Differential Control of Ethylene Responses by GREEN-RIPE and GREEN-RIPE LIKE1 Provides Evidence for Distinct Ethylene Signaling Modules in Tomato1[W][OA]

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The factors that mediate specific responses to the plant hormone ethylene are not fully defined. In particular, it is not known how signaling at the receptor complex can control distinct subsets of ethylene responses. Mutations at the Green-ripe (Gr) and reversion to ethylene sensitivity1 (ret1) loci, which encode homologous proteins of unknown function, influence ethylene responses in tomato (Solanum lycopersicum) and Arabidopsis (Arabidopsis thaliana), respectively. In Arabidopsis, AtRTE1 is required for function of the ETR1 ethylene receptor and acts predominantly through this receptor via direct protein-protein interaction. While most eudicot families including the Brassicaceae possess a single gene that is closely related to AIRTE1, we report that members of the Solanaceae family contain two phylogenetically distinct genes defined by GR and GREEN-RIPE LIKE1 (GRL1), creating the possibility of subfunctionalization. We also show that SGR and SGR1 are differentially expressed in tomato tissues and encode proteins predominantly localized to the Golgi. A combination of overexpression in tomato and complementation of the ret1-3 mutant allele indicates that SGR and SGR1 influence distinct but overlapping ethylene responses. Overexpression of SGR1 in the Gr mutant background provides evidence for the existence of different ethylene signaling modules in tomato that are influenced by GR, GRL1, or both. In addition, overexpression of AIRTE1 in tomato leads to reduced ethylene responsiveness in a subset of tissues but does not mimic the Gr mutant phenotype. Together, these data reveal species-specific heterogeneity in the control of ethylene responses mediated by members of the GR/RETE1 family.

The gaseous plant hormone ethylene influences many aspects of plant development and mediates responses to biotic and abiotic stresses (Abeles et al., 1992). Impaired ethylene biosynthesis and responsiveness can lead to altered cell expansion, cell elongation, senescence, abscission, cell death, fruit ripening, root hair formation, lateral root development, nodulation, and sex determination (Bleecker et al., 1988; Guzmán and Ecker, 1990; Oeller et al., 1991; Lanahan et al., 1994; Tanimoto et al., 1995; Wilkinson et al., 1997; O’Donnell et al., 2001; Xu et al., 2006; Boualem et al., 2008; Penmetsa et al., 2008; Hattori et al., 2009; Wang et al., 2010; Lewis et al., 2011). A framework of the ethylene signaling pathway has been assembled using a combination of genetic and biochemical analyses in Arabidopsis (Arabidopsis thaliana; Kendrick and Chang, 2008; Stepanova and Alonso, 2009; Zhao and Guo, 2011).

In Arabidopsis, ethylene is perceived by a family of five receptors that share homology to bacterial two-component His kinase receptors that are localized to the endoplasmic reticulum (ER) and Golgi membranes and form multimeric complexes thought to be composed of homoreceptor and heteroreceptor dimers (Chang et al., 1993; Hua et al., 1995, 1998; Sakai et al., 1998; Chen et al., 2002, 2010; Dong et al., 2008; Gao et al., 2008). Loss-of-function analyses indicate that the receptors act in a semiredundant manner to negatively regulate ethylene responses, although the ETR1 receptor appears to play more of a significant role in mediating ethylene responses than the other Arabidopsis receptors (Hua and Meyerowitz, 1998, Cancel and Larsen, 2002; Hall and Bleecker, 2003; Wang et al., 2003; Binder et al., 2006; Qu et al., 2007). In the absence of ethylene, the receptors actively suppress downstream responses through direct binding of CONSTITUTIVE TRIPLE RESPONSE1 (CTR1), a Ser/Thr mitogen-activated protein kinase kinase that acts as a negative regulator of the pathway (Kieber et al., 1993; Clark et al., 1998; Gao et al., 2003;
Huang et al., 2003). Upon ethylene binding, a conformational change is thought to occur, leading to receptor inactivation and the release of CTR1-mediated suppression (Huang et al., 2003; Zhao and Guo, 2011). Mutations within the N-terminal ethylene-binding domain of the receptors either inhibit ethylene binding or potentially disrupt the change in conformation following ethylene binding, leading to dominant ethylene-insensitive mutations that cannot be inactivated by ethylene (Wang et al., 2006). ETHYLENE-INSSENSITIVE2 (EIN2) acts genetically downstream of the ethylene receptors and CTR1 and encodes a protein with homology to the natural resistance-associated macrophage protein family of metal ion transporters (Roman et al., 1995; Alonso et al., 1999). EIN2 is localized within the ER, where it interacts with the kinase domain of each receptor in an ethylene-dependent manner (Alonso et al., 1999; Bisson et al., 2009; Bisson and Groth, 2010). Phosphorylation of EIN2 by CTR1 retains EIN2 within the ER but ethylene-dependent dephosphorylation leads to proteolytic cleavage of EIN2, resulting in translocation of the EIN2 C terminus into the nucleus, where it activates EIN3 and ethylene-dependent transcription (Qiao et al., 2012).

ETR1 receptor function requires REVERSION TO ETHYLENE SENSITIVITY1 (AIRTE1), a protein thought to facilitate the conformational change in the receptor following ethylene binding (Resnick et al., 2008). AIRTE1 was identified through a mutant screen to identify suppressors of ethylene insensitivity mediated by the etr1-2 receptor allele of Arabidopsis (Resnick et al., 2006). Loss of AIRTE1 function in Arabidopsis leads to enhanced ethylene responsiveness, whereas overexpression results in reduced sensitivity (Resnick et al., 2006). Genetic analysis indicates that AIRTE1 functions at or upstream of the ethylene receptors but acts independently of the RAN1 copper transporter (Resnick et al., 2006, 2008). In support of this hypothesis, AIRTE1 colocalizes with ETR1 in the ER and Golgi membranes and interacts with the N-terminal region of the ETR1 and ERS1 receptors (Zhou et al., 2007; Dong et al., 2008, 2010). Interestingly, mutations at the rts1 locus suppress ethylene insensitivity mediated by a subset of, but not all, etr1 mutant alleles, and reduced ethylene sensitivity mediated through AIRTE1 overexpression is primarily dependent on the presence of ETR1 and not the other ethylene receptors (Resnick et al., 2006, 2008; Zhou et al., 2007). Together, these data suggest that AIRTE1 acts predominantly through the ETR1 receptor, although the exact mechanisms of this interaction are not fully understood.

Ethylene influences developmental processes in diverse plant species, including those that are not part of the Arabidopsis life cycle (e.g., fleshy fruit development), and several studies have revealed novel phenotypes associated with the manipulation of ethylene biosynthesis or signaling components that would not be amenable to study in Arabidopsis (Oeller et al., 1991; Xu et al., 2006; Boualem et al., 2008; Pennmetsa et al., 2008; Hattori et al., 2009). The Green-ripe (Gr) mutant of tomato (Solanum lycopersicum) displays reduced ethylene responsiveness in a subset of tissues, resulting in plants with impaired fruit ripening, petal senescence, floral abscission, and an altered seedling triple response (Barry et al., 2005). Sequence analysis of the Gr locus identified a 334-bp deletion in the 5′-flanking region of a tomato homolog of AtRTE1 that results in the ectopic expression of SIGR in the Gr mutant but does not alter the sequence of the protein-coding region (Barry and Giovannoni, 2006). In addition, the tomato genome contains two additional genes designated GREEN-RIPE LIKE1 (SIGRL1) and SIGRL2, with the latter showing higher sequence identity to the RTE1 HOMOLOG (RTH) from Arabidopsis (Barry and Giovannoni, 2006; Resnick et al., 2006). Overexpression of SIGR under the control of the cauliflower mosaic virus (CaMV) 35S promoter recreates the Gr mutant phenotype but does not lead to a whole-plant reduction in ethylene responsiveness, suggesting that SIGR modulates a subset of ethylene responses possibly at the posttranscriptional level (Barry and Giovannoni, 2006).

In this study, the role of SIGRL1 and SIGRL2 in ethylene signaling was investigated together with the relationship of SIGR and SIGRL1 to Arabidopsis RTE1. A combination of overexpression in tomato and Arabidopsis reveals that SIGR and SIGRL1 possess unique specificities within the ethylene signaling pathway, whereas SIGRL2 may not play a role in ethylene signaling. Phylogenetic and functional analyses suggest that SIGRL1 and AIRTE1 are more closely related to one another, whereas SIGR is distinct and may represent a gene that is not widely distributed within the eudicot lineage and may be specific to the Solanaceae. SIGR and SIGRL1 are differentially expressed and together have expression patterns that overlap with AIRTE1 expression in Arabidopsis. Reporter-gene fusions indicate that SIGR, SIGRL1, and SIGRL2 proteins are predominantly localized in the Golgi. Together, these data provide evidence for the expansion and subfunctionalization of the ethylene signaling pathway in tomato and support the hypothesis of the existence of distinct ethylene signaling modules in tomato that operate through SIGR and SIGRL1.

RESULTS

Putative SIGR Orthologs within the Eudicot Lineage Are Restricted to the Solanaceae

Previously, we identified SIGR and two additional GR homologs from tomato, designated SIGRL1 and SIGRL2, with phylogenetic analysis, revealing that SIGR and SIGRL1 are more closely related to one another and to Arabidopsis RTE1, whereas SIGRL2 is more divergent and similar to Arabidopsis RTH (Barry and Giovannoni, 2006). Three-way sequence comparisons between SIGR and SIGRL1 with available sequences consistently revealed that SIGRL1 displayed higher sequence homology to genes from other eudicot species than did SIGR (data not shown). Furthermore,
all eudicot species examined, including those with accessible genome sequences, contain only two genes that are either highly similar to \textit{SlGRL1} or \textit{SlGRL2}. Together, these data suggest that \textit{SIGR} is more divergent and may represent a gene that is not widely distributed in eudicot families. In an attempt to identify putative \textit{GR} orthologs, bacterial artificial chromosome (BAC) libraries of three Solanaceae species, eggplant (\textit{Solanum melongena}), pepper (\textit{Capsicum annuum}), and petunia (\textit{Petunia inflata}), were screened with probes to both \textit{SIGR} and \textit{SlGRL1}. Several clones were identified from each library, and restriction mapping coupled with hybridization was used to group clones into families (data not shown). DNA sequencing of representative BAC clones using primers designed to conserved regions of \textit{SIGR} and \textit{SlGRL1} followed by successive rounds of primer walking were utilized to complete the sequence of the BAC clone covering the predicted \textit{GR} and \textit{GRL1} genomic regions. Utilizing this approach, putative \textit{GR} orthologs from pepper, eggplant, and petunia and putative \textit{GRL1} orthologs from eggplant and petunia were identified. The subsequent release of the draft sequence of the potato (\textit{Solanum tuberosum}) genome also revealed the presence of putative \textit{GR}, \textit{GRL1}, and \textit{GRL2} orthologs (Xu et al., 2011). Phylogenetic analysis of the predicted proteins revealed that the putative \textit{GR} orthologs formed a separate subclade that only contained sequences from other Solanaceae species, whereas the putative Solanaceae \textit{GRL1} proteins, while forming a distinct subclade, are more closely related to Arabidopsis \textit{RTE1} and related proteins from other eudicot species (Fig. 1). Furthermore, pairwise sequence comparisons and branch lengths on the phylogenetic tree indicated that the \textit{GRL1}-related proteins are highly similar to one another, ranging between 89% and 95% identical, whereas the putative \textit{GR} proteins are more divergent, ranging between 60% and 89% identity at the amino acid level (Fig. 1; Supplemental Table S2). The phylogenetic relationship of \textit{SIGR} and \textit{SlGRL1} supports the hypothesis that these genes possess distinct roles within ethylene signaling.

Figure 1. Phylogenetic analysis of the GR/RTE1 family of proteins. A neighbor-joining phylogenetic tree derived from a multiple sequence alignment of the deduced full-length amino acid sequences of GR/RTE1-related proteins of plants was constructed using MEGA version 5.0 (Tamura et al., 2011). TMEM222, the human homolog of GR/RTE1, is included as an outgroup. Bootstrap values greater than 80%, derived from 1,000 replicates, are indicated above the nodes. Shaded regions indicate putative Solanaceae GR and GRL1 orthologous groups. Details of the proteins used to construct the phylogeny are provided in Supplemental Table S1.
SlGR, SlGRL1, and SlGRL2 Are Differentially Expressed during Development and in Response to Ethylene

The presence of multigene families facilitates functional plasticity, allowing individual family members to adopt specific or specialized roles. Such subfunctionalization can occur at multiple levels but is often attributed to the divergence of cis-elements leading to differential expression (Force et al., 1999). Previously, using northern-blot analysis, SlGR expression was shown to be low or absent in most tomato tissues, with transcripts displaying maximal accumulation in developing seeds (Bisson et al., 2006). However, a robust and comprehensive view of the expression of SlGR, SlGRL1, and SlGRL2 was not performed. In this study, quantitative reverse transcription (qRT)-PCR was performed using RNA extracted from several tomato tissues and in response to ethylene treatment. SlGR expression is low in most tomato tissues examined, with maximal transcript levels accumulating in the seeds (Fig. 2A). Dissection of the seeds revealed that the majority of this expression is associated with the testa of developing seeds (Fig. 2E). SlGRL1 expression is also predominantly associated with the testa of developing seeds (Fig. 2E) but is also expressed throughout fruit development, with an increase in transcript abundance detected at the breaker stage of fruit ripening (Fig. 2B), in senescing flowers (Fig. 2C), and in response to ethylene treatment (Fig. 2D). SlGRL2 is also widely expressed in tomato tissues, with expression detected in seeds and anthers together with increased expression detected during fruit ripening, but with transcript levels declining slightly following ethylene treatment (Fig. 2, A–D). In contrast to SlGR, where most of the seed expression is associated with the testa, the expression of SlGRL2 is more uniformly distributed across the embryo, testa, and endosperm (Fig. 2E). Together, these data indicate that while transcripts of SlGR, SlGRL1, and SlGRL2 are detectable in all tissues examined, the relative abundance of each differs temporally and spatially throughout development and in response to ethylene.

SlGR, SlGRL1, and SlGRL2 Are Predominantly Golgi-Localized Proteins

Several components of the ethylene signaling pathway are localized within the endomembrane system, and separate studies have reported either dual localization of ArRTE1 within the Golgi and ER membranes or exclusive localization within the Golgi (Chen et al., 2002; Gao et al., 2003; Zhou et al., 2007; Dong et al., 2008; Bisson et al., 2009). To determine the subcellular localization of SlGR and SlGRL1, N-terminal yellow fluorescent protein (YFP) fusions of each protein were constructed and transformed into the ctrl-2/etr1-3 double mutant. Transgenic lines expressing each fusion protein were confirmed by confocal laser scanning microscopy (data not shown). Each YFP fusion protein line was crossed with stably transformed Arabidopsis lines expressing either GFP-HDEL or ST-GFP, which target GFP to the ER and Golgi, respectively (Haseloff et al., 1997; Boevink et al., 1998). The localization of each fusion protein was examined in the cotyledons of the F1 progeny derived from each cross (Fig. 3). These analyses reveal that both SlGR and SlGRL1 are predominantly localized within the Golgi, as each fusion protein colocalized with the ST-GFP marker but not the GFP-HDEL marker (Fig. 3, compare C and F with I and L). In a separate experiment, an N-terminal YFP-tagged version of SlGRL2 was also shown to predominantly colocalize with the ERD2-GFP (Boevink et al., 1998) reporter construct in the Golgi following transient expression in tobacco (Nicotiana tabacum) leaves (Supplemental Fig. S1).

Overexpression of SlGRL1 in Tomato Does Not Recreate the Gr Mutant Phenotype But Influences a Subset of Ethylene Responses

The tissue-specific reduction in ethylene responsiveness observed in the Gr mutant of tomato is mediated through a promoter deletion in SlGR leading to ectopic expression of SlGR, and overexpression of SlGR recreates the Gr mutant phenotype (Bisson and Giovannoni, 2006). Similarly, overexpression of ArRTE1 in Arabidopsis leads to reduced ethylene responsiveness (Lincoln et al., 1987). These data suggest that overexpression of SlGRL1 in tomato may confer reduced ethylene responsiveness. To test this hypothesis, transgenic lines overexpressing SlGRL1 under the control of the CaMV35S promoter were generated. In total, 27 CaMV35S::SlGRL1 primary transformants were recovered from tissue culture. The CaMV35S::SlGRL1 primary transformants ripened normally and did not exhibit the typical inhibition of fruit ripening observed in the Gr mutant and previously developed CaMV35S::SlGR overexpression lines, suggesting functional divergence of these genes (data not shown). Three homozygous independent transgenic CaMV35S::SlGRL1 lines that possess elevated SlGRL1 transcript levels in both fruit and seedlings were selected for further analysis (Fig. 4). These homozygous lines ripened normally, and the expression of the ethylene- and ripening-regulated E4 gene (Lincoln et al., 1987) was similar in fruit of the CaMV35S::SlGRL1 lines and untransformed control fruit, indicating normal ethylene responsiveness (Fig. 4, A–D). Analysis of the seedling triple response in the CaMV35S::SlGRL1 lines indicated that hypocotyl responses to increasing concentrations of 1-aminoacyclopropane-1-carboxylic acid (ACC) are similar to those of wild-type Alisa Craig, although hypocotyl lengths are slightly longer in seedlings grown in the presence of 10 μM ACC, indicating a reduction in ethylene responsiveness (Fig. 4E; Supplemental Table S3). A similar response is observed in hypocotyls of the Gr mutant, whereas hypocotyls of the globally insensitive Never-ripe (Nr) ethylene receptor mutant (Lanahan et al., 1994) display characteristic reduction in ethylene...
Figure 2. Expression of SIGR, SIGRL1, and SIGRL2 during tomato development. A, The relative expression levels of SIGR, SIGRL1, and SIGRL2 in various tomato tissues as determined by qRT-PCR. BK refers to fruit at the breaker stage of development, and RR-peel refers to peel isolated from red-ripe fruits. B, Relative expression levels of SIGR, SIGRL1, and SIGRL2 during tomato fruit development and ripening. Fruit were harvested at various days post anthesis, at the mature green (MG), breaker (BK), and 3 and 7 d post breaker (BK+3 and BK+7) stages. C, Relative expression levels of SIGR, SIGRL1, and SIGRL2 in whole flowers at four stages of development (tight bud, bud opening, anthesis, and senescing) together with expression in floral organs isolated from flowers at anthesis. D, Relative expression levels of SIGR, SIGRL1, and SIGRL2 in leaves of 4-week-old plants treated with 10 μL L⁻¹ ethylene for the specified time points. E, Relative expression levels of SIGR, SIGRL1, and SIGRL2 in whole and dissected seeds isolated from mature green fruits. Experimental details are provided in “Materials and Methods.” For all experiments, data are presented as means ± se of three biological and three technical replicates. Expression values for each gene are presented relative to the expression level in a tissue with a basal level of expression previously normalized to a value of 1. *Statistical analysis is presented for each gene across the tissue, development, or treatment series. Means followed by the same letter are not significantly different at α = 0.05.
responsiveness (Fig. 4E; Supplemental Table S3). Root lengths of the CaMV35S::SlGRL1 lines displayed a partial ethylene-insensitive phenotype, with root lengths intermediate between those observed in control and Nr seedlings that are similar to those observed in the Gr mutant (Fig. 4F). Together, these data indicate that the CaMV35S::SlGRL1 lines possess a triple-response phenotype virtually identical to that observed in the Gr mutant and CaMV35S::SlGR overexpression lines (Fig. 4, E and F; Barry and Giovannoni, 2006).

The Gr mutant and CaMV35S::SlGR overexpression lines display a reduced response to ethylene-induced floral abscission that lies between that observed in control and Nr plants (Barry et al., 2005; Barry and Giovannoni, 2006). Similarly, the CaMV35S::SlGRL1 lines have reduced rates of ethylene-induced floral abscission, comparable to those observed in the Gr mutant (Fig. 4G). Petiole angle in response to ethylene treatment was also determined in the CaMV35S::SlGRL1 lines, indicating a partial ethylene-insensitive phenotype intermediate between the wild type and Nr (Fig. 4H). In contrast, the petiole angle in the Gr mutant and CaMV35S::SlGR lines in response to ethylene is similar to that observed in wild-type seedlings (Fig. 4H; Supplemental Fig. S2). Together, these data indicate that overexpression of SlGRL1 in tomato does not phenocopy the Gr mutant; rather, it influences a subset of ethylene responses that include those observed in the Gr mutant (altered triple response and reduced floral abscission) in addition to those that are distinct (normal fruit ripening and reduced petiole epinasty).

Combining Gr with CaMV35S::SlGRL1 Enhances Ethylene Insensitivity

The data described above indicate that SlGR and SlGRL1 each influences a subset of ethylene responses when overexpressed in tomato (Fig. 4). However, individually, neither is able to confer reduced ethylene responsiveness in all plant tissues; in particular, the response of dark-grown hypocotyls to the ethylene precursor ACC is similar to that observed in wild-type seedlings, with only a slight reduction in ethylene responsiveness observed (Fig. 4E). To determine whether
Figure 4. Phenotypic evaluation of SlGRL1 overexpression lines of tomato. A, Fruit phenotypes of three independent homozygous CaMV35S::SlGRL1 transgenic lines in comparison with the wild type (Ailsa Craig [AC]) and the ethylene-insensitive Nr and Gr mutants. B, Relative expression levels of SlGRL1 in the hypocotyl of CaMV35S::SlGRL1 lines as determined by qRT-PCR. C and D, Relative expression levels of SlGRL1 (C) and the ripening-related gene E4 (D) in the fruit of CaMV35S::SlGRL1 transgenic lines at the breaker + 3 stage of ripening. Data are presented as means of three biological and three technical replicates for each sample. E and F, Hypocotyl and root lengths, respectively of dark-grown AC, Nr, Gr, and CaMV35S::SlGRL1 seedlings germinated and grown in the presence of ACC for 8 d. Data presented are means ± se of at least 17 seedlings (statistical analysis is presented in Supplemental Table S3). G, Percentage floral abscission in CaMV35S::SlGRL1 lines in comparison with AC, Nr, and Gr. Detached flower trusses were immersed in water in conical flasks and treated with 2 μL L⁻¹ ethylene for 72 h. Data presented are means ± se of three independent experiments collected from at least 417 flowers per genotype. H, Petiole epinasty in CaMV35S::SlGRL1 lines in comparison with AC, Nr, and Gr. The adaxial leaf angle of 4-week-old plants treated with 20 μL L⁻¹ ethylene for 16 h was measured. Three leaves for each plant were examined, and at least 11 plants were used for each genotype; the data presented are means ± se. *In all experiments, means followed by the same letter are not significantly different at α = 0.05.
ethylene responses can be further influenced through combining SIGR and SIGRL1, the CaMV35S::SIGRL1 transgene was introduced into the Gr mutant background through a cross, and homozygous lines were recovered through molecular genotyping. In some tissues, the double overexpression line displays a phenotype similar to that observed in either parent. For example, fruit of the Gr × CaMV35S::SIGRL1 line resembles that of the single Gr mutant, showing inhibition of fruit ripening (Fig. 5A). Similarly, upon exposure to ethylene, petiole epinasty in the Gr × CaMV35S::SIGRL1 line is identical to that observed in the CaMV35S::SIGRL1 transgenic line (Fig. 5E). However, in response to ethylene-induced floral abscission and during the seedling triple response, the Gr × CaMV35S::SIGRL1 line has an additive phenotype that is stronger than that observed in either Gr or the CaMV35S::SIGRL1 overexpression line (Fig. 5, B–D). In particular, roots of dark-grown Gr × CaMV35S::SIGRL1 seedlings grown in the presence of ACC display an additive phenotype.

Figure 5. Phenotypic analysis of SIGRL1 overexpression in the Gr mutant background. Phenotypes of Gr × CaMV35S::SIGRL1 in comparison with Ailsa Craig (AC), Nr, and Gr and the CaMV35S::SIGRL1 line (15-7-11) are shown. A, Phenotypes of ripe fruit. B and C, Hypocotyl and root length, respectively, of dark-grown seedlings germinated and grown in the presence of ACC for 8 d. Data presented are means ± se of at least 30 seedlings. Statistical analysis is presented in Supplemental Table S4. D, Percentage of ethylene-induced floral abscission. Data presented are means ± se of two independent experiments collected from at least 318 flowers per genotype. E, The degree of petiole epinasty in response to ethylene treatment was measured in three leaves for each plant from at least 10 individual plants. Data presented are means ± se. Experimental details are available in “Materials and Methods.” *In all experiments, means followed by the same letter are not significantly different at α = 0.05.
overexpression line and with levels of ethylene insensitivity comparable to that observed in the more severe Nr mutant (Fig. 5C).

Overexpression of SIGRL2 in Tomato Does Not Phenocopy the Reduced Ethylene Insensitivity of the Gr Mutant and CaMV35S::SIGRL1 Lines

The role of SIGRL2 and its putative Arabidopsis ortholog, RTH, has not been defined, and phylogenetic analyses indicate that these genes are fairly divergent from SIGR, SIGRL1, and AtRTE1 (Fig. 1). Gene expression analysis indicated that SIGRL2 transcripts increase during fruit ripening but decrease in abundance following ethylene treatment (Fig. 2), suggesting that this gene may play a role in ethylene responsiveness. To test this hypothesis, a set of transgenic lines that overexpress SIGRL2 under the control of the CaMV35S promoter were generated, and four independent homozygous lines with differing transgene expression levels were identified (Supplemental Fig. S3). The phenotype of these lines was indistinguishable from wild-type control plants, and evaluation of ethylene responsiveness in fruit, seedlings, abscission zones, and petioles, which were altered in the Gr mutant and SIGRL1 overexpression lines (Fig. 4), failed to reveal any altered phenotypes when compared with wild-type (Ailsa Craig) control plants. These data suggest that SIGRL2 may not play a role in ethylene signaling, at least during the responses investigated in this study, or that the responses may be subtle and not detected under the experimental conditions utilized.

SIGR Is Unable to Fully Complement the rte1 Mutant Phenotype

The phylogenetic separation of SIGR and SIGRL1, together with the closer relationship of SIGRL1 to Arabidopsis RTE1, suggest that SIGRL1 may be functionally equivalent to AtRTE1 whereas SIGR may possess a slightly different function (Fig. 1). To test this hypothesis on the relationship of SIGR, SIGRL1, and AtRTE1 in more detail, the ability of SIGR and SIGRL1 to complement the rte1-3 allele was examined. N-terminal epitope-tagged versions of SIGR and SIGRL1 under the control of the CaMV35S promoter were transformed in the etr1-2/rte1-3 double mutant, in which ethylene insensitivity is suppressed due to the loss of AtRTE1 function (Resnick et al., 2006). Two homozygous CaMV35S::MYC-SIGRL1 lines were recovered that almost fully complement the rte1-3 allele, restoring ethylene insensitivity in both the hypocotyls and roots of Arabidopsis seedlings to levels almost comparable to those of the etr1-2 allele (Fig. 6, A and C–E). In contrast, three independent lines expressing the CaMV35S::MYC-SIGR transgene did not fully complement the rte1-3 allele (Fig. 6, A, B, D, and E). However, the CaMV35S::MYC-SIGR transgene is active, as transgenic seedlings grown on low concentrations of ACC are able to partially rescue the previously documented ethylene-hypersensitive phenotype of etr1-2/rte1-3 double mutant seedlings (Resnick et al., 2006). For example, in the absence of ACC and in the presence of 0.1 μM ACC, hypocotyl lengths of the CaMV35S::MYC-SIGR lines are longer than those of the etr1-2/rte1-3 double mutant and identical to those observed in Columbia seedlings (Fig. 6D; Supplemental Table S5). However, at higher concentrations of ACC, this difference was eliminated. Similarly, root lengths of the CaMV35S::MYC-SIGR lines grown on low concentrations of ACC are longer than those of the etr1-2/rte1-3 double mutant but remain hypersensitive to ACC compared with the roots of Columbia seedlings (Fig. 6E; Supplemental Table S5). Similar phenotypes were also observed in transgenic lines expressing untagged and YFP-tagged versions of each gene (Supplemental Figs. S4 and S5). These data further illustrate the functional divergence of SIGR and SIGRL1 and suggest that SIGR is not functionally equivalent to AtRTE1, while SIGRL1 is almost able to rescue the rte1 mutant phenotype.

Overexpression of AtRTE1 in Tomato Does Not Confer Reduced Ethylene Responsiveness in All Plant Tissues

Overexpression of AtRTE1 in Arabidopsis leads to reduced ethylene responsiveness (Resnick et al., 2006). To determine whether AtRTE1 is able to influence ethylene responses in tomato, a construct expressing AtRTE1 under the control of the CaMV35S promoter was stably transformed into tomato. Twenty-eight primary transgenic lines were recovered, and all showed a normal fruit-ripening phenotype, suggesting that AtRTE1 is unable to influence ethylene responses in tomato fruit (data not shown). This phenotype was confirmed in two independent homozygous transgenic lines that had high transgene expression levels, normal fruit ripening, and wild-type levels of E4 expression (Fig. 7, A–C). However, the CaMV35S::AtRTE1 transgenic lines display a partial ethylene-insensitive phenotype in both hypocotyls and roots, showing a phenotype that is intermediate between that of wild-type and Nr seedlings (Fig. 7, D and E). Similarly, the CaMV35S::AtRTE1 lines also possess reduced rates of ethylene-induced flower abscission and petiole epinasty compared with those observed in control plants (Fig. 7, F and G). Together, these data suggest that, in general, overexpression of AtRTE1 in tomato more closely resembles the phenotypes observed in the CaMV35S::GRL1 lines than those of the Gr mutant (Fig. 4).

DISCUSSION

Variation in Ethylene Signaling Components and Evidence for Subfunctionalization

The creation and maintenance of gene families through duplication events is a driver of evolution and
increases both the potential for genetic redundancy as well as the opportunity for subfunctionalization leading to functional plasticity (Force et al., 1999; Freeling, 2009). Plasticity is important during the synthesis and perception of plant hormones, which generally control multiple aspects of plant development and responses to environmental perturbation, allowing plants to appropriately regulate hormonal responses. Several components
Figure 7. Phenotypic evaluation of \textit{AtRTE1} overexpression lines of tomato. Phenotypes of \textit{CaMV35S::AtRTE1} lines in comparison with Ailsa Craig (AC) and the ethylene-insensitive \textit{Nr} and \textit{Gr} mutants are shown. A, Phenotypes of the ripe fruit of \textit{CaMV35S::AtRTE1} lines. B and C, Relative expression levels of \textit{AtRTE1} (B) and the ethylene- and ripening-related gene \textit{E4} (C) in fruit of two independent homozygous \textit{CaMV35S::AtRTE1} transgenic lines at the breaker stage of development as determined by qRT-PCR. Data are presented as means ± se of three biological and three technical replicates. D and E, Hypocotyl and root lengths, respectively, of dark-grown seedlings germinated and grown in the presence of ACC. Data presented are means ± se of at least 18 seedlings. Statistical analysis is presented in Supplemental Table S6. F, Percentage of ethylene-induced floral abscission. Data presented are means ± se of two independent experiments collected from at least 352 flowers per genotype. G, Ethylene-induced petiole epinasty in \textit{CaMV35S::AtRTE1} lines. Three leaves for each plant were examined, and at least 12 plants were used for each genotype. Data presented are means ± se. Experimental details are provided in “Materials and Methods.” *In all experiments, means followed by the same letter are not significantly different at α = 0.05.
within the ethylene response pathway are encoded by multigene families that vary in size and composition between species (Bleecker, 1999; Adams-Phillips et al., 2004; Klee, 2004; Rzewuski and Sauter, 2008; Chen and Gallie, 2010). Consequently, subfunctionalization is apparent at multiple steps of the ethylene signaling pathway. For example, EIN3 and EIL1 proteins act semiredundantly to influence separate ethylene responses associated with seedling growth and stem and leaf expansion in Arabidopsis, and individual ETHYLENE RESPONSE FACTOR genes mediate distinct responses to environmental stress (Hattori et al., 2009; An et al., 2010; Zhang et al., 2011). Subfunctionalization also occurs at the level of the ethylene receptors, with specific or more prevalent roles for individual receptors and receptor subfamilies identified, even though, in general, the ethylene receptors act redundantly (Tieman et al., 2000; Binder et al., 2006, 2012; Qu et al., 2007; Plett et al., 2009; Wuriyanthag et al., 2009).

Variation in copy number between species is also evident for the GR/ETE1 family. Among the species investigated in this study, most eudicot species contain a single copy of a gene that is more similar to AtRTE1 and SIGRL1 together with a single copy of the distantly related gene, defined by AIRTH and SIGRL2 (Fig. 1), the latter of which may not influence ethylene responses in tomato (Supplemental Fig. S3). However, in addition, tomato and other members of the Solanaceae family possess a single copy of a gene defined by SGR, which is a phylogenetically distinct paralog of SIGRL1 (Fig. 1; Supplemental Tables S1 and S2). The maintenance of GR and GRL1 in the Solanaceae family may have occurred through subfunctionalization. In support of this hypothesis, SGR and SIGRL1 have overlapping but distinct expression patterns (Fig. 2). SGR expression is relatively low in most of the tissues examined except for the seeds, where transcript abundance peaks in the testa (Fig. 2E). In contrast, SIGRL1 expression is more widely expressed and is ripening and ethylene related (Fig. 2, B and D). In this regard, data available through the e-FP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) reveal that the expression patterns of these genes share expression characteristics with AIRTH. For example, AIRTH1 is expressed in many Arabidopsis tissues, with maximal transcript abundance in developing seed associated with the seed coat and the endosperm, and is enhanced by treatment with ACC. Gene copy number variability in ethylene signaling components creates the potential for the formation of distinct ethylene signaling networks.

Characterization of the Gr mutant and overexpression of SIGR in tomato indicated that this gene influences a subset of ethylene responses in tomato (Barry et al., 2005; Barry and Giovannoni, 2006). However, overexpression of SIGRL1 in tomato causes inhibition of a subset of ethylene responses that overlap but are distinct from those observed in the Gr mutant and CaMV35S::SIGR lines (Fig. 4). Notably, overexpression of SIGRL1 does not lead to inhibition of fruit ripening, which is a feature of the Gr mutant and CaMV35S::SIGR lines (Barry and Giovannoni, 2006). However, the CaMV35S::SIGRL1 lines displayed inhibition of ethylene-induced petiole epinasty, which is not a feature of the Gr mutant or the CaMV35S::SIGR lines (Fig. 4; Supplemental Fig. S2). These data suggest that SGR and SIGRL1 have evolved the ability to influence different subsets of ethylene responses. This is a hypothesis further supported by the differential complementation of the etr1-3 mutant allele by SGR and SIGRL1 (Fig. 6). Similarly, while overexpression of AIRTH1 in tomato leads to reduced ethylene responsiveness in multiple tissues (Fig. 7), it does not result in a whole-plant reduction in ethylene responsiveness, and CaMV35S::AIRTH1 lines do not mimic the Gr mutant phenotype. A recent study on the role of the rice (Oryza sativa) GR/ETE1 family in ethylene signaling indicated that OsRTH1 was able to complement the loss of AIRTH1 function in Arabidopsis and confer reduced ethylene sensitivity in Arabidopsis and rice when overexpressed, whereas OsRTH2 and OsRTH3 appeared not to influence ethylene responsiveness (Zhang et al., 2012). Together, these data point to considerable heterogeneity among the members of the GR/ETE1 family, suggesting that they likely exert their influence on ethylene signaling through specific components of the ethylene pathway and have diverged to the point where no single protein can fully substitute for another. Such differences may be due to evolutionary changes either within the GR/ETE1 proteins themselves or within the ethylene receptors that limit the ability of these proteins to interact, either genetically or biochemically.

Evidence for the Existence of Distinct Ethylene Signaling Modules in Tomato

The presence of different receptor isoforms and the expansion of gene families for multiple components of the ethylene signaling pathway in tomato and other species, coupled with differential expression and/or accumulation of individual signaling components during development or in response to stimuli, are likely to contribute to heterogeneity within ethylene receptor complexes. This heterogeneity may lead to the formation of distinct signaling modules that produce diverse outputs. A model has been proposed in which AirTH1 is required to maintain the ETR1 receptor in the “on signaling state” to inhibit ethylene responses, and biochemical evidence suggests that this occurs through a direct protein-protein interaction (Resnick et al., 2008; Dong et al., 2010).

The ability of SGR and SIGRL1 to influence separate subsets of ethylene responses, when overexpressed in tomato and Arabidopsis, suggests that they may do so through interactions with distinct ethylene receptors or possibly through interactions with the same receptors but with differing affinities. The latter scenario could explain the weak ethylene-insensitive phenotype and partial complementation observed in the etr1-2/rte1-3 double mutant expressing the CaMV35S::MYC-SIGR
Discrepancies in the Subcellular Localization of the GR/RTE1 Family

The ethylene receptors are known to form large heteromeric complexes, and interaction experiments between the ethylene receptors and additional signaling components suggest that these complexes likely contain AtRTE1, EIN2, and CTR1 and occur within the ER and Golgi membranes (Clark et al., 1998; Chen et al., 2002, 2010; Gao et al., 2003, 2008; Bisson et al., 2009; Dong et al., 2010). Our data indicate that SIGR, SIGRL1, and SIGRL2 are localized primarily to the Golgi membranes at steady state, although at present we cannot completely exclude the possibility that these proteins are also localized to additional organelles (Fig. 3; Supplemental Fig. S1). The current model of the ethylene signaling pathway would place the localization of the receptors and their interaction with the GR/RTE1 proteins within the ER, and previous studies have suggested either exclusive localization of AtRTE1, OsRTH1, and OsRTH2 within the Golgi or dual localization within the Golgi and ER (Zhou et al., 2007; Dong et al., 2008; Zhang et al., 2012). These slight discrepancies may be caused by the use of different experimental systems and high-level overexpression of heterologous transgenes that may influence the subcellular localization of proteins. Therefore, it will be important to resolve these discrepancies using additional experimental approaches, as they may have implications for current models of ethylene receptor and GR/RTE1 protein function.

CONCLUSION

The framework of ethylene signaling in plants has arisen through combined genetic and biochemical analyses of seedling responses to ethylene in Arabidopsis. However, evidence is emerging that while many aspects of the ethylene signaling pathway are conserved across species, differences in gene family complement and function exist. This study has identified variation in the control of ethylene responses by members of the GR/RTE1 gene family in tomato, revealing that individual members have evolved distinct abilities to influence ethylene responses and providing evidence for the existence of multiple ethylene signaling modules in tomato. Determination of the exact identity of these modules will require additional experimentation but, based on studies of Arabidopsis (Dong et al., 2010), may involve specific SIGR-ethylene receptor and SIGRL1-ethylene receptor interactions. The sequence variation between SIGR and SIGRL1 and their putative orthologs provides a tool to investigate this hypothesis and the role of these proteins in influencing specific ethylene responses.

MATERIALS AND METHODS

Plant Growth and Treatments

The parental tomato (Solanum lycopersicum) line Ailsa Craig, the ethylene-insensitive mutants Nn and Gr, and CaMV35S::SIGRL1 transgenic lines were described previously (Lanahan et al., 1994; Barry et al., 2005; Barry and Giovannoni, 2006). Plants were grown in peat-based compost supplemented with fertilizer in greenhouses equipped with heating and cooling systems and supplemental lighting either at Cornell University or Michigan State University.

Experiments to evaluate the triple-response phenotype in dark-grown tomato seedlings and floral abscission were performed as described previously (Barry et al., 2005) with the exception that seedlings were measured at 8 d after sowing and flowers were induced to abscise by treatment with ethylene at a concentration of 2 μL−1. Tomato plants for investigating ethylene responses during petiole epinasty were grown in Jiffy-7 Peat Pellets (http://www.hummert.com/) for 4 weeks under 16-h-light/8-h-dark cycles at 28°C and 65% relative humidity. Four-week-old plants were treated with 20 μL−1 ethylene for 16 h, and the degree of the upper angle between the petiole and stem was determined.

Fruit tissues for gene expression analysis were harvested from greenhouse-grown plants at specific days post anthesis and at the mature green, breaker, breaker + 3 d, and breaker + 7 d stages of fruit ripening. Columella and locular gel were removed from the fruits, and the pericarp was frozen in liquid nitrogen and used for subsequent analysis. Whole tomato flowers of different stages and floral organs from flowers at anthesis were collected as described previously (Barry et al., 1996). Tomato seeds were harvested from mature green fruits and stirred in water overnight at room temperature to remove contaminating locular gel. Seeds were dissected into embryo, testa, and endosperm as described previously (Nornogaki et al., 1992). For experiments designed to determine potential ethylene regulation of gene expression, leaflets were harvested from 4-week-old growth chamber-grown plants (see above for details) treated with 10 μL−1 ethylene for the specified time periods.
Seeds of Arabidopsis (Arabidopsis thaliana) ecotype Columbia together with the ethylene signaling mutants etr1-2 and etr1-2/eri1-3 (Resnick et al., 2006) and the fluorescently tagged organelle reporter lines ST-GFP and GFP-FDHE (Haseloff et al., 1997; Boevink et al., 1998) were sown in 1:1:1 Sure mix medium vermiculite:perlite (Michigan Grover Products; www.suremix.com) and exposed to a 3-d cold treatment at 4°C. Seed trays were transferred to a growth chamber at 22°C under 16-h-light/8-h-dark cycles at 145 μmol m⁻² s⁻¹ and 65% relative humidity. Plants were supplemented with fertilizer 0.25× Hoagland solution, pH 5.5. The Arabidopsis triple-response screen was performed using a PhGR primer walking. Putative open reading frames were deduced based on alignment with orthologs (eggplant, 150M15; petunia, 16J10). Sequencing of the genomic regions containing 25 ng of cDNA template. In experiments designed to measure transgene expression, ETR1 was constitutively expressed in Arabidopsis for normalization, as described previously (Expósito-Rodríguez et al., 2006).

Three ordered BAC libraries constructed from partial genomic DNA digests of petunia (Petunia inflata), pepper (Capsicum annuum), and eggplant (Solanum melongena; McCubbin et al, 2000; Wang et al., 2008) were screened with 32P-labeled DNA probes derived from tomato and potato (GTP, GTP, GTP, and GTP, respectively) and the Arabidopsis triple-response screen was performed using a PhGR primer walking. Putative open reading frames were deduced based on alignment with orthologs (eggplant, 150M15; petunia, 16J10). Sequencing of the genomic regions covering either GR or GRL1 orthologs was accomplished by successive rounds of primer walking. Putative open reading frames were deduced based on alignment with tomato and potato (Solanum tuberosum) cDNA clones. CDNA clones corresponding to PpGR and PpGRL1 were amplified by reverse transcription (RT)-PCR from CDNA isolated from petunia ovary.

Total RNA was extracted using the RNAeasy Mini Kit and subjected to column DNease treatment (Qiagen; http://www.qiagen.com). One microgram of RNA was used for RT using the SuperScript III First-Strand Synthesis System (Invitrogen; http://www.lifetech.com/). Gene-specific primers for the analyzed genes were designed by Primer Express 3.0 (Applied Biosystems; http://www.lifetech.com; Supplemental Table S7). PCR was performed with 2X FAST SYBR Master Mix (Applied Biosystems) in a 10-μL volume containing 25 ng of cDNA template. RT-PCR products were digested with restriction enzymes to measure transgene expression levels, only 10 ng of cDNA template was utilized. PCR amplification was performed using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems) at the Research Technology Support Facility of Michigan State University. The qRT-PCR program included a preliminary step of 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The primer efficiency was tested by generating standard curves. Data were analyzed by the comparative ΔΔCT method (Livak and Schmittgen, 2001) using the constitutively expressed GAPDH and CAC genes of tomato and AtUBI10 of Arabidopsis for normalization, as described previously (Expósito-Rodríguez et al., 2008; De Vos and Jander, 2009).

The CaMV35S::SGLR1 construct was assembled as follows. The full-length coding sequence of GRL1 was reamplified from the EST clone cEOD-62516 using the primers GRL1OE-F and GRL1OE-R (Supplemental Table S7), which contain BamHI and SalI linker sites on the forward and reverse primer, respectively. This fragment was cloned into the pCR2.1 vector by TOPO cloning (Invitrogen). The fragment was excised using the restriction enzyme sites in the linkers and ligated downstream of the CaMV35S promoter in the binary vector pB1121, modified by removal of the UbiA coding region by digestion with BamHI and SacI. The CaMV35S::SGLR1 construct was assembled using the same strategy except that a cDNA fragment of SGLR2 was reamplified from the EST clone cEOD-5-M16 using the primers GRL2OE-F and GRL2OE-R. The CaMV35S::GRT1E1 construct was assembled as follows. The full-length coding sequence of ATR1E1 was amplified by RT-PCR from Arabidopsis leaf cDNA using the primers RTE1OE-F and RTE1OE-R, which contain BamHI and SalI linker sites on the forward and reverse primer, respectively. The fragment was cloned into the pCR2.1 vector by TOPO cloning. The clone was digested with SalI, the overhang was rendered blunt with incubation with the Klenow fragment of DNA polymerase I, and the insert was released from the vector by digestion with BamHI. The insert was ligated downstream of the CaMV35S promoter in the binary vector pB1121, previously modified by removal of the UbiA coding region by digestion with SacI followed by polishing with T4 DNA polymerase and subsequent BamHI digestion. All PCR fragments used for construct assembly were amplified using Pfu Ultra DNA polymerase (Agilent Technologies; www.agilent.com), and the fidelity of all constructs was confirmed by DNA sequencing. Transgenic tomato plants were generated through cotyledon-derived explants as Agrobacterium tumefaciens-mediated transformation of the Ailsa Craig line using strain LBA4404 (Fillatti et al., 1987). The presence of transgenes in tomato and the subsequent development of homozygous lines were achieved using a combination of PCR screening and Southern-blot hybridization using probes or markers designed to transgenes or selection markers as described previously (Barry et al., 2005; Barry and Giovannoni, 2006).

Epitope-tagged versions of SGR and SGLR1 were assembled using Gateway cloning technology. Briefly, Gateway entry clones were developed for each gene using primers listed in Supplemental Table S7 to amplify the corresponding gene fragments and insert them into the pENTR:D-TOP vector (Invitrogen). The resultant clones were digested with MluI, which cuts in the vector backbone but not the insert, and the digestion mix was recombinated with the binary vectors pEarleyGate 203 (CaMV35S::MYC-Gateway-ocs 3′) and pEarleyGate 104 (35S-YFP-Gateway-ocs 3′) to create four constructs: CaMV35S::MYC-GR, CaMV35S::MYC-GRL1, CaMV35S::YFP-GR, and CaMV35S::YFP-GRL1. All PCR fragments used for construct assembly were amplified using Pfu Ultra DNA polymerase (Agilent Technologies), and the fidelity of the constructs was confirmed by DNA sequencing. The constructs were transferred into A. tumefaciens strain GV3101 and transformed into Arabidopsis etr1-2/eri1-3 double mutants by floral dip (Clough and Bent, 1998). Transformants were selected by spraying with 0.1% and 0.2% (v/v) Finale herbicide (http://www.bayercropscience.com) at 1 and 2 weeks post germination, respectively. The presence of the transgene was confirmed by PCR, and the development of homozygous lines was achieved using a combination of herbicide selection and PCR screening.

Transient Expression in Tobacco

Transient expression of YFP- and GFP-tagged constructs in tobacco (Nicotiana tabacum ‘Petit Havana’) was performed as described previously (Batroko et al., 2000). Briefly, 3-mL overnight cultures of recombinant A. tumefaciens (strain GV3101) grown at 28°C in YEB medium (per liter: 5 g of beef extract, 1 g of yeast extract, 5 g of Suc, and 0.5 g of MgSO₄·7H₂O, supplemented with 50 μg mL⁻¹ kanamycin and 10 μg mL⁻¹ rifampicin) were harvested by centrifugation at 4,000 g for 5 min, and the pellet was washed and resuspended to an optical density at 600 nm of 0.05 in induction medium (50 mM MES, pH 5.6, 2 mM NaH₂PO₄, 0.05% [w/v] Glc, and 200 μM acetoxyxynorleucine). The ERD2-GFP and YFP-GRL2 cultures were mixed and injected into the abaxial leaf surface of eight 2-week-old plants using 1-mL needleless syringes. Following 48 h of expression, fluorescence of the reporter genes was detected by confocal laser scanning microscopy.

Confocal Laser Scanning Microscopy

Images were captured using an inverted laser scanning confocal microscope (LSM510 Meta; Zeiss) using a Plan- apochromat 63×/1.4 oil objective. Blue-shifted GFP was excited with a 458-nm argon laser line, and fluorescence emission was detected with a 475- to 525-nm band-pass filter. Enhanced-YFP was excited with a 514-nm argon laser line, and fluorescence emission was detected with a 520- to 555-nm band-pass filter. Fluorescence signal was acquired using the line-switch mode of the microscope. Image handling and processing were performed with the Zeiss AIM software of the microscope and Adobe Photoshop.
DNA Sequence and Phylogenetic Analysis

DNA sequences were assembled using Sequencher version 4.7 (GeneCodes; http://genecodes.com). Sequences used for comparisons and phylogenetic analysis were downloaded from organism-specific databases or from GenBank (http://www.ncbi.nlm.nih.gov/GenBank/; Supplemental Table S1). A neighbor-joining phylogenetic tree was constructed from a multiple sequence alignment of the deduced full-length amino acid sequences of selected GR homologs using MEGA version 5.0 software (Tamura et al., 2011).

Statistical Analysis

Statistical analyses were performed using SAS (SAS Institute; www.sas.com). The genotypic constituents were evaluated by Student’s t test and least square means.

The sequences described in this paper have been deposited in GenBank under accession numbers JQ659027 to JQ659031.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Golgi localization of YFP-SIGR2 in tobacco epidermal cells.

Supplemental Figure S2. Petiole responses to ethylene in CaMV35S::SIGR lines.

Supplemental Figure S3. Analysis of ethylene responses in CaMV35S::SIGR2 lines.

Supplemental Figure S4. Complementation of rti1-3 by untagged versions of SIGR and SIGR1.

Supplemental Figure S5. Complementation of rti1-3 by YFP-tagged versions of SIGR and SIGR1.

Supplemental Table S1. GR/RT1-related genes used in phylogenetic analysis.

Supplemental Table S2. Percentage amino acid identity between GR- and GRL1-related proteins from selected Solanaceae species.

Supplemental Table S3. Statistical analysis of the seedling triple-response assay in CaMV35S::SIGR1 lines of tomato.

Supplemental Table S4. Statistical analysis of the seedling triple-response assay in GR × CaMV35S::SIGR1 lines of tomato.

Supplemental Table S5. Statistical analysis of the seedling triple-response assay in CaMV35S::MYC-SIGR and CaMV35S::MYC-SIGR1 lines of Arabidopsis.

Supplemental Table S6. Statistical analysis of the seedling triple-response assay in CaMV35S::AIRTE1 lines of tomato.

Supplemental Table S7. Oligonucleotide primers used in this study.

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