the ethylene-binding domain as being important for ethylene perception. Mutations in this domain inhibit the perception of ethylene, resulting in an ethylene-insensitive phenotype (Lanahan et al. 1994; Wilkinson et al. 1995; Okabe et al. 2011). The function of the ethylene receptor kinase domain is well known to act as a sensor for environmental signals. Evidence of its kinase activity has been demonstrated in tobacco (Zhang et al. 2004; Zhou et al. 2006) and with the *Arabidopsis ETR1* gene (Gamble et al, 1998). Another domain of ethylene receptor genes, the response regulator domain, stimulates downstream signalling events (Blecker and Pattersen, 1997; Wang et al. 2002). In many previous studies, mutant analysis was used to provide evidence of the functional role of each ethylene receptor gene and for each individual domain of these genes. Among those domains, only the functional role of the ethylene receptor GAF domain has not been clearly established (Klee and Tiemen, 2002).

This study characterized two ethylene receptor gene mutants, namely, *Sletr4-1,* which has a mutation in the region between the transmembrane and GAF domains, and *Sletr5-1,* which has a mutation in the GAF domain, to demonstrate the functional roles of *SlETR4* and *SlETR5*. By examining the effects of these mutations on plant phenotypes, it may be possible to identify the function of the region between the transmembrane and GAF domains in *SlETR4* and of the GAF domain in *SlETR5*.

Materials and methods

Screening of mutant alleles by TILLING

The TILLING method was used to screen for mutations in ethylene receptor genes in tomato M_2 EMS mutant lines. The screen was carried out as described by Okabe et al. (2011). Briefly, DNA samples were collected from 3,052 and 1,536 EMS-mutagenesis M_2 lines for the first screen and additional screening, respectively (Watanabe et al. 2007; Saito et al. 2011; Okabe et al. 2011). Therefore, a total of 4,588 populations were screened. A Maxwell 16 DNA Purification Kit (Promega, USA) was used to extract genomic DNA. DNA from eight lines was mixed in a single well of a 96-well plate to generate DNA superpools. PCR amplification was performed with a gene-specific primer system, using IRD700 and IRD800-labeled primers, and a universal primer system using unlabelled gene-specific primers attached to the T7 (CGCGTAATACGACTCACTATAG) or SP6 (CATACGATTTA

GGTGACACTATAG) sequence at the 5' end. Electrophoresis was carried out to confirm that PCR amplification was successful. Then, 3-7 µl of PCR products was mixed with sterilized water to a total volume of 10 µl and subjected to SlENDO1 digestion and TILLING screening using an LI-COR DNA analyser (LI-COR, USA) (Okabe et al., 2011).

Selection of homozygous TILLING mutants in bulked M_3 populations

The homozygous mutant lines *Sletr4-1* and *Sletr5-1* were selected from a bulked M_3 population. TILLING primers were used to distinguish homozygous mutant alleles from wild type. Then, 400–500 ng of PCR product was digested with SIENDO1, and the digested fragments were visualized by standard 1.5-2.0% agarose gel electrophoresis followed by SYBR Safe DNA gel staining (Invitrogen, USA) (Okabe et al., 2011). The homozygous M_3 plants were cultivated to obtain M_4 plants, which were then used for further characterization.

Ethylene triple response analysis

The ethylene triple response was examined in the homozygous mutant lines by the additional of exogenous ethylene at desired concentrations (0, 0.5, 1, 2.5, and 5 ppm). Seeds were sterilized for 20 minutes by soaking in 10% commercial bleach plus detergent (Kitchen Haiter, Kao, Tokyo Japan) and then rinsed with sterilized water three times for 5 minutes each (Okabe et al., 2011). Exogenous ethylene was injected to the sealed seeds as described by Mubarok et al. (2015).

Qualitative and quantitative plant morphological analysis

Wild type Micro-Tom (WT-MT) and the homozygous *Sletr4-1* and *Sletr5-1* mutant lines were germinated on wet paper in the dark at 25 °C for three days. Germinated seed were transplanted into rock wool, 5 cm x 5 cm x 5 cm in size (Toyobo, Osaka, Japan), and grown in a growth chamber under the following conditions: 25 °C, 55% relative humidity (RH), and supplemented with 15.000 lm m^{-2} with SON-T lamps (Philips, 400 Watt) for 16 hours daily. During plant growth several observations were made, including the phenotypic characteristics of the leaves, flowers and fruit, flowering time and time to breaker. Flowering time was the days from germination of seed to first flowering and time to breaker was the days from flowering to fruit at breaker (Br) stage.

Fruit shelf life analysis

The date of the Br stage was recorded to determine when fruit should be harvested. To analyse fruit shelf life, red fruits were harvested at the same maturation stage, Br+7 days, which was designated as 0 day post storage (DPS). All investigated fruits were stored under similar conditions, with a temperature of 22 ± 2 °C and 80% humidity on the laboratory bench. The fruit shelf life was determined by counting the number of days from the beginning of storage until approximately 10% of the fruit skin was wrinkled or black spots were observed (Mubarok et al., 2015).

Analysis of the fruit firmness, total soluble solids (TSS) and titratable acidity (TA).

Fruit firmness, TSS and TA were measured to evaluate the effect of the mutation in two ethylene receptor genes, *SlETR4* and *SlETR5*. The fruit firmness was measured using TA.XT Express Texture Analyser (Stable Micro Systems Ltd., UK). TSS was used to estimate the sugar level, and TA was used to estimate the organic acid level. Fruits at pink stage (P/Br+4) were used to analyse TSS and TA. TSS was measured using a refractometer PAL-J (Atago, Tokyo, Japan) and TA was measured by titration of 0.1 N sodium hydroxide up to a pH of 8.1 as described by Mubarok et al. (2015).

Genotyping of *Sletr4-1* and *Sletr5-1* mutant alleles

Homozygous and heterozygous *Sletr4-1* and *Sletr5-1* mutant alleles were distinguished from wild type alleles by cleaved amplified polymorphic sequence (CAPS) analysis. PCR amplification from each gene was performed with the following primers: *SlETR4*-CAP forward (5'-TTTATGCTG AAAAAGAAGACTTGGGATCCT-3') and *SlETR4*-CAP reverse (5'-CTGGATCACTTCTCGGGA TAGG-3'), yielding a 284-bp *SlETR4* PCR product, or *SlETR5*-CAP forward (5'-AGGAAGTCAC TTGATAAGCACAC-3') and *SlETR5*-CAP reverse (5'-TTGAAGTCCGAAGCACGAAGCAGTGG CAGC-3'), yielding a 326-bp *SlETR5* PCR product. To detect the *Sletr4-1* and *Sletr5-1* alleles, PCR products were digested with *Xsp*I (Takara, Japan), and *Pvu*II (Takara, Japan), respectively.

Segregation analysis

The inheritance patterns of *Sletr4-1* and *Sletr5-1* were investigated by crossing the mutant lines with WT-MT, and then the F_2 population was observed to determine the segregation ratio of mutant and wild type phenotypes. The F_2 population of mutant alleles was segregated based on specific

characteristics as an effect of ethylene response. The segregation ratio of each mutant line was scored using different ethylene response characteristics. *Sletr4-1* was scored based on the seedling sensitivity to 5 ppm of exogenous ethylene (sensitive vs. insensitive), while *Sletr5-1* was identified based on fruit size compared to the WT-MT (similar/big or small). The inheritance pattern was estimated based on the χ^2 value, at which the values were significant at the level of 5%.

Gene expression analysis of ethylene receptor gene

Gene expression analysis was performed using qRT-PCR to quantify the relative expression of six ethylene receptor genes: *SlETR1*, *SlETR2*, *SlETR3/NR*, *SlETR4*, *SlETR5,* and *SlETR6* This analysis was conducted as follows: first, RNA was extracted from leaves and fruits at different stages of fruit maturation: immature green (IMG/flowering+15 days), mature green (MG/flowering+30 days), breaker (Br), pink (P/Br+3 days), red (R/Br+10 days), and mature red (MR/Br+20 days). Total RNA was purified from up to 100 mg per sample using an RNeasy Mini kit (Qiagen) according to the supplier's instructions. Contamination from genomic DNA was removed using a RNase-Free DNase Set (QIAGEN), and the total RNA concentration was measured with a NanoDrop 2000C Spectrophotometer. Single strand cDNA was synthesized from 1-2 µg of total RNA using a SuperScript^{TM} II 1st strand cDNA Synthesis Kit (Takara, Japan). qRT-PCR for each target gene was performed on a Takara Thermal Cycler Dice Real-Time system using SYBR Premix Ex Taq II (Takara, Shiga, Japan) using the primer pairs (Supplementary Table 2Reactions were performed with the following conditions: pre-denaturation at 94 °C for 30 seconds followed by 40 cycles of denaturation at 95 °C for 5 seconds, primer annealing at 60 °C for 10 seconds, and extension at 72 °C for 15 seconds. The SAND gene was used as an internal control to normalize mRNA levels (Rodriguez et al. 2008).

Gene expression analysis of ethylene signalling genes

Gene expression analysis was performed using qRT-PCR to examine the expression levels of *SlCTR1, SlEIN2, SlEIL1, SlEIL2, SlEIL3, SlERF.B3, ERF.E1* and *ERF.E4*. Total RNAs from mature green (MG/flowering+30 days) and Pink/P (Br+4 days) were extracted by using ISOLATE II RNA Plant Kit (Bioline, BIO-52077). And then, total RNA amount was determined by NanoDrop 2000C Spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized from 1 µg RNA of total RNA by using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). qRT-

PCR was performed with Takara Thermal Cycler Dice Real-Time system using SYBR Premix Ex Taq II (Takara, Shiga, Japan) using the primers (Supplementary Table 2). At least three independent experiments were performed by using three biological replicates.

Results

Identification of novel ethylene receptor *Sletr4-1* and *Sletr5-1* mutant alleles

In a previous study, the TILLING method was performed to identify mutations in 10 genes involved in fruit ripening, softening and GABA metabolism (Okabe et al. 2011). In two rounds of screening, with a total of 4,588 EMS-mutagenesis lines, multiple alleles were found for each gene. Among them, two *SlETR4* mutant alleles and five *SlETR5* mutant alleles were identified. The mutations in each of these lines, including *Sletr4-1* and *Sletr5-1,* result in amino acid substitutions at a variety of positions within the *SlETR4* and *SlETR5* ethylene receptor genes (Supplementary Table 1). The *Sletr4-1* mutation results in the acid substitution G154S between the transmembrane domain and the GAF domain. The amino acid substitution in *Sletr5-1,* R278Q, is within the GAF domain (Figure 1 and Supplementary Fig. 1 - 2).

Two ethylene receptor mutant alleles, *Sletr4-1* and *Sletr5-1,* show altered ethylene triple responses.

Sletr4-1 and *Sletr5-1* mutant seedlings were exposed to a range of exogenous ethylene concentrations for 7 days. *Sletr1-1* and wild type seedlings were used as positive and negative controls, respectively. Figure 2 shows the phenotypic characteristics of the ethylene triple response as a response to the presence or absence of exogenous ethylene. In all treated seedlings, except for *Sletr1-1*, exogenous ethylene in the range of 0.5 - 5 ppm dramatically reduced hypocotyl and root elongation, but the extent of this reduction varied among the lines. Under ethylene-free conditions, significant reductions of hypocotyl and root length were observed in *Sletr5-1,* with values 1.59 and 0.5 cm lower than those in the WT-MT, respectively (Figure 2). On the other hand, when *Sletr5-1* mutants were treated with up to 5 ppm exogenous ethylene, hypocotyl and root elongation were inhibited to a comparable extent as in WT-MT seedlings. In *Sletr4-1* seedlings treated with 0.5 – 5 ppm of exogenous ethylene, the hypocotyl and root length were significantly longer than those in the WT-MT by 0.67 and 0.58 cm, respectively. Although *Sletr4-1* had longer hypocotyls and roots than did WT-MT seedlings, they were not as long as those in *Sletr1-1.* The hypocotyl and root length of *Sletr4-1* were 17.73 and 32% shorter than those of *Sletr1-1,* respectively (Figure 2).

Different plant characteristics were observed in the *Sletr5-1* mutant line.

Alterations in plant morphology were only observed in the *Sletr5-1* mutant line. Relative to WT-MT, *Sletr5-1* mutant plants and their leaves were narrower, their fruits were smaller, and fewer fruits were set, most *Sletr5-1* flowers wilted and dropped prematurely, so only a few flowers successfully set fruit (Figure 3 and 4). Statistical analysis showed that the time to flowering was delayed by 3 days in *Sletr4-1* compared to WT-MT, whereas *Sletr5-1* and WT-MT flowered at a comparable time. Significant reductions in fruit diameter, fruit weight and the fruit/flower ratio were observed in *Sletr5-1,* with values of 1.05 cm (6%), 0.46 g (18.5%), and 11.3 (60%) lower than WT-MT, respectively (Figure 5). Time of fruit to breaker can be used as an indicator for fruit ripening. This study revealed that mutation in *SlETR4* and *SlETR5* did not significantly effect on the time of breaker. The time of breaker of *Sletr4-1* and *Sletr5-1* mutant alleles was comparable with the WT-MT (Figure 5).

Mutation in the *Sletr4-1* allele affects the fruit TSS and TA

TSS and TA were analysed at pink stages of fruit maturation. Significant reduction of TSS value was only detected in the pink red fruit of *Sletr4-1* mutant alleles as an effect of *SlETR4* mutation. TSS value of *Sletr4-1* mutant was significantly lower compared with WT-MT with the value of 5.12 and 5.23 °Brix, respectively for *Sletr4-1* and WT-MT. On the other hand, the mutation in *Sletr5-1* mutant did not change the value of TSS that has a comparable value compared with WT-MT (Figure 6). Besides the TSS value, *Sletr4-1* mutation significantly effect on the increasing TA value with the value of 2.3%, but the effect of *Sletr4-1* and *Sletr5-1* mutation did not affect fruit firmness that has comparable with WT-MT (Figure 6.).

Mutations in the *SlETR4* and *SlETR5* genes altered fruit shelf life.

Fruit shelf life analysis was performed by counting the number of days of storage until symptoms of reduced quality were observed on the fruit skin, such as black spots or wrinkling of more than 10% of the total fruit skin area (Mubarok et al. 2015). Statistical analysis showed that, whereas the reduction in WT-MT fruit quality occurred at 20 DPS, it occurred 3 days earlier in *Sletr5-1* (Figure 6 and 8). On the other hand, the *Sletr4-1* mutant exhibited a slight improvement in fruit shelf life compared

with WT-MT. However, the effect of the *Sletr4-1* mutation was too minor to improve fruit shelf life, as it only improved fruit shelf life by 2 days compared to that of WT-MT (Figure 7).

Sletr4-1 and _Sletr5-1_ mutants exhibited recessive inheritance patterns

The inheritance patterns of characteristics of interest were observed in F_2 populations of the *Sletr4-1* and *Sletr5-1* mutant lines. The *Sletr4-1* F_2 population comprised 36 sensitive and 15 less sensitive seedlings ($\chi^2 = 0.53$), while the *Sletr5-1* F_2 population comprised 23 plants producing large fruit and 9 plants producing small fruit ($\chi^2 = 0.17$) (Table 1). Because the mutant to wild-type segregation ratios of the F_2 populations were approximately 1:3 for both *Sletr4-1* and *Sletr5-1*, we suggest that the *Sletr4-1* and *Sletr5-1* mutant phenotypes are monogenic recessive traits.

The Relative expression of ethylene receptor genes varied among the mutant lines and fruit maturation stages

The expression of six ethylene receptor mutants (*SlETR1 – SlETR6*) was investigated in the WT-MT, *Sletr4-1,* and *Sletr5-1* lines in leaves and at different fruit maturation stages. Our data showed that the relative expression of the ethylene receptor genes was similar among the investigated plants. The relative expression of *SlETR1*, *SlETR2* and *SlETR5* was stable during fruit maturation, and only *SlETR5* was up-regulated in leaves. High expression of *NR* and *SlETR4* was detected at the onset of ripening when fruit reached the breaker stage, whereas high expression of *SlETR6* was detected in MG (Figure 8). During fruit development, *NR* was the highest expressed, especially in Br fruit, with an relative expression 14 to 29-fold higher than that of IMG fruit. Based on statistical analysis, the relative expression of *NR* was down-regulated by 1.83-fold in Br-stage in *Sletr4-1* mutants and up-regulated by 2.81-fold in *Sletr5-1* relative to WT-MT relative expression (Figure 8). Similar to *NR*, the relative expression of *SlETR4* was down-regulated in *Sletr4-1,* while it was up-regulated in *Sletr5-1* mutant, although these differences from WT-MT were not statistically significant, except for *Sletr4-1* in R fruit. As for the other receptor genes, the relative expression of *SlETR5* was significantly reduced in *Sletr4-1* and *Sletr5-1* leaves by 2.08- and 2.89-fold, respectively (Figure 8). The relative expression of ethylene signalling gene, namely *constitutive triple-response 1* (*SlCTR1*), *Ethylene insensitive 2 (SlEIN2), EIN3*-like genes (*SlEIL1, SlEIL2,* and *SlEIL3*), *Ethylene response factors* (*SlERF.B3, SlERF.E1* and *SlERF.E4*) which are positive regulators of ethylene signalling, have been identified at two stages of fruit maturation

(Leclercq et al. 2002; Shimozaki et al. 2015; Klay et al. 2018). They showed a great change of expression of those genes compared with WT-MT as an effect of *SlETR4* and *SlETR5* gene mutations. In *Sletr4-1* mutant alleles, mutation significantly reduced the relative expression of *SlEIN2*, *SlEIL2*, *SlEIL3* and *SlERF.E4* that are detected in P fruit, whereas in Br fruit there has a reduction in the relative expression of *SlEIL1* and *SlERF.E4*. On the other hand, *Sletr5-1* mutation significantly increased the relative expression of *SlEIL2* and *SlERF.B3* at Br fruit (Figure 9). In addition, gene expression levels of CTR1 in *Sletr4-1* and *Sletr5-1* significantly decreased at Br fruit compared with WT-MT (Figure 9).

Discussion

The ethylene response has been widely studied in tomato plants, and the function of ethylene receptor genes has been determined by characterizing the phenotypes of several mutants, such as *Nr, Sletr1-1* and *Sletr1-2*. Okabe et al. (2011) showed that mutations in the first or second transmembrane domain of the *SlETR1* gene, in the *Sletr1-1* or *Sletr1-2* mutant lines, respectively, resulted in an insensitive or reduced response to ethylene. An ethylene-insensitive phenotype was also observed in *Nr* mutants. These results indicate that the *SlETR1* and *NR* genes have functions in the regulation of ethylene sensitivity. The functions of other ethylene receptor genes, such as *SlETR4* and *SlETR5*, have not yet been reported. Here, we demonstrated the functional roles of *SlETR4* and *SlETR5* by characterizing and identifying two ethylene receptor mutants, namely, *Sletr4-1* and *Sletr5-1*.

The preliminary observations of this study showed that mutations in *SlETR4* and *SlETR5* result in altered ethylene sensitivity. Changes in ethylene sensitivity were observed in the ethylene triple response and in fruit shelf life (Figure 2 and 7). These data showed that mutation in *SlETR4* slightly reduces ethylene sensitivity, thereby improving fruit shelf life, whereas mutation in *SlETR5* slightly increases ethylene sensitivity and thus reduces fruit shelf life (Figure 7).

Ethylene receptors are divided into three domains. Okabe et al. (2011) showed that *Sletr1-1* and *Sletr1-2* respectively possess amino acid substitutions P51L and V69D in the first and second transmembrane regions, resulting in strong and moderate ethylene-insensitive phenotypes (Okabe el al. 2011). The P51L substitution of *Sletr1-1* corresponds to the amino acid substitution P36L in *Nr* and *Arabidopsis etr2-1* (Sakai et al. 1998). Based on those results, the transmembrane region is important for ethylene binding, whereas the functions of the other ethylene receptor domains, such as the GAF domain,

have not yet been clearly determined. The amino acid substitution of *Sletr4-1,* G154S, is between the transmembrane and GAF domains, while the amino acid substitution of *Sletr5-1,* R278Q, is within the GAF domain. Thus, these two mutants are useful materials for elucidating the complex mechanisms of ethylene signalling and the ethylene receptors in tomato, especially for the GAF domain.

The ethylene triple response can be used as an indicator to characterize ethylene sensitivity. Our study showed that exogenous ethylene in the range of 0.5 - 5 ppm dramatically reduced hypocotyl and root elongation, though the effect varied between the two mutant lines. Compared to WT-MT, *Sletr4-1* seedlings had increased hypocotyl and root elongation under ethylene exposure. In contrast, *Sletr5-1* exhibited reduced hypocotyl and root length in the absence of exogenous ethylene (Figure 2). Many studies have argued that the primary characteristics of the ethylene triple response are inhibition of hypocotyl elongation, expansion of the hypocotyl base and inhibition of primary root elongation in response to ethylene exposure (Crocker et al. 1913; Guzman and Ecker, 1990).

In contrast with *Sletr4-1* and *Sletr5-1, Sletr1-1* exhibited no reduction in root or hypocotyl elongation. Okabe et al. (2011) showed that *Sletr1-1* is not responsive to exogenous ethylene up to 10 ppm. In *Sletr4-1*, although 5 ppm of exogenous ethylene significantly increased the hypocotyl and root length, both lengths were significantly lower than in *Sletr1-1* (Figure 2). This slight increase in hypocotyl and root length in *Sletr4-1* seedlings exposed to ethylene indicated that the *Sletr4-1* has slightly reduced ethylene sensitivity, despite its response being weaker than that of *Sletr1-1*. Compared to WT-MT, *Sletr5-1* had shorter hypocotyls and roots, as well as reduced fruit shelf life, suggesting an increased ethylene sensitivity in this mutant line.

Ethylene controls many growth and development processes, such as responses to biotic and abiotic stress, germination, flower development, ripening and senescence. Mutations in the *SlETR4* or *SlETR5* ethylene receptor gene did not change the appearance of the whole plant and also fruit (Figure 3, 4 and 7). In addition to their qualitative characteristics, we also characterized the quantitative characteristics of these mutants. Among the investigated ethylene receptor mutants, all showed different fruit characteristics. The *Sletr4-1* mutant exhibited a delay in flowering time and time to breaker, whereas *Sletr5-1* showed reduced fruit diameter, fruit weight and fruit/flower ratio (Figure 5). We hypothesize that the reduction in the fruit/flower ratio in *Sletr5-1*, of up to 60%, is due to increased ethylene sensitivity in this mutant, which affects flower and fruit development. By visual investigation during flower development, most *Sletr5-1* mutant flowers grew abnormally and underwent premature wilting and

dropping; therefore, fruit did not set completely (Figure 4). Several studies have shown that ethylene induces flower senescence or abscission, resulting in early flower wilting (Jones et al. 2001; Evensen, 1991; Cameron and Reid, 2001). A similar premature flower senescence phenotype has been observed in the *Nr*, *LeETR4* and *LeETR6* antisense lines (Kevany et al. 2007; Tieman et al. 2000). The mutant phenotypes of *Sletr4-1* (seedling response) and *Sletr5-1* (fruit size) were inherited by progeny as monogenic recessive traits, as observed in F_2 populations (Table 1).

The change of TSS and TA occurs during fruit ripening. This study revealed the mutation in *SlETR4* of *Sletr4-1* mutant allele significantly reduced the value of TSS and TA. However, the mutation in *Sletr5-1* mutant allele did not change the value of TSS and TA. During ripening process, there has change of sugar, organic acid and other compounds related to fruit flavour. The change of TSS and TA mostly used as an indicator to estimate sugar and organic acids, respectively that associated with fruit sweetness and sourness, respectively (Defilippi et al. 2004). The highest TSS value and lowest TA value were observed in red tomato fruit. TSS increases during fruit maturation due to the conversion of starch into sugar and also the hydrolysis process of polysaccharides (hemicellulose and pectin) in cell wall that induced by ethylene (Crouch, 2003; Baldwin and Biggs, 1988). Mutation in *Sletr4-1* mutant allele significantly reduced the ethylene sensitivity that effects on the reduction of TSS value and increasing the TA value (Figure 6). Reduction in TSS content during fruit ripening also observed in *Nr* and *nor* mutants (Hobson, 1980; Rodríguez et al. 2010). Contrasting study was observed in hybrid lines of *Sletr1-2* mutant alleles that has comparable value of TSS compared with WT-MT F1 (Mubarok et al., 2015). During ripening process, the increase of sugar content corresponds with the reduction of TA (Winsor et al., 1962). Similar study was shown in this study that showed during the ripening process, TSS was increasing and TA was decreased (Figure 6). Decrease in TA content is caused by the degradation of organic acids due to effect of ethylene and respiration process in tomatoes (Defilippi et al., 2004). Mutation in *Sletr4-1* mutant allele resulted the increase of TA content in P stage, but did not change its value on MG and P stages. The change of TA might be due to the decrease of ethylene sensitivity that effect on the inhibition of organic acids degradation.

Ethylene regulates several aspects of plant growth and development, such as fruit development and ripening (Abeles et al. 1992). The presence of ethylene accelerates fruit ripening and reduces fruit shelf life. Several studies have successfully isolated and characterized ripening mutants with mutations in ethylene receptor genes, such as *never ripe* (*Nr*)*, Sletr1-1*, and *Sletr1-2* (Lanahan et al. 1994; Wilkinson

et al. 1995; Okabe et al. 2011). These mutants show reduced ethylene sensitivity. A reduction in ethylene sensitivity was also observed in the *Sletr4-1* mutant line, which resulted in a fruit shelf life up to 2 days longer than that of the wild type (Figure 8). For fresh market purposes, extending fruit shelf life by 2 days is only beneficial for nearby markets. Prolonged fruit shelf life is important for long-distance transportation to markets, fruit storage, and handling (Mubarok et al. 2015; Mubarok et al. 2016). In contrast with the *Sletr4-1* mutant line, *Sletr5-1* exhibited an accelerated fruit ripening process. Under normal postharvest storage conditions (22°C) and without exogenous ethylene, *Sletr5-1* fruits decayed faster than wild type fruit, leading to a shelf life 3 to 4 days shorter than that of wild type (Figure 7). This acceleration of the ripening process in *Sletr5-1* mutants is similar to the effect of ethylene, in which treatment with exogenous ethylene accelerates the ripening process.

The ethylene sensitivity of the mutant lines, which was observed as alterations in the ripening process, was correlated with the expression of ethylene receptor genes. Gene expression was investigated during fruit maturation (IMG, MG, Br, P, R and MR) and in leaves. The results of the present study showed that *SlETR1*, *SlETR2* and *SlETR5* are expressed in leaves and consistently throughout fruit maturation, while *SlETR5* was up-regulated in leaves (Figure 8). Our results are consistent with those of Lashbrook et al. (1998), who showed that *LeETR1* and *LeETR2* are expressed at a consistent level in all tissues throughout development. They also demonstrated that *NR* expression is up-regulated at the breaker stage. That result supports our finding that the *NR* gene was up-regulated at the breaker stage, though its expression level varied among the mutant lines. A reduced level of *NR* expression was observed in the *Sletr4-1* mutant, indicating that the *Sletr4-1* mutation delayed ripening. Meanwhile, an increased level of *NR* was observed in *Sletr5-1* at the onset of ripening (Br fruits), indicating that the *Sletr5-1* mutation accelerated the ripening process. As a result, fruit shelf life was longer in *Sletr4-1* mutants and shorter in *Sletr5-1* (Figure 8). Based on this result, we have confirmed that *NR* is important for the ripening process.

According to the gene expression data, the expression of *SlETR4* in the *Sletr4-1* background was higher than in WT-MT, indicating that the stronger response of *Sletr5-1* to ethylene explains its early ripening phenotype, although this mutant phenotype was observed under ethylene-free conditions In contrast, Kevany et al. (2007) found that a reduction in the expression of *LeETR4* and *LeETR6* caused an early-ripening phenotype in both *LeETR4* and *LeETR6* antisense lines. In the current study, the high expression of *SlETR4* and *SlETR6* explains the early ripening phenotype of *Sletr5-1*. In the same study

mentioned above, the authors also observed an increase in the expression levels of *SlETR4* and *SlETR6* after ethylene treatment (Kevany et al. 2007). In conclusion, a mutation in the GAF domain increased ethylene sensitivity in the *Sletr5-1* mutant line, resulting in early ripening and reduced hypocotyl and root length under ethylene-free conditions. On the other hand, a mutation between the transmembrane and GAF domains of *SlETR4* (*Sletr4-1* mutant allele) led to reduced ethylene sensitivity, with delayed ripening and slight increases in hypocotyl and root lengths in the presence of ethylene. Moreover, the expression of *NR* was down-regulated in *Sletr4-1* and up-regulated in *Sletr5-1* at the breaker stage, marking the onset of the ripening process. Gene expression analysis showed that *NR* is up-regulated in *Sletr5-1* but down-regulated in *Sletr4-1*, suggesting a functional role of three ethylene receptors in ethylene signalling and, for the first time, demonstrating a function for the GAF domain of ethylene receptors.

The expression of ethylene signalling genes have been observed to check the effect of *Sletr4-1* and *Sletr5-1* mutations. Mutation occurred in the region between transmembrane domain and GAF domain of *SlETR4* resulted in the reduction of ethylene sensitivity of *Sletr4-1* mutant corresponds with the reduction of relative expression of ethylene signalling genes such as *SlCTR1*, *SlEIN2, SlEIL1, SlEIL3,* and *SlERF.E4* (Figure 9). Down regulation of those genes in *Sletr4-1* mutant allele resulted in the reduction of ethylene sensitivity such as increase of root and shoot length under ethylene treatment and increased the fruit shelf life (Figure 2 and 7). However, in *Sletr5-1* that improve ethylene sensitivity only *SlEIL2* and *SlERF.B3* that increase the expression of these genes (Figure 9). It has been well established that ethylene signalling gene; *SlCTR1* acts in down-stream of ethylene receptors, while *SlEIN2, EIN3*-like genes, and *SlERF* gene family act as a positive regulators of ethylene signalling (Kieber et al., 1993). Yang et al., (2013) stated that the reduction of expression of *CTR1, EIN2A, EIL4* and *ERF*s genes results in the reduction of ethylene sensitivity by improving fruit shelf life of apple. The similar study reported by Alonso et al. (1999) and Tieman et al. (2001) that the loss function of *EIN2* due to mutations resulted in an insensitive ethylene phenotype and the down-regulation of *EIL*s expression results in the reduction of ethylene sensitivity.

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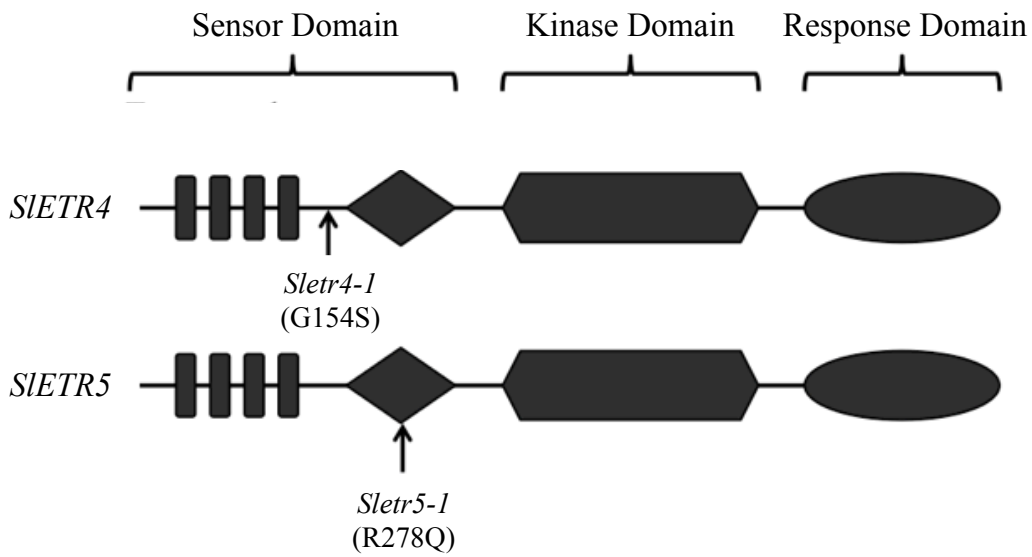
550 **Figure Captions**



551

552 Figure 1. Location of two ethylene receptor mutations. The *Sletr4-1* mutant allele results in the amino
553 acid substitution G154S, between the transmembrane and GAF domains of the *SlETR4* gene. The *Sletr5-*
554 *1* amino acid substitution, R278Q, is within the GAF domain of *SlETR5*.

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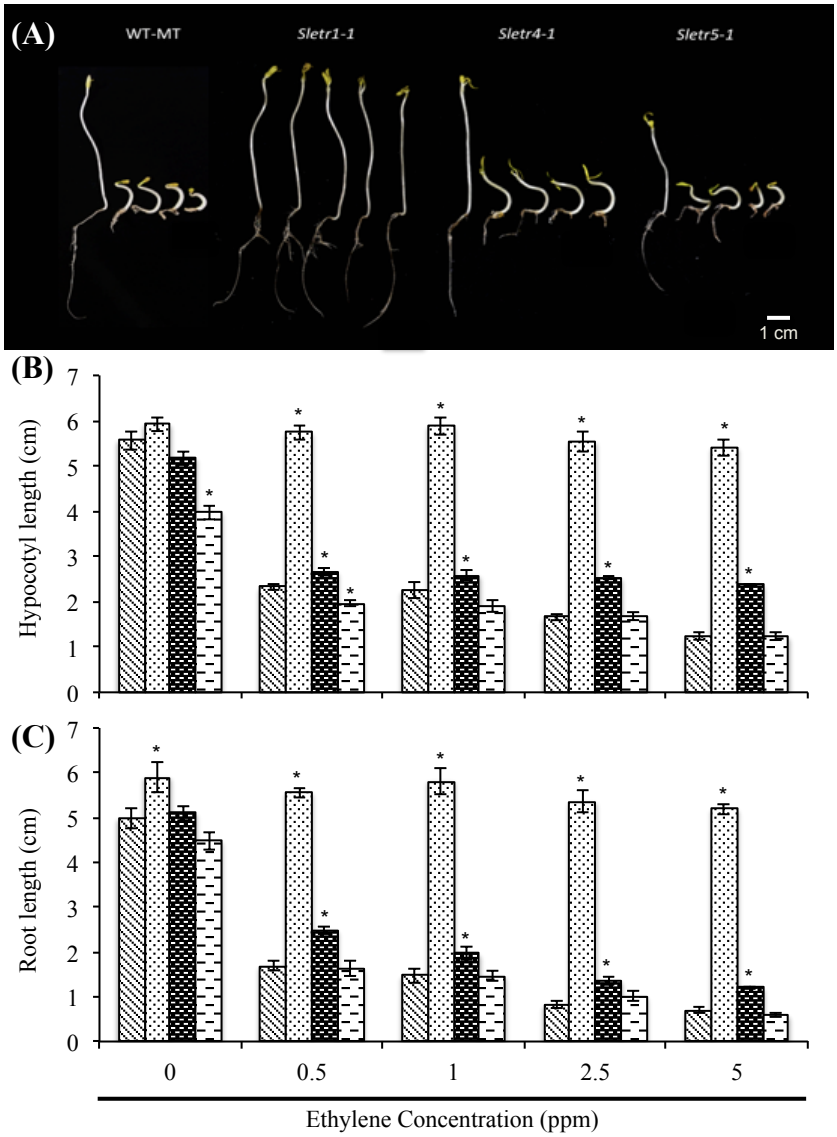


Figure 2. Ethylene triple response of ethylene receptor mutants. Seedlings were incubated with 0–5 ppm of exogenous ethylene for 7 days. A) Images of seedlings in response to exogenous ethylene exposure of 0–5 ppm. B) and C) Quantitative analysis of hypocotyl length and root lengths of two ethylene receptor mutants, respectively, with *Sletr1-1* and WT-MT as positive and negative controls. Values represent the mean ± SE (n=8) followed by asterisk indicate values significantly different from the control (WT-MT) at $p<0.05$, according to Student's t-test.

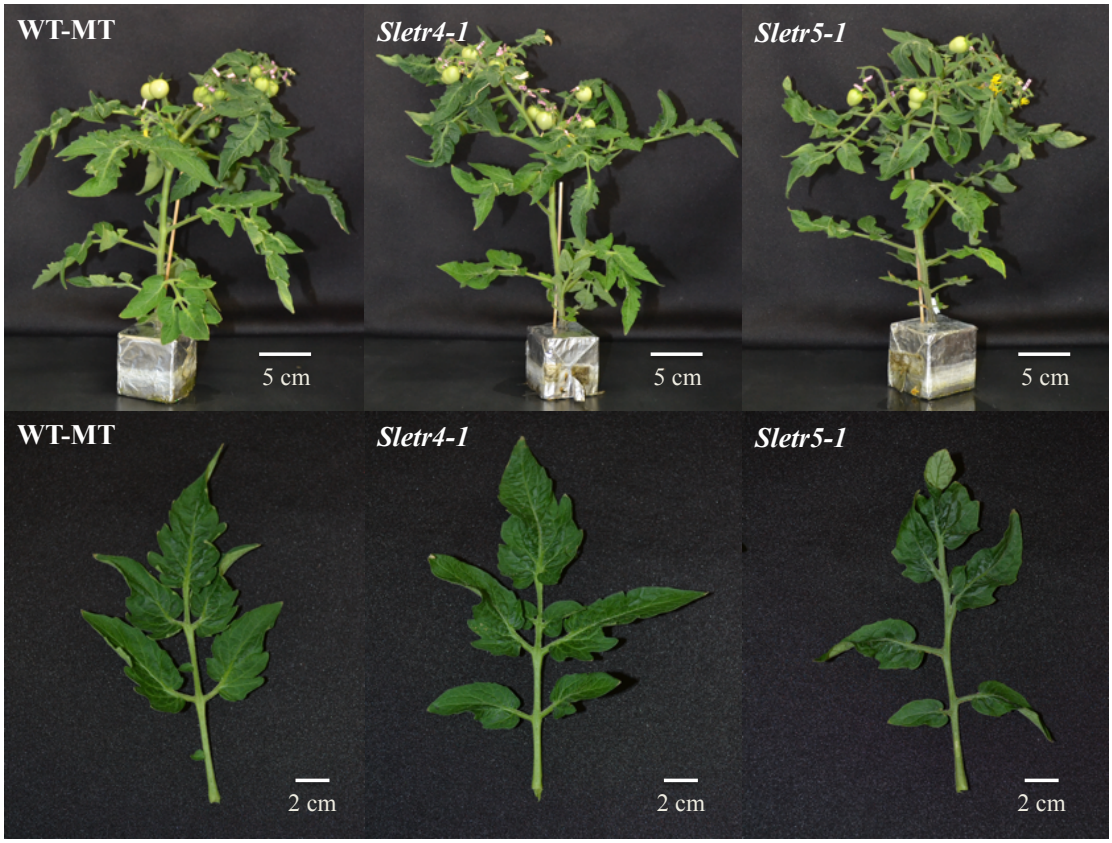


Figure 3. The phenotypes of two ethylene receptor mutant alleles, *Sletr4-1* and *Sletr5-1*. Representative images showing the appearance (upper) and leaves (lower) of the two ethylene receptor mutants. The plant and leaves were taken at 60 days after sowing.

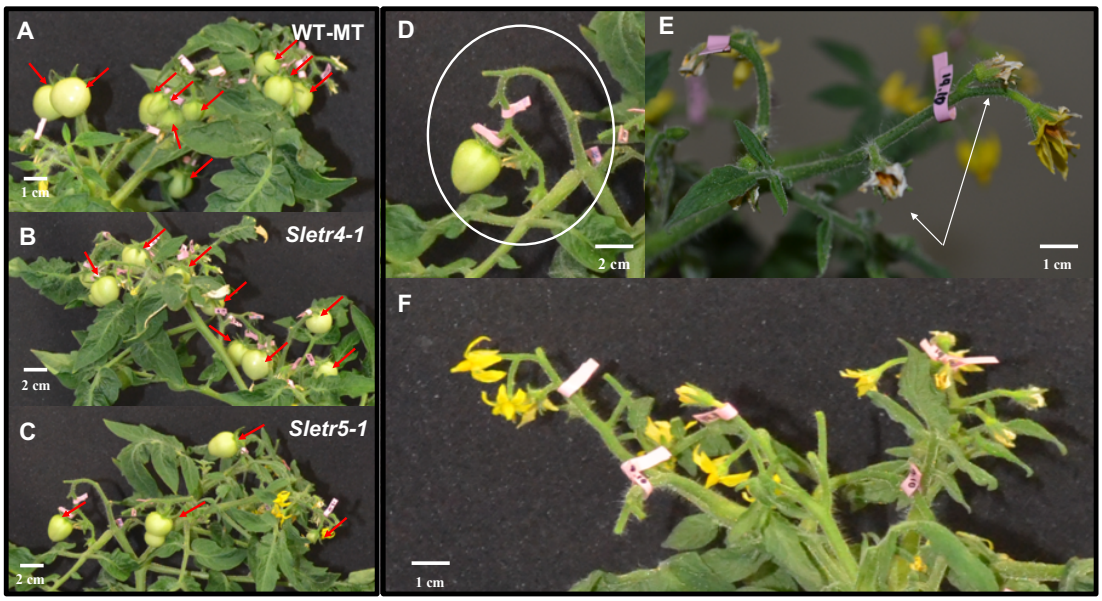


Figure 4. Representative images showing the appearance of fruit for A) WT-MT, B) *Sletr4-1* and C) *Sletr5-1* at 60 days after sowing, arrow indicates the number of formed fruit that shows the number of fruit of WT-MT is much more than *Sletr5-1* alleles and fewer fruits were set in *Sletr5-1*. D) the number of formed fruit from a stalk in *Sletr5-1* at 60 days after sowing due to the failure of fertilization. E) and F) most *Sletr5-1* flowers wilted and dropped prematurely, arrows indicate the wilted flower prematurely, so only a few flowers successfully set fruit, the pictures were taken at 50 days after sowing.

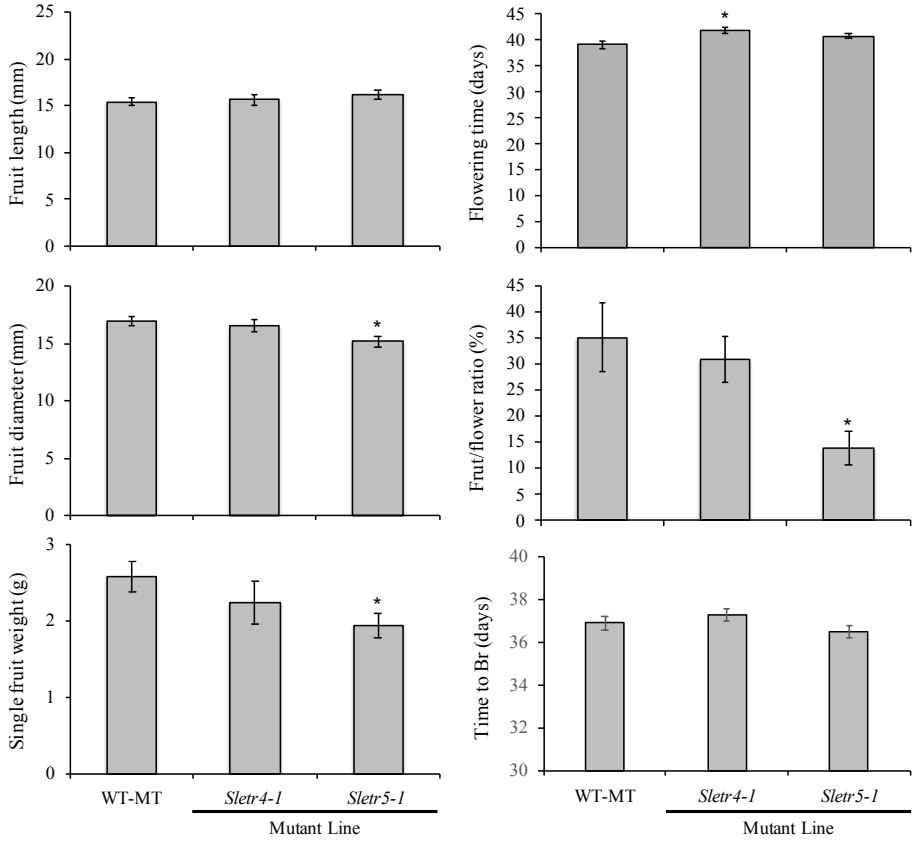
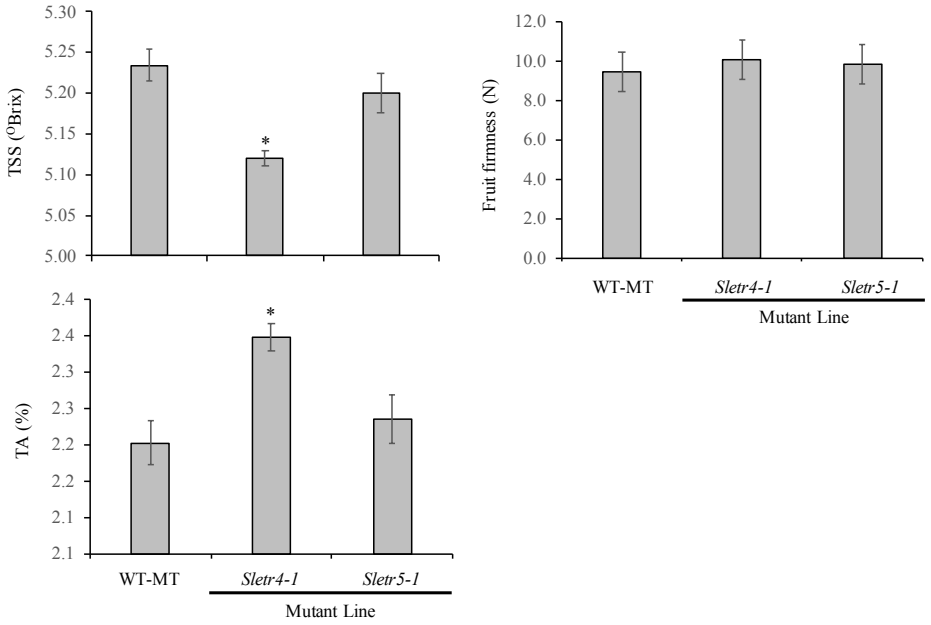


Figure 5. Fruit characteristics of two ethylene receptor mutant lines, *Sletr4-1,* and *Sletr5-1*. Values represent the mean ± SE (n=15), and asterisks indicate values significantly different from the control (WT-MT) at $p<0.05$, according to Student's t-test.



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587 Figure 6. The effect of *Sletr4-1* and *Sletr5-1* mutation on the change of TSS and TA during fruit
588 maturation. Values represent the mean ± SE (n=4 for TSS and TA, n=12 for Fruit firmness), and asterisks
589 indicate values significantly different from the control (WT-MT) at $p<0.05$, according to Student's t-test.

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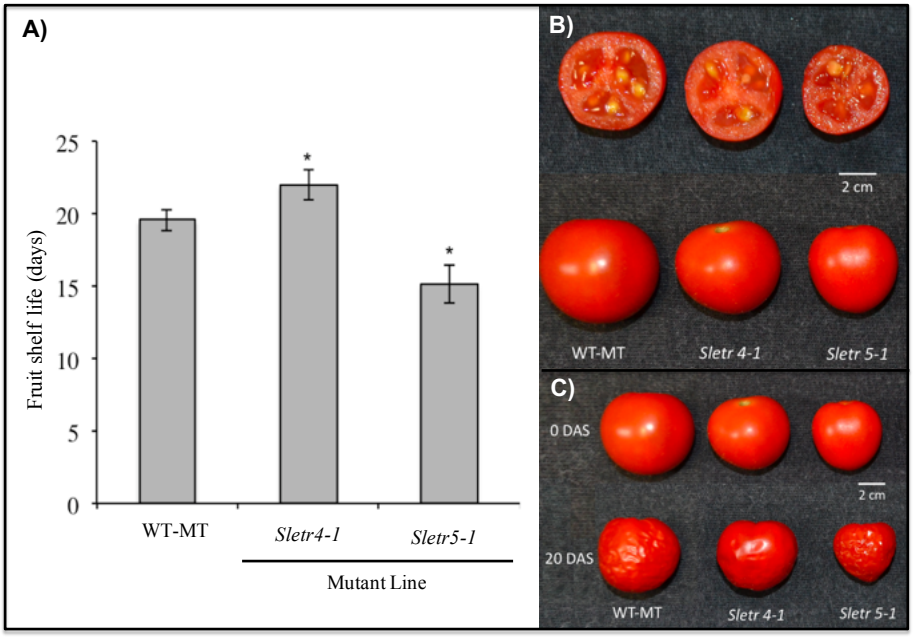
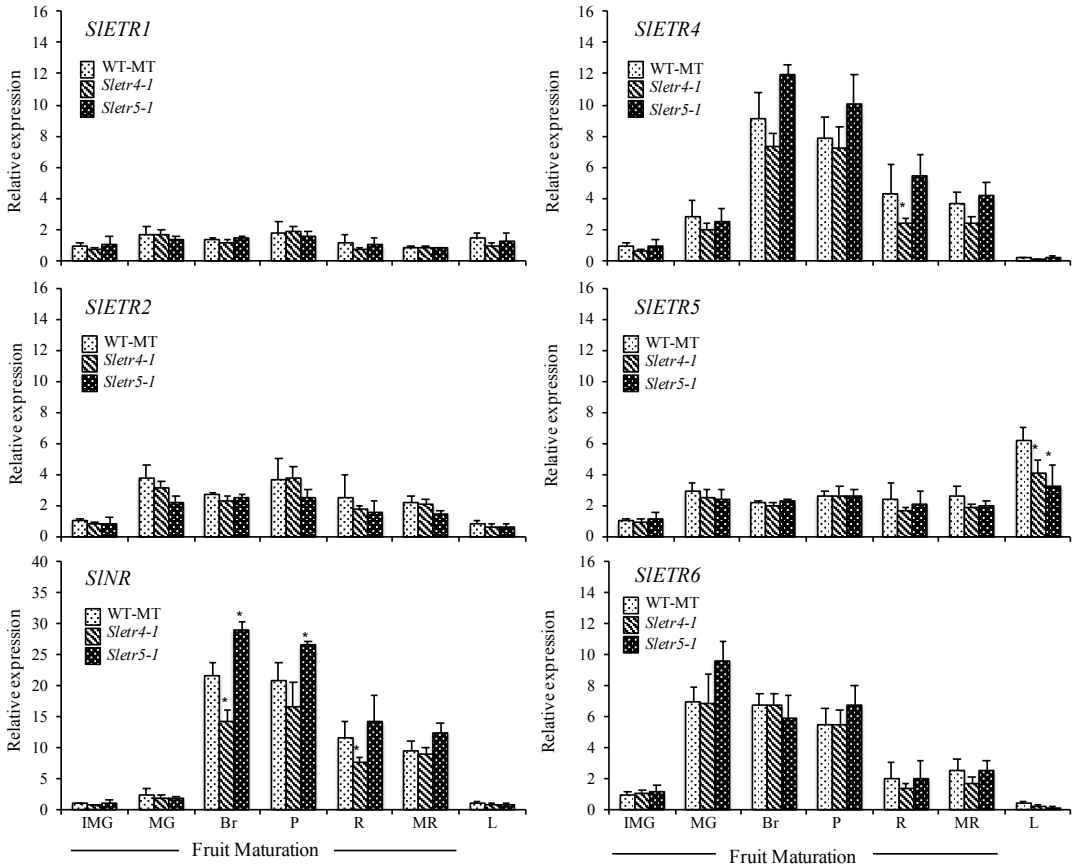


Figure 7. A) Fruit shelf life of two ethylene receptor mutant lines. Values represent the mean ± SE (n=24), and asterisks indicate values significantly different from the control (WT-MT) at $p<0.05$, according to Student's t-test. Representative images showing the appearance of B) fruit the two ethylene receptor mutants C) the fruit shelf life for 20 days of postharvest storage under normal room condition.



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602 Figure 8. Relative expression of ethylene receptor genes (*SlETR1, SlETR2, NR, SlETR4, SlETR5,* and
603 *SlETR6*) at different fruit maturation stages and in the leaves of two ethylene receptor mutant lines
604 (*Sletr4-1*, and *Sletr5-1*) with wild type as a control. Fruits were harvested at 6 stages of fruit maturation:
605 immature green (IMG), mature green (MG), Breaker (Br), pink (P), red (R), and mature red (MR). Data
606 are presented as the mean ± SE (n=3), and asterisks indicate values significantly different from the
607 control (WT-MT) at $p<0.05$, according to Student's t-test.

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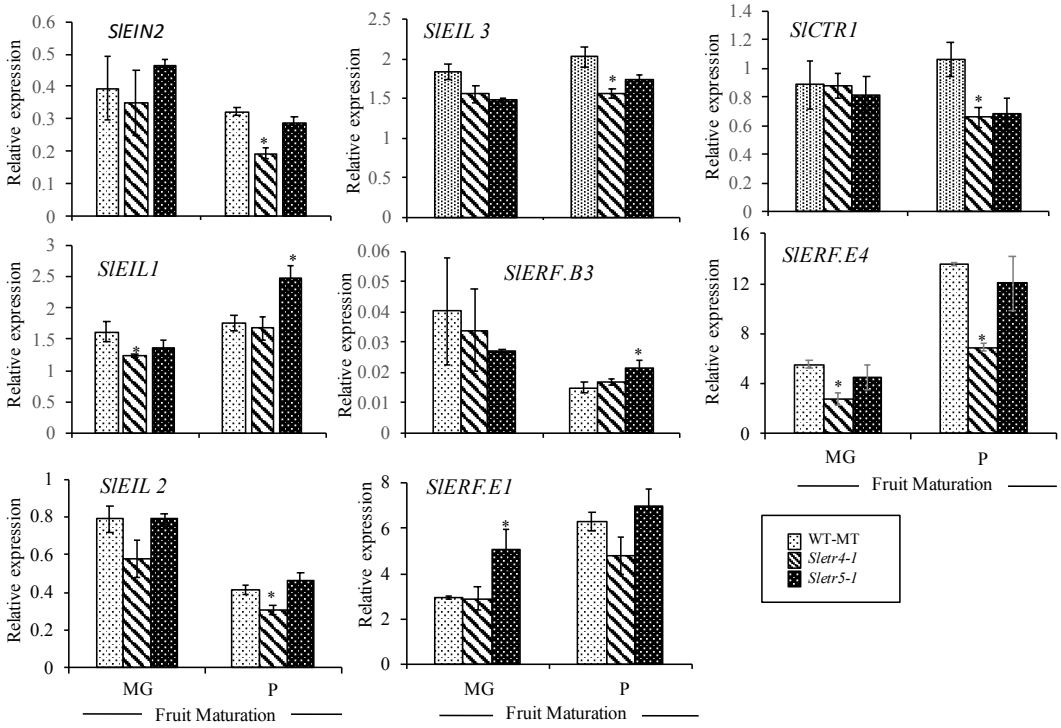

Figure 9. Relative expression of ethylene receptor genes (*SlCTR1, SlEIN2, SlEIL1, SlEIL2, SlEIL3, SlERF.B3, SlERF.E1* and *SlERF.E4*) at two different fruit maturation stages (MG and P) of two ethylene receptor mutant lines (*Sletr4-1*, and *Sletr5-1*) with WT-MT as a control. Data are presented as the mean ± SE (n=3), and asterisks indicate values significantly different from the control at $p<0.05$, according to Student's t-test.

619 Table 1. Inheritance pattern of the two ethylene receptor mutant alleles

Populations[1]	F2[2] Segregations (mutant : WT-MT)	χ^2 value[3]	Inheritance pattern[4]
Sletr4-1 x WT-MT	15 : 36	0.53	Monogenic recessive
Sletr5-1 x WT-MT	9 : 23	0.17	Monogenic recessive

620 [1] *Sletr4-1* and *Sletr5-1* mutant alleles were crossed with WT-MT
621 [2] The number of progeny exhibiting the indicated phenotype in the F2 population. *Sletr4-1* (WT-MT:
622 ethylene sensitive, mutant: ethylene insensitive), and *Sletr5-1* (WT-MT: large fruit, mutant: small fruit).
623 [3] χ^2 values were calculated for the F2 populations.
624 [4] Inheritance patterns were estimated based on the χ^2 value. The values were significant at the level of
625 5%.
626
627

MLRTLASALLVLSFFVSLSAADNGFPRCNCDDEGFWSIESILECQKISDLFIAIAYFSIPIELLYFVSC

SNFPFKWVLFQFIAFIVLCGMTHLLNFWTYYGQHPFQLMLALTIFKVLTALVSFATAITLITLFPMLLK

VKVREFMLKKKTWDL**G**REVGLIKMQKEAGWHVRMLTQEIRKSLDRHTILYTTLVELSKTLDLHNCAVWK

PNENKTEMNLIHELRDSSFNSAYNLPIPRSDPDVIQVKESDGVKILDADSPLAVASSGGSREPGAVAAI

RMPMLKVSNFKGGTPELVPECYAILVLVLPSEQGRSWCSQEIEIVRVVADQVAVALSHAAILEESQHMR

ETLEEQNRALEQAKQDALRASQARNAFQMVMSHGLRRPMHSILGLLSLLQDEKLGNEQRLLVDSMVKTS

NVVSTLIDDVMDTSTKDNGRFPLEMRYFQLHSMIKEAACLAKCLCAYRGYNISIEVDKSLPNHVLGDER

RVFQVILHMVGNLLKDPNGGLLTFRVLPESVSREGIGGAWRTRRSNSSRDNAYIRFEVGTSNNHSQPEG

TMLPHYRPKRCSKEMDEGLSFTVCRKLVQLMQGDIWVIPNPEGFDQSMAVVLGLQLRPSIAIGIPEYGE

SSDHSHPHSLLQGVKVLLADYDDVNRAVTSKLLEKLGCSVSAVSSGRDCIGVLSPAVSSFQIVLLDLHL

PDLDGFEVTMRIRKFGSHNWPLIVGLTATADENVTGRCLQIGMNGLIRKPVLLPGIADELQRVLLRGSR

MM

S (*Sletr4-1*)

Supplementary Figure. 1. The amino acid sequence of the tomato ethylene receptor *SlETR4*. Solid and dotted horizontal lines indicate the transmembrane sub-domain and the GAF sub-domain, respectively.

MLAMLRLLFLVLLISLVIISVSANDGEFFNCCDEDGFWSIHTILDCQKVSDFFIAVAYFSIPLELLYFI

SRSNLPFKWVLVQFIAFIVLCGLTHLLNGWTYNPHPSFQLILSLTVAKILTALVSCATAITLLTLIPLL

LKIKVRELFLAQNVLELDQEVGMMKKQTEASMHVRMLTHEIRKSLDKHTILYTTLVELSKTLKLQNCAV

WMPNESRSQMNLTHELSPSSAAESHRSLSINDPDVLEITKNKGVRILRQDSVLAASSSGGSGEPCAVAA

IRMPLLRASDFKGGTPELVDTRYAILVLVLSSVDERVWSYDEMEIVEVVADQVAVALSHATVLEESQTM

REKLEMRNRVLQQAQENAMKASQARTSFQKVMNNGMRRPMHSILGLLSIFQDEKASSDQRMIVDTMVKT

STVLSTLINDAMEISAKDDGRFPVEMKPFQLHLLVREASCLVKCLCVYKGFGFSTDVPTSLPNQVMGDE

KRTFQVLLHMVGHLLNVSIGKGSVIFRVVLETGAETGNDKVWGTRRPSTTDEYVTIKFEIEVSLEGSQS

DSSISTIHFGGRRHNSKEVTEGLSFNMCKKLVQMMQGNIWMSSNAQGHAQGMTLILRFQKQSSFRKRMF

EYRNPLEQPISSTMFRGLHVLLTDDDDVNRLVTRKLLEKLGCQVTAVSTGFQCLSALGPSLTTFQVLIL

DLQMPEMDGYEVALRVRKFRSRSWPLIIALTASSEEQVWEKCLQVGMNGLIRKPVLLQGLADELQRLLQ

RGGGGDGL

Q (*Sletr5-1*)

Supplementary Figure. 2. The amino acid sequence of the tomato ethylene receptor *SlETR5*. Solid and dotted horizontal lines indicate the transmembrane sub-domain and the GAF sub-domain, respectively.

662 Supplementary Table 1. Identified mutations in the *SlETR4* and *SlETR5* genes

Gene	Sample ID	Nucleotide Change	Effects
SlETR4	1	G →A	G154S
	2	G →A	V261I
SlETR5	1	C →T	Q368stop
	2	G →A	G267R
	3	G →A	R278Q
	4	G →A	E320=

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665 Supplementary Table 2. List of primers used for qRT-PCR

Gene Name	Primer Name	Sequence	Sources
SlCTR1	*CTR1_Fw*	CATCCTCTTTCTTACTGTGAGAAAATTTAGA	Leclercq et al., 2002
	CTR1_Rv	CATTTCCCTGTATAAAAACGTTCAGTT	
SlETR1	*SlETR1_Fw*	TTTTTGGCCACGATGGGAT C	In this paper
	SlETR1_Rv	ACTGTGGGTCAATGATGCAG	
SlETR2	*SlETR2_Fw*	CGTCGCG TATCTCTTTTTCCG	In this paper
	SlETR2_Rv	GCAACAGTGGATCGAAGCAG	
SlNR	*SlNR_Fw*	CGGAACATTCAATCTTCATGGC	In this paper
	SlNR_Rv	ACGTTTTGCATCACCC ACAG	
SlETR4	*SlETR4_Fw*	TGTGTGCAGAAAGCTGGTTC	In this paper
	SlETR4_Rv	ATT GATGGCCGCAGTTGAAG	
SlETR5	*SlETR5_Fw*	TCACTTTGGTGGAAGAAGGC	In this paper
	SlETR5_Rv	TGGGCATTCGAGGACATCC	
SlETR6	*SlETR6_Fw*	TGCTCCTCCAACATACGACA	In this paper
	SlETR6_Rv	ACAATCACAGCCATGCCTTG	
SlEIN2	*SlEIN2_Fw*	ATGACAGGGATGATGGAGATTCG	Gao, et al., 2016
	SlEIN2_Rv	TATGACCCCGGACCATCAGA	
SlEIL1	*SlEIL1-Fw*	AGGCTCCAACGACAACTTCC	Shinozaki et al., 2015
	SlEIL1-Rv	ATCCAATGCTAGGTAGATTTCCG	
SlEIL2	*SlEIL2-Fw*	CGGCTGATGACTTGACTTTCC	Shinozaki et al., 2015
	SlEIL2-Rv	AAGACAACTGGCTTGACCTCCT	
SlEIL3	*SlEIL3-Fw*	AGCCTGCCTCAGCAACAAA	Shinozaki et al., 2015
	SlEIL3-Rv	TGAACGGGGAACCGAATC	
SlERF.B3	*Sl-ERF.B3_Fw*	CGGAGATAAGAGATCCAAGTCGAA	Klay, et al. 2018
	Sl-ERF.B3_Rv	CTTAAACGCTGCACAATCATAAGC	
SlERF.E1	*Sl-ERF.E1_Fw*	GTTCCTCTCAACCCCAAACG	Klay, et al. 2018
	Sl-ERF.E1_Rv	TTCATCTGCTCACCACCTGTAGA	
SlERF.E4	*Sl-ERF.E4_Fw*	AGGCCAAGGAAGAACAAGTACAGA	Klay, et al. 2018
	Sl-ERF.E4_Rv	CCAAGCCAAACGCGTACAC	
EXPRESSED	*EXPRESSED_Fw*	GCTAAGAACGCTGGACCTAATG	Choi et al, 2018
	EXPRESSED_Rv	TGGGTGTGCCTTTCTGAATG	

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