EXPLORING THE ROLE OF THE GR/RTE1 FAMILY IN ETHYLENE SIGNALING

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ABSTRACT

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The gaseous plant hormone ethylene influences many aspects of plant development and mediates responses to biotic and abiotic stresses. A framework of the ethylene signaling pathway has been assembled using a combination of genetic and biochemical analysis in *Arabidopsis* (*Arabidopsis thaliana*) although this pathway is not completely understood. Furthermore, ethylene influences developmental processes and responses to environmental challenges that are not part of the *Arabidopsis* life cycle. Mutation at the *Green-ripe* (*Gr*) and *reversion to ethylene sensitivity 1* (*rte1*) loci, which encode homologous proteins of unknown biochemical function, influence ethylene responses in tomato (*Solanum lycopersicum*) and *Arabidopsis thaliana,* respectively. In *Arabidopsis*, RTE1 is required for function of the ETR1 ethylene receptor and acts predominantly through this receptor isoform via direct protein-protein interaction. In tomato, mutation at the *Gr* locus causes ectopic expression of *GR* leading to reduced ethylene responsiveness in a subset of tissues, which can be recapitulated by over-expression of *GR* driven by the *CaMV35S* promoter. The tomato genome contains two additional *GR* homologs designated *GREEN RIPE LIKE 1* (*SlGRL1*) and *GREEN RIPE LIKE 2* (*SlGRL2*), whose function, and role in ethylene signaling remain unknown.

In this study, the potential role of *SlGRL1* and *SlGRL2* in ethylene signaling was investigated together with their relationship to *SlGR* and *RTE1* of *Arabidopsis*. *SlGR, SlGRL1* and *SlGRL2* are differentially expressed during development and in response to ethylene treatment and each protein is predominantly localized in the Golgi. A combination of over-expression in tomato and

complementation of the *rte1-3* mutant allele indicates that *SlGR* and *SlGRL1* influence distinct ethylene responses suggesting the existence of separate ethylene-signaling modules in tomato that are influenced either individually by *Sl*GR or *Sl*GRL1 or together by both proteins. In contrast, over-expression of *SlGRL2* in tomato did not reveal any altered ethylene-related phenotypes suggesting that this gene may not be involved in ethylene signaling. Interestingly, over-expression of *AtRTE1* in tomato leads to reduced ethylene responsiveness in a subset of tissues, which more closely resemble the *SlGRL1* lines than *SlGR* lines.

Phylogenetic and sequence analysis indicated an expansion of the *GR/RTE1* family within the Solanaceae family of the eudicot lineage. Typically eudicot species contain a single gene closely related to *AtRTE1* and *SlGRL1*. In contrast, members of the Solanaceae family contain a second more divergent gene, defined by *SlGR* that appears to be restricted to this plant family. Furthermore, putative *GR* orthologs are relatively divergent when compared to putative *GRL1*orthologs of the Solanaceae family leading to the hypothesis that they may exhibit altered functional properties. This hypothesis was confirmed through over-expression of putative *GR* orthologs in tomato and through complementation of the *rte1-3* mutant allele. Utilizing a comparative approach, a series of amino acid residues were identified that correlate with the ability of the Solanaceae GR and GRL1 proteins to complement the *rte1* mutant phenotype and these were tested through the expression of synthetic constructs which carry altered amino acids leading to the identification of a set of 10 amino acids that are important for the ability of Solanaceae GR and GRL1 orthologs to complement the *rte1* mutant phenotype. Together, these data provide considerable new insight into the role of the *GR/RTE1* family in controlling ethylene responses in plants.

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LIST OF ABBREVIATIONS

1-MCP – 1-methylcyclopropene

35S – Cauliflower mosaic virus 35S promoter

ACC – 1-aminocyclopropane-1-carboxylic acid

ACO – ACC oxidase

ACS – ACC synthase

bar – BASTA resistant

BIFC – Bimolecular fluorescence complementation

BLAST – Basic Local Alignment Sequence Tool

bp – base pairs

Col-0 – *Columbia* ecotype 0

C-terminus – Carboxy terminus

e.g. – example

EMS – Ethyl methanesulfonate

ER – Endoplasmic reticulum

GFP – Green fluorescent protein

MET – methionine

NCBI – National Center Biotechnology Information

N-terminus – Amino terminus

PCR – Polymerase Chain Reaction

UBQ – Ubiquitin

YFP – Yellow fluorescent protein

Chapter 1 Introduction - Exploring the role of the GR/RTE1 family in ethylene signaling

Advances in plant hormone biology

Plant hormones are small molecules that regulate multiple aspects of plant growth and development together with a variety of responses to biotic and abiotic stresses. Nine major classes of plant hormones have been identified and characterized in depth: abscisic acid (ABA), ethylene, cytokinins (CKs), auxins, gibberellins (GAs), jasmonoyl-isoleucine (JA-Ile), brassinosteroids (BRs), salicylic acid (SA), and strigolactones (SLs) (Browse, 2005; Vert *et al.*, 2005; Loake and Grant, 2007; Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). Over the past 20 years, significant progress has been made in understanding the mechanisms of plant hormone biosynthesis and signaling. Much of this progress is due to the adoption of *Arabidopsis thaliana* as a model system for studying plant hormone biology. In particular, the ability to perform genetic screens for altered hormone responses on large numbers of mutagenized seeds coupled with the ability to rapidly progress from mutant phenotype to isolation of candidate gene has revolutionized understanding of the mechanisms plant hormone signaling and biosynthesis (Meyerowitz and Pruitt, 1985; Estelle and Somerville, 1986; Meyerowitz, 1987, 2001; Browse, 2009; Lin et al., 2009; Morant et al., 2010).

Although each hormone has varied roles and mediates different responses, several share common biosynthetic origins and action mechanisms. For example, ethylene, auxin, salicylic acid and jasmonoyl-isoleucine are derived from the amino acids methionine, tryptophan, phenylalanine, and isoleucine, respectively (Wichner and Libbert, 1968; Owens *et al.*, 1971; Hanson and Kende, 1976; MauchMani and Slusarenko, 1996; Staswick and Tiryaki, 2004). In addition, strigolactones and abscisic acid are isoprenoids derived from carotenoids (Parry and Horgan, 1992; Tan *et al.*, 1997; Matusova *et al.*, 2005). Common regulatory processes also control

aspects of hormone signaling and biosynthesis. For example, protein phosphorylation plays a role in the signaling of several hormones including abscisic acid, brassinosteroids, cytokinin and ethylene (Kieber et al., 1993; Eckardt, 2005; Mahonen et al., 2006; Fujii et al., 2007; Yoo et al., 2008). The ubiquitin-proteasome system has also emerged as an important regulatory mechanism that controls the perception, signaling and biosynthesis of nearly all the major plant hormones and often acts at multiple steps within these pathways (Moon *et al.*, 2004; Santner and Estelle, 2010; Wang and Deng, 2011). F-box proteins serve directly as hormone receptors in the case of auxin and JA-Ile, as components of the receptor complex for gibberellins, or at the control of turnover of receptors or additional signaling components in the case of ethylene and strigolactones and ABA (Stirnberg *et al.*, 2002; Guo and Ecker, 2003; Potuschak *et al.*, 2003; Wang *et al.*, 2004; Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005; Zhang *et al.*, 2005; Griffiths *et al.*, 2006; Stone *et al.*, 2006; Thines *et al.*, 2007; Christians *et al.*, 2009; Qiao *et al.*, 2009).

Although classic physiological experiments defined specific roles for individual hormones, it is widely accepted that plant hormones do not act in isolation but are interrelated by synergistic or antagonistic cross-talk. This crosstalk between hormones can occur at the level of biosynthesis, transport and signal transduction. For example, ethylene, jasmonate, and auxin are involved in the apical hook development. Jasmonate inhibits hook formation in a COI1-dependent manner (Ellis and Turner, 2002). Ethylene causes apical hook development by increasing *HOOKLESS1 (HLS1)* mRNA level (Lehman *et al.*, 1996; Li *et al.*, 2004). HLS1 decreases AUXIN RESPONSE FACTOR 2 (ARF2) protein level, which is an auxin response transcription factor, required for apical hook formation (Li *et al.*, 2004). Similarly, GA and ABA have antagonistic

effects on cell expansion which is mediated through GA and ABA alteration in stability of the DELLA regulatory protein, whereby ABA increases the stability of the DELLA protein RGA, while GA promotes its degradation (Achard *et al.*, 2006). As a result, ABA suppresses GA-induced cell expansion. Multiple hormones are also known to interact to stimulate the onset of senescence and fruit ripening (Fischer, 2012). For example, ethylene, ABA and JA-Ile are known to stimulate the onset of senescence whereas cytokinins and GA treatment can delay the onset of senescence.

The role of ethylene in plant development

Manipulation of ethylene to influence crop quality has been used for centuries. The ancient Egyptians would gash figs in order to stimulate ripening whereas the Chinese would burn incense in closed rooms to enhance the ripening of pears (Abeles *et al.*, 1992). In the nineteenth century, it was observed that leaks from illuminating coal gas caused defoliation of trees. In 1901, Neljubov discovered that the active component of these leaks was ethylene, demonstrating that ethylene caused the horizontal growth of etiolated pea seedlings, compared with the typical vertical growth of seedlings in air (Abeles *et al.*, 1992). Doubt discovered that ethylene stimulated abscission in 1917 and in 1924, Denny demonstrated that smoke from kerosene combustion in lanterns used to de-green citrus fruits contained ethylene as the active ingredient and demonstrated that ethylene is a fruit-ripening agent that can act at very low concentrations (Doubt, 1917; Denny, 1924). Ethylene synthesis by plant material was first demonstrated in apple fruit suggesting that ethylene may have a natural role in plant tissues rather than simply acting as an exogenous compound that can elicit responses in plant tissues (Gane, 1934). In

1962, it was reported that applied ethylene hastens mango ripening even after the climacteric has begun (Burg and Burg, 1962). These results demonstrated that fruit ripening is related with the production and action of ethylene.

Ethylene is now known to regulate a wide range of physiological responses (Abeles *et al.*, 1992; Bleecker and Kende, 2000; Guo and Ecker, 2004). For example, ethylene influences leaf epinasty, induces lateral cell expansion, inhibits nodulation, promotes leaf and flower senescence, breaks seed and bud dormancy in some species, induces fruit ripening and abscission (Goodlass and Smith, 1979; Abeles *et al.*, 1992; Lee and Larue, 1992; Bleecker and Kende, 2000; Guo and Ecker, 2004). Ethylene promotes internode and petiole elongation of submerged aquatic species (Kende *et al.*, 1998; Sauter, 2000). An agriculturally important example is rice (*Oryza sativa*). Submergence retards ethylene diffusion (Jackson, 1985). Ethylene enhances plant sensitivity to GA causing cell elongation and cell division, and suppresses ABA accumulation, which is a negative regulator of shoot elongation (Hoffmann-Benning and Kende, 1992; Azuma et al., 1995). As a result, submergence induces rapid internode elongation of submerged rice (Kende *et al.*, 1998; Benschop *et al.*, 2006). Ethylene induces adventitious root formation in leaves and vegetative stems, and suppression of ethylene sensitivity reduces adventitious root formation in petunia leading to cuttings that have reduced rooting capacity and poor horticultural performance (Phatak et al., 1981; Robbins et al., 1983; Gubrium et al., 2000). In *Arabidopsis*, ethylene induces root hair formation at atypical positions of the root and inhibitors of ethylene action, i.e., silver ions (Ag+) or the ethylene biosynthesis inhibitor, aminoethoxyvinylglycine (AVG) inhibit root hair formation (Masucci and Schiefelbein, 1994; Tanimoto et al., 1995; Cao et al., 1999; Dolan, 2001). Ethylene induces flowering in pineapple (*Ananas comosus Merr*.) and

its relatives (Burg and Burg, 1966). In agricultural applications, pineapple trees are normally sprayed with an ethylene-releasing compound, e.g. 2-chloroethanephosphonic acid, to synchronize flowering (Cooke and Randall, 1968). Ethylene also influences sex expression in some species, particularly cucurbits. For example, ethylene promotes formation of female flowers in cucumber, melon, and squash (McMurray and Miller, 1968; Rudich et al., 1969; Augustine et al., 1973; Takahashi et al., 1983; Papadopoulou et al., 2005). The developmental fate of flowers in these plants is not only affected by the exposed ethylene amount at the time of sex determination, but also affected by the perceptiveness sensitivity of specific floral organs to ethylene (Yin and Quinn, 1992, 1995; Little et al., 2007). In melon, ethylene perception sensitivity of the stamen (or petal) primordial, is critical in promoting femaleness (carpel development) (Little et al., 2007). Ethylene is also well known to regulate responses to biotic stresses (for example, pathogen infection) and abiotic stresses, such as wounding, hypoxia, ozone, chilling, or freezing (Abeles *et al.*, 1992; Bleecker and Kende, 2000; Guo and Ecker, 2004).

The role of ethylene in fruit ripening and its control in horticultural crops

During fruit ripening, a series of biochemical changes occur, which typically include softening, starch hydrolysis, sugar accumulation, formation of brightly colored pigments, production of aroma volatiles and the disappearance of organic acids and phenolic compounds, and loss of chlorophyll (Adams-Phillips et al., 2004; Giovannoni, 2004; Barry and Giovannoni, 2007). These biochemical changes convert an unpalatable fruit into one that is desirable for animal consumption and therefore facilitate seed dispersal.

Depending on the ability to undergo a transient increase in respiration and ethylene biosynthesis at the onset of fruit ripening, fruits are classified as climacteric or non-climacteric (Biale, 1964; Abeles et al., 1992). Climacteric fruits, including apple, banana, peach, and tomato, undergo a significant transient increase in respiration that signifies the onset of ripening (Burg and Burg, 1962, 1965). Treatment with ethylene can stimulate the respiratory climacteric and the rate of ripening in climacteric fruits and climacteric fruits are able to ripen off the vine (Burg and Thimann, 1959; Adato and Gazit, 1977; Karikari et al., 1979; Woodrow and Rowan, 1979). In contrast, non-climacteric fruits which include citrus, grapes and strawberries do not ripen off the vine and show no significant increase in respiration and ethylene production during ripening (Biale, 1964; Aharoni, 1968; Abeles et al., 1992). However, when exposed to exogenous ethylene, some non-climacteric fruits show enhanced respiration rates and can respond to ethylene through changes in gene expression and biochemical properties, including pigment changes (Stewart and Wheaton, 1972; Purvis and Barmore, 1981; Goldschmidt et al., 1993; El-Kereamy et al., 2003; Katz et al., 2004).

Several physiological, biochemical and genetic studies have illustrated the important role of ethylene in regulating fruit ripening. Fruit ripening is inhibited by ethylene synthesis inhibitors including AVG and Aminooxyacetic acid (AOA) and ethylene action inhibitors including Ag+ and 1-methylcyclopropene (1-MCP) (Sisler and Serek, 1997; Rogiers *et al.*, 1998; Saltveit, 2005; Kumar *et al.*, 2009). Suppression of ethylene biosynthesis by antisense RNA to 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) also represses fruit ripening in tomato and other species (Hamilton et al., 1990; Oeller et al., 1991;

Picton et al., 1993; Ayub et al., 1996; Flores et al., 2001; Flores et al., 2002; Dandekar et al., 2004; Silva et al., 2004; Lopez-Gomez et al., 2009; Hao et al., 2011). The tomato ethylene receptor mutant *Never-ripe* (*Nr*) displays a dramatic inhibition of fruit ripening and over-expression of a dominant negative mutant form of the *Arabidopsis ETR1* ethylene receptor also inhibits fruit ripening in tomato (Wilkinson et al., 1995; Wilkinson et al., 1997).

Variation in ethylene biosynthesis and signaling also impacts fruit ripening in apple and peach and melon. For example, *MdACS1-1* and *MdACS1-2* are alleles *ACS1* that are present in the Golden Delicious cultivar of apple (Sunako *et al.*, 1999). A short interspersed DNA element (SINE) is specific in the 5' region of *MdACS1-2*, which prevents *MdACS* expression at the onset of apple fruit ripening (Sunako *et al.*, 1999). As a result, apple cultivars that are homozygous for the *MdACS1-2* allele have reduced *ACS1* expression, lower ethylene production, and enhanced storage properties (Harada *et al.*, 2000; Oraguzie *et al.*, 2004; Sato *et al.*, 2004). In addition, a second apple *ACS* gene, *MdACS3*, also influences fruit ripening in apple (Wang *et al.*, 2009). Three *MdACS3* alleles have been identified in apple. *MdACS3a* represents the functional allele and *MdACS3a-G289V* and *Mdacs3a* are non-functional (Wang *et al.*, 2009). In *MdACS3a-G289V*, an amino acid substitution (glycine-289/valine) lies within the active site of the enzyme and is catalytically inactive whereas *Mdacs3a* is a null allele whose molecular basis remains unclear. Apple cultivars homozygous or heterozygous for the mutant alleles, show reduced ripening-related gene expression and maintain fruit firmness (Wang *et al.*, 2009). For example, Kitaro and Koukou that contain *Mdacs3a/MdACS3a-G289V*, have much lower ethylene production and longer fruit shelf life than Kotaro and Gala that contain *MdACS3a/MdACS3a-G289V* (Wang *et al.*, 2009).

Similar natural variation in ethylene biosynthesis occurs in peach cultivars carrying recessive *stony hard* (*hd*) mutation, *PpACS1* expression is eliminated in *hd* varieties during ripening causing reduced softening (Haji *et al.*, 2001; Tatsuki *et al.*, 2006). Although the mechanism underlying the *hd* phenotype is unclear, it is possible that a SNP or small deletion or insertion may disrupt a ripening-specific transcription factor binding site in the *PpACS1* promoter or that the *hd* phenotype may be caused by a mutation in a ripening-specific transcription factor (Tatsuki *et al.*, 2006). Melon is a phenotypically diverse fruit crop. Cantaloupe type of melons are climacteric fruit, whereas Honey Dew type of melons are nonclimacteric, and produce little or no ethylene during ripening (Miccolis and Saltveit, 1991; Hadfield *et al.*, 1995). Genetic analysis of a population of recombinant cantaloupe Charentais × PI 161375 (a nonclimacteric melon) inbred lines indicated that fruit abscission and ethylene production were controlled by two recessive independent loci designated *Abscission layer (Al)-3* and *Al-4* (Zheng and Wolff, 2000; Perin *et al.*, 2002). The relationship between these two loci and ethylene production and sensitivity remains to be defined.

Controlling the effects of ethylene is important in horticultural crops both to stimulate ripening and improve quality, but also to control the rate of ripening and prevent over-ripening, senescence and postharvest deterioration (Abeles *et al.*, 1992; Bleecker and Kende, 2000; Barry and Giovannoni, 2007). Ethylene treatment is used commercially to facilitate uniform ripening in tomato, banana, and melon; degreening of citrus fruits. However, it is more common to reduce the effects of ethylene to limit postharvest deterioration and extend shelf-life of horticultural produce by adopting handling and storage strategies that reduce ethylene biosynthesis and / or

responsiveness including low temperature and controlled atmosphere storage, which involves decreasing oxygen concentration and increasing carbon dioxide concentration during storage which reduces respiration rate, ethylene biosynthesis and decreases sensitivity to ethylene (Kader *et al.*, 1989; Beaudry, 1999; Dilley, 2006). Furthermore, the ethylene action inhibitor 1-MCP is now widely used to block ethylene responses to prolong apple storage and is being experimentally evaluated in other crops (Sisler, 2006; Watkins, 2006; Barry and Giovannoni, 2007).

The regulation of ethylene biosynthesis

Ethylene is synthesized from methionine (MET) by a 3-step process (Adams and Yang, 1979; Kende, 1993; Bleecker and Kende, 2000). The first step converts MET to *S*-adenosylmethionine (SAM) which is catalyzed by the enzyme SAM synthetase at the expense of ATP (Chou and Talalay, 1972; Ravanel *et al.*, 1998). The second step converts SAM to 1-aminocyclopropane-1-carboxylic acid (ACC) and 5'-methylthioadenosine (MTA), catalyzed by ACC synthase (ACS) (Yu *et al.*, 1979; Bleecker *et al.*, 1986). MTA is reutilized for the synthesis of methionine *via* the Yang cycle, which allows for the regeneration of methionine which may be particularly important in tissues that synthesize large quantities of ethylene when demand for MET is high (Miyazaki and Yang, 1987). In the third step, ACC is oxidized to ethylene (C_2H_4), CO_2, and hydrogen cyanide (HCN) by ACC oxidase (ACO) (Hamilton *et al.*, 1991). The HCN is detoxified to β-cyanoalanine by β-cyanoalanine synthase to prevent toxicity of accumulated cyanide during high rates of ethylene synthesis (Peiser *et al.*, 1984; Yip and Yang, 1988). Ethylene synthesis is normally limited by the supply of the immediate precursor, ACC, which is

formed by ACS. SAM synthetase, ACS, and ACO, are encoded by multigene families in all plant species examined, allowing the plant flexibility to differentially regulate ethylene biosynthesis at different stages of development or in response to individual stimuli (Sato and Theologis, 1989; Yip *et al.*, 1992; Zarembinski and Theologis, 1994; Whittaker *et al.*, 1997; Bleecker and Kende, 2000). Indeed, many experiments have revealed differential temporal and spatial expression of *ACS* and *ACO* gene families in different tissues at various stages of development and in response to different environmental stimuli (Rottmann *et al.*, 1991; Barry *et al.*, 1996; Blume and Grierson, 1997; Nakatsuka *et al.*, 1998; Barry *et al.*, 2000; Liu and Zhang, 2004; Chae and Kieber, 2005; Peng *et al.*, 2005; Sell and Hehl, 2005; Wang *et al.*, 2005).

Notably, two systems of ethylene production exist in plant tissues. System 1 operates during vegetative growth and in immature fruits and flowers and is under negative feedback regulation by ethylene (autoinhibitory). In contrast, system 2 operates during the ripening of climacteric fruits and during flower senescence in some plant species and is under positive feedback regulation by ethylene (autocatalytic) (McMurchie et al., 1972). Autocatalytic ethylene synthesis may operate to facilitate rapid ripening and senescence to complete the life cycle of those tissues as efficiently as possible. Research on the tomato fruit ripening system has provided insight into the molecular mechanisms that operate during system 1 and system 2 ethylene biosynthesis. For example, *LeACS1A* and *LeACS6* are responsible for system 1 ethylene biosynthesis in green tomato fruit and ethylene treatment inhibits their expression (Barry *et al.*, 2000). *LeACS1A* and *LeACS4* function in the transition between system 1 and system 2 and both *LeACS2* and *LeACS4* are involved in system 2 ethylene biosynthesis and are positively regulated by ethylene (Olson et al., 1991; Rottmann et al., 1991; Yip et al., 1992; Lincoln et al., 1993; Nakatsuka et al., 1998;

Barry et al., 2000). The ACC oxidase genes, *LeACO1* and *4*, are also involved in system 2 ethylene production (Nakatsuka *et al.*, 1998). The MADS box transcription factor RIPENING INHIBITOR (RIN) is required for the normal ripening of tomato fruit, including autocatalytic ethylene synthesis mediated by increased expression of *LeACS2* and *LeACS4* (Tigchelaar *et al.*, 1978; Vrebalov *et al.*, 2002; Ito *et al.*, 2008; Fujisawa *et al.*, 2011). RIN directly binds to the promoters of *LeACS2* and *LeACS4* to regulate their expression during fruit ripening (Ito et al., 2008; Fujisawa et al., 2011).

In addition to regulation at the transcriptional level, ethylene biosynthesis can also be regulated post-translationally at the level of enzyme activity and protein stability (Liu and Zhang, 2004; Chae and Kieber, 2005). For example, *ethylene overproducer 1* (*eto1*) is a recessive mutation that possesses a triple response phenotype due to increased production of ethylene (Guzman and Ecker, 1990; Wang *et al.*, 2004). *ETO1* encodes a BTB-TPR (broad-complex, tramtrack, bric-a-brac/tetratricopeptide repeat) protein that targets *Arabidopsis* ACS5 for protein degradation through the 26S proteasome (Wang *et al.*, 2004). Therefore, loss-of-function mutants at the *eto1* locus overproduce ethylene due to increased stability of ACS5 (Chae *et al.*, 2003). The dominant *eto2* and *eto3* mutants also increase ethylene production in *Arabidopsis* by increasing the stability of ACS proteins (Chae *et al.*, 2003). The *eto2* mutant is caused by a single nucleotide insertion in the *ACS5* gene, resulting in a frameshift that alters the last 12 residues of the ACS5 protein. The *eto2-1* mutation does not alter the expression of *ACS5* although ethylene production is 20-fold higher than in wild type, suggesting that the increased ethylene production is the result of altered enzyme activity or protein stability (Vogel *et al.*, 1998). Similarly, the *eto3* mutant is

due to a missense mutation in the C-terminal of ACS9 that increases protein stability (Chae *et al.*, 2003).

Insight into the mechanism of the *eto2-1* mutation was derived from studies of the tomato *Le*ACS2, which is phosphorylated in response to wounding (Tatsuki and Mori, 2001). The phosphorylation site of *Le*ACS2 (Ser-460) is equivalent to the site of *Arabidopsis* ACS5 (Ser-400), which is mutated in *eto2-1* (Tatsuki and Mori, 2001). It is reported that *Le*CDPK2, and other protein kinases that are still unknown, is activated by the increase of stress stimuli and is involved in the phosphorylation of Ser-460 in *Le*ACS2 (Tatsuki and Mori, 2001; Kamiyoshihara et al., 2010). The phosphorylation of *Le*ACS2 by protein kinases leads to ACS accumulation and subsequent ethylene production in tomato (Tatsuki and Mori, 2001; Kamiyoshihara et al., 2010). Ethylene overproduction in *eto2-1 Arabidopsis* mutant may be caused by the phosphorylation of the mutated ACS5.

Ethylene signaling in *Arabidopsis*

Genetic Screens for Identifying Ethylene Signaling Mutants

Phenotypic screens for altered ethylene responsiveness, performed on mutagenized populations of *Arabidopsis*, have defined the mechanisms that regulate ethylene perception and signaling. In the presence of ethylene, dark-grown *Arabidopsis* seedlings show inhibition of root and hypocotyl elongation, increased radial swelling of the hypocotyl and exaggeration of the apical

hook, phenotypes collectively known as the triple response (Bleecker *et al.*, 1988; Guzman and Ecker, 1990).

The triple response has been widely used in different genetic screens to identify mutants with compromised ethylene responses. For example, several mutants have been reported which are insensitive to ethylene and therefore lack the triple response phenotype in the presence of exogenous ethylene (Bleecker *et al.*, 1988; Guzman and Ecker, 1990; Kieber and Ecker, 1993; Roman *et al.*, 1995). A variation of this screen identified mutants that possess a constitutive triple response in the absence of ethylene leading to the identification of mutants involved in ethylene signaling and biosynthesis (Guzman and Ecker, 1990; Kieber *et al.*, 1993). Characterization of these mutants has led to the identification of many genes involved in ethylene signaling and analysis of epistatic interactions has facilitated placement of these genes within a signaling pathway (Kieber *et al.*, 1993; Roman *et al.*, 1995; Chao *et al.*, 1997).

Ethylene Receptors in Arabidopsis and ethylene perception

Five ethylene receptors are present in *Arabidopsis*: ETHYLENE RESPONSE 1 (ETR1), ETHYLENE RESPONSE SENSOR 1 (ERS1), ETHYLENE RESPONSE 2 (ETR2), ETHYLENE RESPONSE SENSOR 2 (ERS2), and ETHYLENE INSENSITIVE 4 (EIN4) (Bleecker *et al.*, 1988; Hua *et al.*, 1995; Hua *et al.*, 1998; Sakai *et al.*, 1998). The receptors are functionally redundant as single loss-of-function receptor mutants lack evident phenotypes, with the exception of *etr1-7* that shows relatively slow hypocotyl elongation (Cho and Yoo, 2007). Double, triple, and quadruple loss-of-function mutants show constitutive ethylene responses

when grown in air, indicating that binding of ethylene inactivates receptor function rather than activates ethylene signaling, and also implying the ethylene receptors act as negative regulators of the ethylene response pathway (Hua and Meyerowitz, 1998). The ethylene receptors are similar in structure to bacterial two-component receptor kinases (Chang *et al.*, 1993). They possess a modular structure which includes N-terminal transmembrane domains containing the ethylene binding domain, a GAF domain, which may be involved in mediating heteromeric interactions among the receptors, and signal output domains in the C-terminal region, which include a histidine kinase domain (or Ser/Thr kinase domain) and in some cases a receiver domain (Chang *et al.*, 1993; Schaller and Bleecker, 1995; Gamble *et al.*, 1998; Gao *et al.*, 2008). ERS1 and ERS2 lack the receiver domain at the C-terminus.

The ethylene receptors are divided into two subfamilies. Subfamily-1 (ETR1 and ERS1) has three N-terminal membrane-spanning domains and a conserved histidine kinase domain at the C-terminus (Chang *et al.*, 1993; Hua *et al.*, 1995; Gamble *et al.*, 1998). Subfamily-2 receptors (ERS2, ETR2 and EIN4) have an extra N-terminal hydrophobic domain, and possess Ser/Thr kinase activity (Moussatche and Klee, 2004). However, ERS1, a subfamily-1 receptor, has both histidine kinase and Ser/Thr kinase activity (Moussatche and Klee, 2004). In *Arabidopsis*, subfamily-1 receptors have stronger effects on ethylene responses than subfamily-2. For example, loss-of-function of subfamily-1 receptors in *Arabidopsis* results in a stronger constitutive ethylene-response phenotype in air-grown plants than loss of subfamily-2 receptors (Hua and Meyerowitz, 1998; Hall and Bleecker, 2003; Wang et al., 2003; Qu et al., 2007).

Ethylene receptors localize to the endoplasmic reticulum (ER) and Golgi membranes, (Chen et al., 2002; Ma et al., 2006; Dong et al., 2008). Localization to the ER and Golgi membrane may allow the ethylene signaling to interact with other pathways. Cytokinin receptors are also histidine kinases and structurally related with ethylene receptors (Inoue *et al.*, 2001; Ueguchi *et al.*, 2001; Yamada *et al.*, 2001). Recent experiments using cytokinin binding assays, fluorescent protein fusions, and biochemical fractionation revealed that the cytokinin receptors are also localized in the ER (Wulfetange *et al.*, 2011). Localization of both receptors in the ER may facilitate the cross talk between cytokinin and ethylene. However, ethylene receptors may also localize to other cellular compartments. For example, NTHK1, an ethylene receptor in tobacco, has been reported to localize to the plasma membrane (Xie *et al.*, 2003). Ethylene binding does not appear to affect subcellular localization of receptors (Chen *et al.*, 2002).

Binding of ethylene to the receptors is mediated by a copper cofactor (Rodriguez *et al.*, 1999). The ethylene receptor ETR1 can form a disulfide-linked homodimer, with two receptor monomers binding to a single copper ion (Rodriguez *et al.*, 1999). RESPONSE TO ANTAGONIST 1 (RAN1) delivers copper to the ethylene receptors (Hirayama *et al.*, 1999; Woeste and Kieber, 2000). Mutations at the *ran1* locus lead to non-functional ethylene receptors that lack a copper ion leading to constitutive ethylene responses (Hirayama *et al.*, 1999; Woeste and Kieber, 2000).

Seven amino acids (I62, C65, H69, D25, Y32, I35, and P36) within the first and second transmembrane helices of each receptor monomer are thought to be involved in chelating the copper ion based on analysis of *etr1-1*(C65Y), *etr1-3*(A31V), *etr1-4*(I62F) mutant alleles, and

additional site-directed mutagenesis (Chang et al., 1993; Schaller and Bleecker, 1995; Hall et al., 1999; Rodriguez et al., 1999; Wang et al., 2006). The mutated receptors are locked in the signaling state, leading to ethylene-insensitive plants, so mutations within the ethylene binding domain typically lead to dominant ethylene insensitivity. These types of dominant point mutations have been isolated in the *ETR1*, *ETR2*, and *EIN4* genes. Similar mutations introduced into *ERS1* and *ERS2* also confer dominant insensitivity (Hua *et al.*, 1995; Hua *et al.*, 1998).

As mentioned above, ETR1 functions as a disulfide-linked homodimer, and Cys-4 and Cys-6 are the sites that form disulfide bonds in the homodimer (Schaller *et al.*, 1995). Formation of a disulfide-linked ERS1 homodimer has also been detected and the existence of heterodimers proposed (Schaller et al., 1995; Hall et al., 2000; Cancel and Larsen, 2002; Liu et al., 2010). These heteromeric receptor interactions require the GAF domain of the receptors (Gao *et al.*, 2008). Moreover, results in transiently transformed tobacco cells and in yeast mating-based split-ubiquitin system indicated that all five *Arabidopsis* ethylene receptors form both homodimers and heterodimers in all possible combinations (Grefen *et al.*, 2008).

Control of the receptors can occur at the transcriptional level as the expression of some receptors (*ERS1*, *ETR2* and *ERS2*) is responsive to ethylene (Hua *et al.*, 1998). In addition, ETR2 may also be a target for degradation by the 26S proteasome in response to ethylene binding (Chen *et al.*, 2007).

An endomembrane localized ethylene signaling complex

Recent research has revealed the existence of an ethylene signaling complex located within the endomembrane system that includes the ethylene receptor ETR1 together with at least three components that physically and genetically interact with this receptor isoform, including CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), ETHYLENE INSENSITIVE 2 (EIN2), and REVERSION TO ETHYLENE SENSITIVITY 1 (RTE1) (Clark et al., 1998; Chen et al., 2002; Dong et al., 2008; Bisson et al., 2009).

CTR1 encodes a protein with homology to serine threonine MAP KINASE KINASE KINASEs (MAP3Ks) which acts downstream of the ethylene receptors as a negative regulator of ethylene signaling (Kieber *et al.*, 1993; Hua *et al.*, 1995; Roman *et al.*, 1995). CTR1 interacts with the histidine kinase domain and receiver domains of the ethylene receptors and shows preferential interaction with ETR1, which may possibly explain why ETR1 appears to have a more prominent role in signaling than the other ethylene receptors (Clark *et al.*, 1998; Hall and Bleecker, 2003; Wang *et al.*, 2003).

RTE1 is also required for the function of the ETR1 receptor and moreover appears to be specific for the function of this particular receptor isoform (Resnick *et al.*, 2006; Zhou *et al.*, 2007; Rivarola *et al.*, 2009). *RTE1* encodes a membrane bound protein of unknown biochemical function with an N-terminus that lies in the cytoplasm and a C-terminus in Golgi, or both ER and Golgi (Zhou et al., 2007; Dong et al., 2008; Dong et al., 2010). The *rte1* mutant was identified as a suppressor of the *etr1-2* mutant allele which is a weak dominant receptor allele that does not disrupt ethylene binding (Resnick *et al.*, 2008). Mutant alleles at the *rte1* locus suppress ethylene insensitivity conferred by the *etr1-2* mutant allele as well as additional dominant ethylene-

insensitive alleles (Resnick *et al.*, 2006; Zhou *et al.*, 2007; Resnick *et al.*, 2008). However, *rte1* is unable to suppress all dominant *ETR1* alleles, nor alleles that confer dominant ethylene insensitivity in additional ethylene receptors (Resnick *et al.*, 2006; Zhou *et al.*, 2007; Rivarola *et al.*, 2009).

Over-expression of *RTE1* causes a weak ethylene-insensitive phenotype which is dependent of the presence of the ETR1 receptor and can be restored by expression of the ETR1 N-terminus (Zhou *et al.*, 2007). RTE1 promotes ETR1 signaling by a physical association between RTE1 and the ETR1 N-terminus, an interaction which is not disrupted by ethylene treatment (Dong *et al.*, 2010). Together, these data indicate that RTE1 action is specific for the ETR1 receptor and moreover is specific for certain alleles of *ETR1*, the significance of which is not understood (Resnick *et al.*, 2006; Zhou *et al.*, 2007; Rivarola *et al.*, 2009). The *Arabidopsis* genome carries a second homolog of *RTE1*, known as *RTE1-HOMOLOG* (*AtRTH*), although the function of this gene is unknown. *At*RTH was reported to be localized to the ER and nucleus, which is different from the ER and Golgi localization of RTE1(Zhang et al., 2012).

EIN2 is a positive regulator of the ethylene signaling pathway that acts genetically downstream of CTR1(Roman *et al.*, 1995). The *ein2* loss-of-function mutant is completely ethylene insensitive, which indicates that EIN2 is a central regulator of ethylene signaling (Guzman and Ecker, 1990). *EIN2* encodes a 12-pass transmembrane protein, which is similar to the NRAMP family of metal transporters, with an N-terminal transmembrane domain and a C-terminal membrane extrinsic domain (Alonso *et al.*, 1999). Over-expression of the *EIN2* C-terminus in *Arabidopsis* leads to a constitutive ethylene response, which suggests that the EIN2 C-terminus

functions in signal transduction although the true biochemical function of EIN2 is unknown (Alonso *et al.*, 1999). The EIN2 protein accumulates upon ethylene exposure and in the *ctr1-1* mutant, but EIN2 does not accumulate in the *etr1-1* mutant background (Qiao *et al.*, 2009). The degradation of EIN2 is triggered by two ethylene-regulated F-box proteins EIN2 TARGETING PROTEIN 1 (ETP1) and EIN2 TARGETING PROTEIN 2 (ETP2) (Qiao *et al.*, 2009).

EIN2 is also located at the ER membrane where it interacts with all five ethylene receptors in *Arabidopsis* (Bisson *et al.*, 2009). The interaction between ETR1 and EIN2 is modulated by the autokinse activity of ETR1 (Bisson and Groth, 2010). EIN2 interacts with all ethylene receptors (Bisson and Groth, 2010). Since subfamily-2 has a different kinase domain with subfamily-1 receptors, its mechanism of interaction with EIN2 may be different. The interaction may be mediated by phosphorylation of serine or threonine residues in subfamily-2 receptors or by heterodimers between subfamily-1 receptors and subfamily-2 receptors, but these possibilities remain to be determined (Bisson and Groth, 2010). In the absence of ethylene, the EIN2 at ER membrane shows CTR1 kinase-dependent phosphorylation, while the presence of ethylene induces dephosphorylation of EIN2 at S645, which leads to proteolytic cleavage at S645. As a result, the carboxyl-terminal of EIN2 (EIN2-C') is released and rapidly translocates to the nucleus and activates the components in the downstream of signaling (Qiao et al., 2012).

A newly identified EIN2-interacting protein EIN2 C-terminus Interacting Protein 1 (ECIP1) is thought to function together with subfamily-2 receptors (ETR2 and EIN4) and EIN2, although the specific mechanism is unclear (Lei *et al.*, 2011). ECIP1 is mainly localized in the cytoplasm and acts as a negative regulator of ethylene signaling and salt tolerance (Lei *et al.*, 2011). Loss-

of-function of ECIP1 resulted in enhanced ethylene responsiveness, increased plant survival rate under salt stress, reduced cotyledon size, but lower seed germination under the salt stress (Lei *et al.*, 2011). Further research is required to define the exact function of ECIP1 and its role in ethylene signaling.

Tetratricopeptide Repeat Protein1 (TRP1) is another protein, which interacts with receptor ERS1 in the yeast two-hybrid system and in vivo immuno-precipitation pull-down assays (Lin et al., 2009). It is highly expressed in the vascular tissue, anthers and pollen, abscission zone, and accumulates following ethylene treatment (Lin et al., 2009). Over-expression of *TRP1* in wild type *Arabidopsis* resulted in dwarfed plants with reduced fertility, altered leaf/silique morphology, and enhanced expression of *AtChiB* (Lin et al., 2009). Although over-expression of *TRP1* in the *etr1-1* mutant altered aspects of the mutant phenotypes, it did not change the dominant ethylene insensitivity. The biochemical function of TRP1 is unclear, but it may function as an adaptor to facilitate receptor degradation (Lin et al., 2009).

Ethylene signaling events in the nucleus

ETHYLENE INSENSITIVE 3 (EIN3) and EIN3-LIKE (EIL) proteins, which consist of five members (EIL1-5), act downstream of EIN2 (Chao *et al.*, 1997). EIN3 and EILs are members of a plant specific family of transcription factors that accumulate in the nucleus (Chao *et al.*, 1997; Solano *et al.*, 1998). EIN3 and EIL1 play significant roles in ethylene signaling as the *ein3/eil1* double mutant displays almost complete ethylene insensitivity in the triple response assay (Alonso *et al.*, 2003). Furthermore, over-expression of either EIN3 or EIL1 in wild-type and the

ein2 mutant confers a constitutive ethylene response at all stages of development (Chao *et al.*, 1997). EIN3 and EIL1 are partially redundant (Chao *et al.*, 1997; Alonso *et al.*, 2003). EIL1 mainly functions in the leaf and stem of adult plants, but EIN3 largely regulates ethylene responses in seedlings (An *et al.*, 2010).

As with EIN2, the EIN3/EIL proteins accumulate after ethylene treatment (Guo and Ecker, 2003; Potuschak *et al.*, 2003) and EIN3/EIL protein stability is regulated by two F-box proteins, known as EIN3 BINDING F-BOX 1 (EBF1) and EIN3 BINDING F-BOX 2 (EBF2) (Guo and Ecker, 2003; Potuschak *et al.*, 2003; An *et al.*, 2010). EBF1 and EBF2 are partially redundant. EBF1 mainly functions in the absence of ethylene and during the early stages of seedling responses to ethylene, and EBF2 affects the later stages of the triple response and the recovery stage after ethylene removal (Binder *et al.*, 2007). Although *EBF1* and *EBF2* display genetic redundancy, they also possess independent functions. *EBF2*, but not *EBF1*, is involved in negative feedback regulation of EIN3 through direct binding of EIN3 to the *EBF2* promoter which activates the *EBF2* expression promoting EIN3 degradation (Konishi and Yanagisawa, 2008).

The expression of *EBF1* and *EBF2* and their protein accumulation are also tightly regulated. *EBF1* and *EBF2* transcript levels are regulated by ETHYLENE-INSENSITIVE5 (EIN5), which encodes a 5'→3' exoribonuclease that actively promotes *EBF1* and *EBF2* mRNA decay (Olmedo *et al.*, 2006). EBF proteins may also be regulated by 26S proteasome. It was reported that EBF proteins are very stable in the *ein2* mutant and EIN2 C -terminus interacts with a negative regulator of the SCF complex (An et al., 2010). According to these data, EBF1 and

EBF2 proteins may be subject to an EIN2 mediated autoubiquitination process and degraded by as yet unknown F-box proteins.

A second pathway has also been proposed that operates independently of EIN2 to stabilize EIN3 by way of a phosphorylation cascade involving MKK9-MPK3/MPK6 (Yoo et al., 2008). EIN3 has two phosphorylation sites (T174 and T592), which appear to have opposing functions in mediating EIN3 stability (Yoo et al., 2008). In the absence of ethylene, CTR1 inactivates MKK9-MPK3/MPK6, T592 is phosphorylated, by an unknown protein kinase and EIN3 is degraded. In the presence of ethylene, CTR1 is inactivated and repression of MKK9-MPK3/MPK6 is released leading to phosphorylation of T174 and EIN3 stabilization (Yoo et al., 2008). The role of this proposed bifurcated MAPK cascade in regulating ethylene responses through EIN3 stability is still a matter for debate in the scientific literature. Several independent studies have demonstrated that ethylene signaling lies downstream of MPK6 activation and that the MKK9-MPK3/MPK6 module may function in the stability of ACS2 and ACS6 to control ethylene biosynthesis (Liu and Zhang, 2004; Joo *et al.*, 2008; Xu *et al.*, 2008; Bethke *et al.*, 2009; An *et al.*, 2010).

EIN3 functions as a dimer and binds to the PRIMARY ETHYLENE RESPONSE ELEMENT (PERE) in the promoters of ETHYLENE RESPONSE FACTOR 1 (ERF1), ETHYLENE RESPONSE DNA BINDING FACTOR (EDF)1, 2, 3, 4, and other early ethylene response genes (Solano *et al.*, 1998). EIN3/EILs has been implicated in the regulation of several genes involved in diverse processes including those involved in light signaling, biotic stress defense, chlorophyll

biosynthesis, and ethylene signaling (Solano *et al.*, 1998; Konishi and Yanagisawa, 2008; Chen *et al.*, 2009; Zhong *et al.*, 2009; Boutrot *et al.*, 2010).

ERF1 and other ERFs are members of the APETALA2 (AP2)/ERF superfamily of transcription factors, which is one of the largest groups of transcription factors in plants (Hao *et al.*, 1998; Brown *et al.*, 2003; Wessler, 2005). According to the number of AP2/ERF domains, the AP2/ERF superfamily is divided into ERF, AP2, and related-to-ABI3/VP1 (RAV) families (Sakuma *et al.*, 2002; Nakano *et al.*, 2006). In *Arabidopsis*, 145 genes are predicted to encode AP2/ERF proteins and more than 80% of these genes belong to the ERF family (Sakuma *et al.*, 2002). The ERF family is further classified into two subfamilies: dehydration-responsive element binding protein (DREB) subfamily and ERF subfamilies. The DREB subfamily proteins interact with a CCGAC sequence (Jiang *et al.*, 1996), and are involved in plant development, hormonal signal transduction and response to abiotic stress (Hsieh *et al.*, 2002; Narusaka *et al.*, 2003; Qin *et al.*, 2008). The ERF subfamily proteins typically bind to an AGCCGCC sequence, referred to as the GCC box, and are involved in plant defense, stress signaling, plant development, and ethylene signaling responses (Ohme-Takagi and Shinshi, 1995; Yang *et al.*, 2005; Onate-Sanchez *et al.*, 2007; Pre *et al.*, 2008). *Arabidopsis* has 122 predicted ERF genes (Nakano *et al.*, 2006). The N-terminus of ERF subfamily proteins contains the conserved DNA binding domain whereas the C-terminus is more divergent and may mediate signaling specificity (Hao *et al.*, 1998). As ERFs are encoded by a large gene family and constitute the last step in the ethylene signaling pathway prior to downstream target genes, they potentially contribute to functional diversity of ethylene responses, possibly mediating tissue, development or stimulus-specific transcription. Each ERF protein normally has more than one function. For example, The ERF1

protein regulates the expression of various genes including *PDF1-2*, *prb-1b* (*PR1*), *β-1*, 3-glucanase (PR2), chitinase (PR3), and osmotin (PR5) etc (Ohme-Takagi and Shinshi, 1995; Buttner and Singh, 1997; Zarei et al., 2011).

The ERF subfamily of proteins function by either activating or repressing the expression of target genes through the interaction with the GCC box (Fujimoto *et al.*, 2000). In *Arabidopsis*, ERF1, 2 and 5 are transcriptional activators and ERF3, 4, 7, 10, 11 and 12 repress GCC-box-containing genes (Fujimoto *et al.*, 2000; Ohta *et al.*, 2000). Recently, reports have suggested that some ERF subfamily proteins also regulate genes through additional sequence motifs within promoters. For example, ERF Required for Nodulation (ERN), which is an ERF protein and is required for nodulation, binds specifically to the NF-box (Andriankaja *et al.*, 2007). *Arabidopsis* RAP2.6 binds to both GCC box and the CE1 element (TGCCACCGG), which is commonly present in the promoter region of many stress-related genes (Zhu *et al.*, 2010). ERF proteins also regulate gene expression by protein-protein interactions between an ERF protein and elicitor-responsive element binding factors. For example, AtEBP, which is an ERF protein, interacts with an octopine synthase (*ocs*) element binding factors (OBFs), a class of basic-region leucine zipper (bZIP) transcription factors (Buttner and Singh, 1997). In addition to mediating ethylene responses, ERFs are also involved in controlling ABA, JA and SA mediated transcription, suggesting that they may function in crosstalk between these plant hormone signaling pathways (Gutterson and Reuber, 2004). For example, at least five ERFs, including ERF1, ERF2, ERF3, ERF4, and RAP2.10, are induced by JA, suggesting that these components may be mediators of crosstalk between the JA and ethylene signaling pathway (Brown *et al.*, 2003; Lorenzo *et al.*, 2003).

In agreement with a dual role in both JA and ethylene signaling, manipulation of *ERF* expression in plants leads to phenotypes related to plant defense (Onate-Sanchez and Singh, 2002; Lorenzo *et al.*, 2003; Gutterson and Reuber, 2004; McGrath *et al.*, 2005). For example, *ERF1* over-expression in *Arabidopsis* leads to a stunted plant phenotype with etiolated seedlings grown in air displaying shortening and thickening of roots and hypocotyls but no exaggeration of the apical hook (Solano *et al.*, 1998). *ERF1* over-expression lines also display increased resistance to several necrotrophic fungi, including *B. cinerea* and *P. cucumerina* (Berrocal-Lobo *et al.*, 2002). Similarly, over-expression of *ERF14* in *Arabidopsis* leads to reduced plant size, loss of seed set, and enhanced defense-related gene expression whereas loss-of-function mutants display increased susceptibility to *Fusarium oxysporum* (Onate-Sanchez *et al.*, 2007). In contrast, *erf4* loss-of-function mutants are more resistant to *F. oxysporum* (McGrath *et al.*, 2005).

A model for ethylene signaling in Arabidopsis

Ethylene signaling is negatively regulated (Hua and Meyerowitz, 1998). In the absence of ethylene, the ethylene receptors directly bind to CTR1, which is also a negative regulator of ethylene signaling (Clark *et al.*, 1998; Cancel and Larsen, 2002; Gao *et al.*, 2003). RTE1 binds to ETR1 N-terminus to promote ETR1 signaling (Dong *et al.*, 2010). This results in a complex that includes RTE1, an ethylene receptor dimer, and CTR1. The association between receptors and CTR1 may prevent receptor interaction with EIN2. EIN2 is turned over by the F-box proteins ETP1/2 and ethylene signaling is inhibited (Qiao *et al.*, 2009). In the presence of ethylene, the interaction between ethylene and ethylene receptors induces a conformational change (Binder *et*

al., 2010). CTR1 is released and inactivated, which allows interaction of the kinase domain of the receptors with EIN2 (Bisson *et al.*, 2009; Bisson and Groth, 2010). This leads to an additional complex comprised of RTE1, a receptor dimer, and EIN2. Subsequently, the S645 of EIN2 is dephosphorylated, leading to releasing of C-terminus of EIN2(EIN2-C') (Qiao et al., 2012). The released EIN2-C' rapidly translocates to the nucleus and activates EIN3/EILs proteins, and in the end, the ethylene responsive target genes are transcribed (Chao et al., 1997; Yanagisawa et al., 2003; Alonso and Stepanova, 2004; Yoo et al., 2008; Qiao et al., 2012). Control of ethylene responses can be achieved at multiple levels including turnover of EIN3/EIL proteins through the action of two F-box proteins, EBF1 and EBF2 (Guo and Ecker, 2003; Potuschak *et al.*, 2003; An *et al.*, 2010).

Alternative pathways that bypass CTR1 and EIN2 that may function through a MKK9-MPK3/MPK6 module to control EIN3 protein levels have also been proposed, although discussion of the role of this module is debated in the literature (Hua and Meyerowitz, 1998; Larsen and Chang, 2001; Liu and Zhang, 2004; Joo *et al.*, 2008; Xu *et al.*, 2008; Yoo *et al.*, 2008; Bethke *et al.*, 2009; An *et al.*, 2010).

Ethylene receptor signaling in diverse plant species

Hormone signaling pathways are generally conserved in higher plant species and components of the ethylene signaling pathway have been isolated and functionally characterized from many plant species (Sato-Nara *et al.*, 1999; Takahashi *et al.*, 2002; Guo and Ecker, 2004; Kendrick and Chang, 2008; Ma *et al.*, 2010; Tatsuki, 2010). However, some developmental processes or responses to environmental signals in which ethylene is influential are unique to specific groups of plants, *e.g.*, the ripening of fleshy fruits, nodulation, and elongation in response to flooding.

As such, both ethylene biosynthesis and signaling mechanisms together with their corresponding physiological outputs may vary between plant species, extending or challenging concepts developed using *Arabidopsis* as a model system. Conservation of ethylene perception and signaling between diverse species is probably best illustrated by transgenic expression of a dominant mutant form of the *Arabidopsis etr1-1* receptor in many plant species including petunia, tomato, carnation, *Campanula carpatica* and tobacco. These experiments have demonstrated that multiple ethylene responses in diverse species can be controlled by a single heterologous transgene (Wilkinson *et al.*, 1997; Knoester *et al.*, 1998; Bovy *et al.*, 1999; Sriskandarajah *et al.*, 2004). However, differences are emerging in the mechanisms that contribute to ethylene signaling in diverse plant species. In particular, tomato, petunia and rice serve as model systems for investigating the role of ethylene during the ripening of fleshy fruits, flower senescence and elongation under flooding conditions, respectively.

Tomato is a powerful model system for studying the role of ethylene in plant growth and development. Primarily, ethylene research in tomato has focused on its role in regulating fruit ripening, but a substantial body of research has also investigated the role of ethylene in abscission, senescence, response to flooding, wounding and various biotic stresses (Roberts *et al.*, 1984; Tucker *et al.*, 1984; McNamara and Mitchell, 1991; Abeles *et al.*, 1992; Lanahan *et al.*, 1994; Barry *et al.*, 2005; Barry and Giovannoni, 2006). There are six known ethylene receptors in tomato, *Le*ETR1, *Le*ETR2, NR (also referred to as *Le*ETR3), *Le*ETR4, *Le*ETR5, and *Le*ETR6, while there are only five in *Arabidopsis* (Wilkinson *et al.*, 1995; Payton *et al.*, 1996; Zhou *et al.*, 1996; Lashbrook *et al.*, 1998; Tieman and Klee, 1999; Klee, 2004). *Le*ETR1, *Le*ETR2, and NR belong to subfamily-1, and *Le*ETR4, *Le*ETR5, and *Le*ETR6 belong to

subfamily-2. NR is the only member of the tomato ethylene-receptor that lacks a receiver domain (Tieman and Klee, 1999). *Le*ETR1 and *Le*ETR2 are more closely related to *Arabidopsis* ETR1, NR is similar to the ERS1 (Hua *et al.*, 1995), whereas *Le*ETR4 , *Le*ETR5 and *Le*ETR6 are more closely related to ETR2 and EIN4 (Tieman and Klee, 1999). NR was the first tomato ethylene receptor to be identified, and the *Nr* mutant is ethylene insensitive in all tissues examined (Lanahan *et al.*, 1994; Wilkinson *et al.*, 1995). In summary, the tomato ethylene receptors differ in number and type between tomato and *Arabidopsis*.

Tomato receptor genes have distinct patterns of gene expression (Klee, 2002). For tomato receptor genes, both *LeETR1* and *LeETR2* are ubiquitously expressed, although *LeETR1* is expressed at approximately a 5-fold higher level than *LeETR2* (Zhou *et al.*, 1996; Lashbrook *et al.*, 1998). *LeETR2* expression is transiently suppressed by ozone treatment, and returns to the same level as at the beginning of ozone exposure (Moeder *et al.*, 2002). *NR* is detected in floral ovaries and ripening fruit (Lashbrook *et al.*, 1998). *LeETR4* and *LeETR5* have a higher expression level in flowers and during fruit development than in vegetative tissues (Tieman and Klee, 1999). Notably, *LeETR4* contributes approximately 90% of the putative receptor expression in green fruit and 50% of the putative receptor expression in ripening fruit (Tieman and Klee, 1999). For *Arabidopsis*, the RNA levels of the five genes are generally low and ubiquitous, with similar expression patterns in most of the tissues, although minor differences exist in their expression patterns in each tissue (Hua *et al.*, 1998; Binder, 2008).

Ethylene-related phenotypes in single loss-of-function mutants are masked by over-expression of one or more other ethylene receptors in tomato, but not in *Arabidopsis*. In antisense *NR* tomato

plants where *NR* expression is reduced by 90%, *LeETR4* was over-expressed four-fold higher than wild type compensating for the loss of *NR* (Tieman *et al.*, 2000). However, this type of compensation does not occur *LeETR4* antisense lines (Tieman *et al.*, 2000).

Rice is a model plant species for investigating ethylene signaling in monocot species. In rice, five ethylene receptor genes have been identified, which encode two subfamily-1 receptors *Os*ERS1, *Os*ERS2 and three subfamily-2 receptors *Os*ETR2, *Os*ETR3, and *Os*ETR4 (Cao *et al.*, 2003; Watanabe *et al.*, 2004; Yau *et al.*, 2004). Similarly, *OsETR2* expression is induced by flooding, IAA, ethylene, and GA, suggesting that this receptor may be responsible for the hypoxia adaptation responses in rice (Watanabe *et al.*, 2004; Yau *et al.*, 2004). Strikingly, there is no ETR1-type ethylene receptor in rice (Cao *et al.*, 2003; Watanabe *et al.*, 2004; Yau *et al.*, 2004).

Petunia serves as a model for ethylene sensitive flower senescence research. There are four putative ethylene receptors in petunia, but only two *Ph*ERS1 and *Ph*ETR2, are characterized (Wang and Kumar, 2007). *Ph*ETR2 regulates timing of anther dehiscence (Wang and Kumar, 2007). *PhETR2* mRNA increases following wounding and salt treatment, whereas *PhERS1* mRNA does not (Wang and Kumar, 2007). *PhERS1* knock-down lines possess a wild-type phenotype, suggesting some genetic redundancy between the petunia ethylene receptors (Wang and Kumar, 2007).

In general, a more pronounced role for subfamily-2 ethylene receptors is evident in tomato, rice, and petunia compared to *Arabidopsis*. In tomato, reduction in the expression level of *LeETR4*

using an antisense transgene leads to a constitutive ethylene related phenotype, leading to increased epinasty of petioles and leaves, premature senescence of flowers, accelerated fruit ripening, and enhanced ethylene sensitivity in dark grown seedlings (Tieman *et al.*, 2000). *LeETR6* antisense lines have similar phenotypes to *LeETR4* antisense lines, including accelerated ripening, epinastic leaf growth, and premature flower senescence (Kevany *et al.*, 2007). Similarly, over-expression and silencing of the rice subfamily-2 receptor *OsETR2* caused phenotypic changes in flowering time, ethylene responsiveness, seed weight and starch accumulation (Wuriyanghan *et al.*, 2009). Knock-down lines in *OsETR2*, *OsETR3*, and *OsERS2* display enhanced ethylene sensitivity and early flowering (Wuriyanghan *et al.*, 2009). Furthermore, silencing of the subfamily-2 receptor, *PhETR2* in petunia led to stomium degeneration and anther dehiscence prior anthesis (Wang and Kumar, 2007). In contrast, loss-of-function of subfamily-1 ethylene receptors in *Arabidopsis* results in more dramatic phenotypic consequences related to ethylene hypersensitivity (Hall and Bleecker, 2003; Wang *et al.*, 2003; Qu *et al.*, 2007). Increased reliance on subfamily-2 receptors may be wide spread in a number of plant species as ETR1-type receptors are also absent in wheat and maize (Ma and Wang, 2003; Gallie and Young, 2004).

Downstream ethylene signaling components in diverse plant species

In addition to variation in the composition of ethylene receptor families and the different emphasis on the functional characteristics of subfamily-1 and subfamily-2 receptors, functional characterization of downstream signaling components in species other than *Arabidopsis* is revealing increased complexity. While a single CTR1 protein is encoded by the *Arabidopsis*

genome; multiple *CTR1*-like genes are present in other species (Adams-Phillips et al., 2004; Yin et al., 2008; Ma et al., 2010; Manzano et al., 2010). For example, three *CTR1*-like genes are present in tomato, *LeCTR1*, *LeCTR3*, and *LeCTR4* and each is expressed in multiple tissues with *LeCTR1* displaying increased transcript abundance in response to ethylene treatment (Leclercq et al., 2002; Adams-Phillips et al., 2004). Each tomato *CTR1*-like gene possesses a differential capability to complement the *Arabidopsis ctr1-8* mutant allele. For example, *LeCTR3* fully complements the *ctr1-8* allele in both seedlings and adult plants (Adams-Phillips et al., 2004). In contrast, *LeCTR4* is able to fully complement *ctr1-8* allele in adult plants but fails to fully complement the seedling phenotype whereas *LeCTR1* fails to complement either the seedling or rosette phenotype of *ctr1-8* (Adams-Phillips et al., 2004). A fourth *CTR1*-like gene, *LeCTR2*, is present in tomato (Lin, 1998; Adams-Phillips et al., 2004). However, phylogenetic analysis indicates that *LeCTR2* is more closely related to *Arabidopsis* ENHANCED DISEASE RESISTANCE 1 (EDR1), which is a negative regulator of disease resistance and ethylene-induced senescence (Frye et al., 2001; Adams-Phillips et al., 2004). Over-expression of the *LeCTR2* N-terminus in tomato resulted in enhanced susceptibility to infection by the fungal pathogen *Botrytis cinerea*, due to stronger induction of pathogenesis-related genes such as *PR1b1* and *chitinase B* (Lin *et al.*, 2008). *Le*CTR2 has also been implicated in ethylene signaling. *Le*CTR1, *Le*CTR3, and *Le*CTR4 proteins interact with the tomato subfamily-1 receptors, whereas *Le*CTR2 only interacts with *Le*ETR1 and *Le*ETR2 (Lin *et al.*, 2008; Zhong *et al.*, 2008). Furthermore, interaction of *Le*CTR1, *Le*CTR3, and *Le*CTR4 with the NEVER-RIPE receptor leads to recruitment of each CTR protein to the ER (Zhong *et al.*, 2008).

EIN2 in tomato was also been isolated, and like other *EIN2s*, *LeEIN2* is also a single copy gene (Zhu *et al.*, 2006). Down-regulation of *LeEIN2* in tomato inhibits expression of ethylene-related genes and ripening-related genes including *POLYGALACTURONASE* and *TOMLOXB*, and further inhibited fruit ripening and sensitivity (Zhu *et al.*, 2006; Hu *et al.*, 2010). In petunia, *PhEIN2* mediates ethylene signaling involved in a wide range of physiological processes including, seedling responses to ethylene, flower senescence, fruit ripening, formation adventitious root and root hairs (Shibuya *et al.*, 2004). Expression of *PhEIN2* mRNA is spatially and temporally regulated and is decreased by ethylene treatment in petals but not in seedlings (Shibuya *et al.*, 2004). Notably, *EIN2* expression is not affected by treatment with ethylene in *Arabidopsis* and rice (Alonso *et al.*, 1999; Jun *et al.*, 2004; Shibuya *et al.*, 2004). The *Medicago truncatula* ortholog of *EIN2*, *MtEIN2/MtSkl1*, is a negative regulator of bacterial infection and nodule formation in legumes in response to symbiotic rhizobia (Penmetsa *et al.*, 2008). Over-expression of the C-terminal domain of *MtEIN2/MtSkl1* is sufficient to block nodulation responses suggesting that ethylene inhibits nodule formation (Penmetsa *et al.*, 2008).

Three *EILs* have been functionally characterized in tomato, *LeEIL1–3,* and they function redundantly (Tieman et al., 2001). Reduced expression of all these three genes in transgenic tomato resulted in delayed fruit ripening, decreased flower abscission, senescence, and leaf epinasty (Tieman et al., 2001; Fu et al., 2005). In addition, the over-expression of *LeEIL1* can partially restore ripening in the *Nr* tomato mutant (Chen *et al.*, 2004). In melon, two *EILs* (*CmEIL1* and *CmEIL2*) have been characterized and found to regulate expression of *CmACO1* (Huang et al., 2010).

Two tomato F-box genes, *SlEBF1* and *SlEBF2* were reported, but the targets of *Sl*EBF1 and *Sl*EBF2 are currently unknown (Yang *et al.*, 2010). The expression of *SlEBF1* and *SlEBF2* is up-regulated during the transition from bud to anthesis, and then decreases dramatically at the post-anthesis stage, expression increases at the onset of fruit ripening and is induced by ethylene in seedlings (Yang *et al.*, 2010). Silencing of individual *SlEBF* genes does not cause dramatic phenotypes due to functional redundancy, but silencing both *SlEBF1* and *SlEBF2* expression causes a constitutive ethylene response phenotype, defects in fertility, growth inhibition, accelerated plant senescence and fruit ripening (Yang *et al.*, 2010).

ERF genes that act downstream of ethylene signaling, also play specialist roles in the response of rice to submergence. Two *ERF* genes, *SNORKEL1 (SK1)* and *SNORKEL2 (SK2)*, are responsible for submergence-escape response in deepwater rice (Hattori *et al.*, 2007; Hattori *et al.*, 2008; Hattori *et al.*, 2009). Under deepwater conditions, ethylene accumulates in the plant. Increased ethylene activates *SK1* and *SK2* genes, induces GA biosynthesis genes, and represses genes involved in ABA biosynthesis (Hattori *et al.*, 2011). As a result, internodes elongate and submergence is avoided by rapid stem growth. A third *ERF* gene, *SUB1A*, confers submergence-tolerance response in lowland rice (Fukao *et al.*, 2006; Xu *et al.*, 2006). *SUB1A* shares high similarity with *SK1* and *SK2*, but functions independently (Nagai *et al.*, 2010). SUB1A restricts the degradation of SLENDER RICE1 (SLR1) and SLR1-LIKE1 (SLRL1), which are DELLA repressor proteins in GA signaling (Xu *et al.*, 2006; Fukao and Bailey-Serres, 2008). At the same time, Sub1A inhibits ethylene biosynthesis (Fukao *et al.*, 2006). As a result, Plants carrying SUB1A are stunted that avoid energy consumption associated with stem elongation under water

stress conditions. Normally, they could survive in water for a few weeks, and then restart to grow after the stress.

GR/RTE1 family proteins regulate a subset of ethylene responses

GREEN RIPE (GR) is a novel protein that regulates a subset of ethylene responses in tomato and is a homolog of *Arabidopsis* RTE1 (Barry and Giovannoni, 2006). The *Gr* mutant displays ethylene insensitivity during fruit ripening, floral senescence, abscission, and root elongation during the triple response. However, dark-grown hypocotyls and petiole retain a typical wild type response to ethylene (Barry *et al.*, 2005). The *Gr* mutant phenotype is caused by a 334 bp deletion in the 5'-UTR and 5'-flanking region of *GR*, but does not disrupt the predicted protein coding region (Barry and Giovannoni, 2006). *GR* transcripts accumulate to higher levels in the *Gr* mutant background compared to wild type controls suggesting that the basis for the mutant phenotype is ectopic expression of *Gr*. This hypothesis was confirmed through over-expression of *GR* under the control of the *CaMV35S* promoter. *GR* over-expression lines recreated the *Gr* mutant phenotype but did not lead to whole plant ethylene-insensitivity indicating the GR control of ethylene responses occurs at the level of the protein function (Barry and Giovannoni, 2006).

GR encodes a protein of 243 amino acids with a molecular mass of ≈27.9 kDa and belongs to a family of conserved proteins that have two or three predicted transmembrane spanning domains that includes the ethylene signaling component RTE1 (Barry and Giovannoni, 2006; Resnick *et al.*, 2006). The tomato genome contains two additional *GR* homologs designated *GREEN RIPE LIKE 1* (*GRL1*) and *GREEN RIPE LIKE 2* (*GRL2*), the function of which remain unknown

(Barry and Giovannoni, 2006). GR shares 53% and 37% of amino acid identity with GRL1 and GRL2, respectively (Barry and Giovannoni, 2006). GR possesses a motif MXCXXC and an MXXXM motif (where X is any hydrophobic residue, M is methionine and C is cysteine), which are in C terminus and in a predicted membrane-spanning domain, respectively (Barry and Giovannoni, 2006). These motifs may function in the binding of copper ions or copper transport activity, but these two motifs are not conserved in other homologs (Barry and Giovannoni, 2006).

Recently the involvement of GR/RTE1 family proteins in mediating ethylene responses in rice was reported (Zhang et al., 2012). Over-expression of *OsGRL1a/OsRTH1*, a homolog of *RTE1*, complemented the *rte1-2* loss-of-function and conferred whole-plant ethylene insensitivity when over-expressed in *Arabidopsis* and rice (Zhang et al., 2012). However, over-expression of *OsGRL1b/OsRTH2* and the *RTH* ortholog *OsGRL2/OsRTH3* did not influence ethylene responsiveness. Both *Os*GRL1a/*Os*RTH1 and *Os*GRL1b/*Os*RTH2 appear to be localized to the Golgi and ER membranes whereas *Os*GRL2/*Os*RTH3 is localized to the ER and the nucleus (Zhang et al., 2012).

Hypothesis

Previous research by our laboratory identified a role for the *Sl*GR protein in controlling ethylene responses in tomato although the exact biochemical function of this protein remains unknown. Furthermore, the role of the *Sl*GR homologs, *Sl*GRL1 and *Sl*GRL2 remain unknown. The objective of this research is to explore the potential role of these proteins in mediating ethylene responses and to define their relationship to one another.

We hypothesize that *SlGR* and *SlGRL1* are involved in modulating ethylene responses in tomato but that their specific roles have diverged to influence distinct subsets of responses. Furthermore, differences in the primary amino acid sequence of these proteins may determine functional specificity.

To test this hypothesis a combination of transgene expression in tomato and *Arabidopsis* of wild type and mutant transgenes has been performed together with a comparative approach utilizing putative GR orthologs to attempt to identify amino acid residues and domains important for the function of these proteins.

Thesis rationale and overview

The work presented here expands our current knowledge of the role of the GR/RTE1 family in ethylene signaling. *AtRTE1* functions in ethylene signaling. The *rte1* mutant was identified as a suppressor of the *etr1-2* mutant allele (Resnick et al., 2006; Zhou et al., 2007; Resnick et al., 2008). *At*RTE1 promotes ethylene receptor ETR1 signaling by a physical association between *At*RTE1 and the ETR1 N-terminus, an interaction which is not disrupted by ethylene treatment (Dong et al., 2010). There are three homologs of *At*RTE1 in tomato; GREEN RIPE (*Sl*GR), GREEN RIPE LIKE 1 (*Sl*GRL1) and GREEN RIPE LIKE 2 (*Sl*GRL2) (Barry and Giovannoni, 2006). Previous research indicated that over-expression of *SlGR* influences a subset of ethylene responses in tomato (Barry and Giovannoni, 2006). In Chapter 2, the potential role of *SlGRL1* and *SlGRL2* over-expression in influencing ethylene responsiveness in tomato was examined together with ability of *AtRTE1* to influence ethylene responses in tomato. In addition, the ability

of *SlGR* and *SlGRL1* to complement the loss of *rte1* in *Arabidopsis* was also tested. The results from these experiments suggest that *Sl*GR and *Sl*GRL1 function in ethylene signaling, although they function differently to influence distinct yet over-lapping ethylene responses suggesting the existence of distinct ethylene signaling modules in tomato. Over-expression of *At*RTE1 in tomato influences ethylene responses although the phenotypes more closely resembled those of *Sl*GRL1 over-expression rather than *Sl*GR over-expression. In contrast, over-expression of *Sl*GRL2 in tomato suggested that this gene is not involved in ethylene signaling.

Chapter 3 investigated the role of putative functional domains and amino acids in *Sl*GR and *Sl*GRL1 using a combination of natural variation in putative Solanaceae orthologs together with site-directed mutagenesis. Firstly, amino acids hypothesized to be important for functional differences between *Sl*GR and *Sl*GRL1 were identified utilizing diversity within putative GR orthologs. Several amino acids were identified that correlated with the ability of putative GR and GRL1 orthologs to complement the *rte1* mutant of *Arabidopsis*. Secondly, the role of the C-terminus of *Sl*GR and *Sl*GRL1 in controlling ethylene responsiveness was explored. Although studies of *At*RTE1 and *Sl*GR function suggest an important role for the C-terminus of these proteins in mediating ethylene responses, mutagenesis and deletion of highly conserved amino acid residues within the C-terminus of *Sl*GRL1 did not disrupt the ability of *Sl*GRL1 to complement the *rte1-3* allele.

Chapter 4 addresses the potential differential expression of *SlGR*, *SlGRL1* and *SlGRL2* characterization and localization of GR/RTE1 family proteins. The expression level of *SlGR*, *SlGRL1* and *SlGRL2* are differentially expressed in different tissues of tomato. *SlGR* is highly

expressed in seeds, especially in testa, with very little expression detected elsewhere in tomato. *SlGRL1* expression is also predominantly associated with the testa of developing seeds but is also expressed throughout fruit development with an increase in transcript abundance detected at the breaker stage of fruit ripening, in senescing flowers and in response to ethylene treatment and following wounding. *SlGRL2* is also widely expressed in tomato tissues with high expression levels detected in seeds and anthers together with increased expression detected during fruit ripening. 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. Slightly conflicting data have appeared in the literature regarding the sub-cellular localization of *At*RTE1, with different reports suggesting either Golgi localization or dual localization to both the ER and the Golgi (Zhou et al., 2007; Dong et al., 2008). The localization of SlGR, *Sl*GRL1 and related Solanaceae homologs was determined to test the hypothesis that differential control of ethylene responsiveness by *Sl*GR and *Sl*GRL1 is mediated by different subcellular localization of these proteins. These data revealed that *Sl*GR, *Sl*GRL1, *Sl*GRL2, *Ph*GR, *Ph*GRL1, and *Sm*GR are all localized in the Golgi thereby refuting the hypothesis. Together, these data provide considerable new insight into the role of the *GR/RTE1* family in controlling ethylene responses in plants.

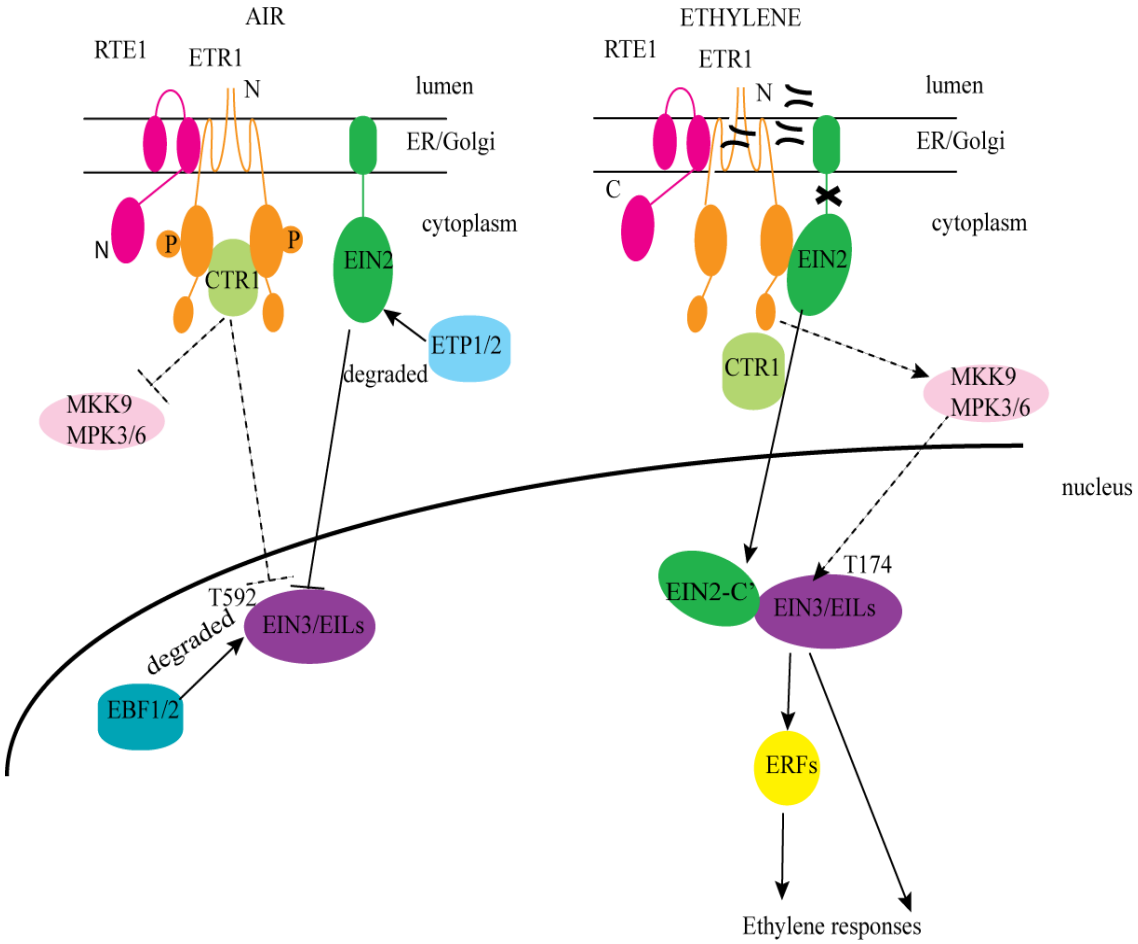

Figure 1.1 A model of the proposed ethylene signaling pathway in *Arabidopsis* in the presence and absence of ethylene. Pathway connections that are currently disputed in the literature are indicated by dashed lines. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

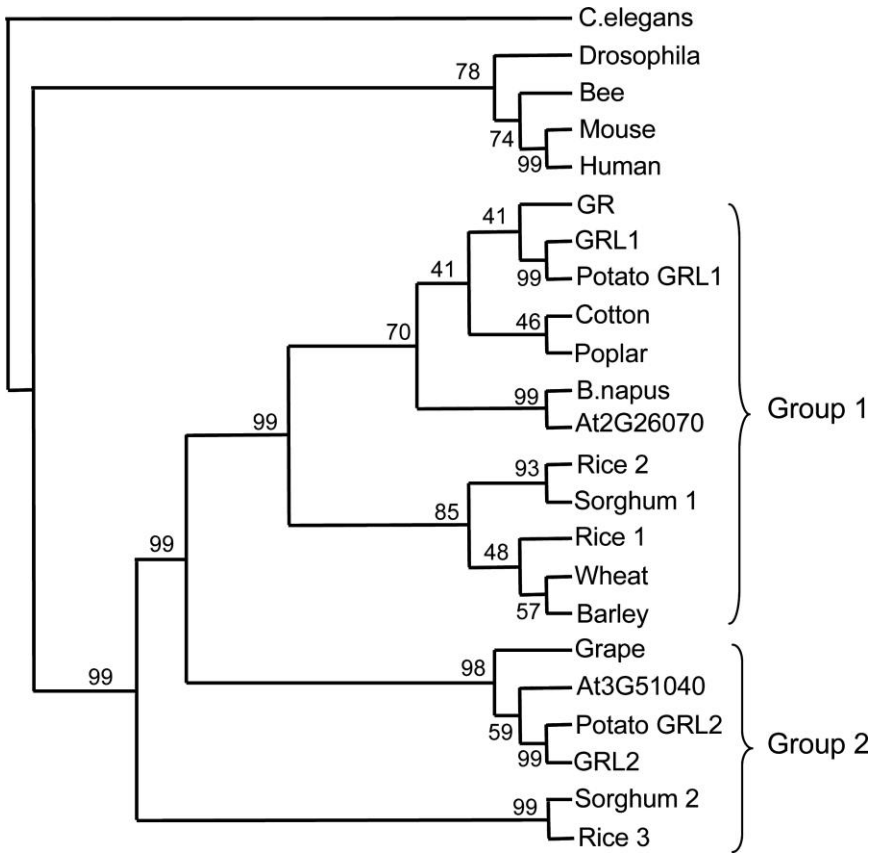

Figure 1.2 Phylogenetic analysis of GR/RTE1 family proteins (Barry and Giovannoni, 2006). At2G26070 and At3G51040 correspond to RTE1 and RTH of *Arabidopsis*, respectively.

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Chapter 2 Functional divergence of *SlGR*, *SlGRL1*, *SlGRL2* and *AtRTE1*

Abstract

The factors that mediate specific responses to the plant hormone ethylene during development or in response to individual stimuli are not fully defined. In particular, it is not known how, or whether, signaling at the level of the receptor complex can control distinct subsets of ethylene responses. Mutation at the *Green-ripe* (*Gr*) and *reversion to ethylene sensitivity 1* (*rte1*) loci, that encode homologous proteins of unknown function, influence ethylene responses in tomato (*Solanum lycopersicum*) and *Arabidopsis thaliana*, respectively. In *Arabidopsis*, RTE1 is required for function of the ETR1 ethylene receptor and acts specifically through this receptor isoform via direct protein-protein interaction. The *rte1* mutant was identified as a suppressor of the *etr1-2* mutant allele which is a weak dominant receptor allele that does not disrupt ethylene binding. The tomato genome contains two additional *GR* homologs designated *GREEN RIPE LIKE 1* (*SlGRL1*) and *GREEN RIPE LIKE 2* (*SlGRL2*). A combination of over-expression in tomato and complementation of the *rte1-3* mutant allele indicates that *SlGR* and *SlGRL1* influence distinct ethylene responses suggesting the existence of separate ethylene-signaling modules in tomato. In addition, over-expression of *AtRTE1* in tomato leads to reduced ethylene responsiveness in a subset of tissues, which more closely resemble the *SlGRL1* lines than *SlGR* lines. In contrast, *SlGRL2* over-expression lines possess a wild type phenotype and ethylene responsiveness appeared normal. These data suggest that *SlGRL2* probably does not play a role in ethylene signaling. Together, these data reveal heterogeneity in the control of ethylene responses mediated by members of the *GR/RTE* family in tomato and in *Arabidopsis*.

Introduction

Ethylene is a plant hormone that affects plant growth and development and responses to biotic and abiotic stresses (Bleecker and Kende, 2000; Guo and Ecker, 2004). A framework of ethylene signaling has been assembled using a combination of genetic and biochemical analysis in *Arabidopsis* (Kendrick and Chang, 2008; Stepanova and Alonso, 2009; Zhao and Guo, 2011). In *Arabidopsis*, ethylene is perceived by five ethylene receptors, ETHYLENE RESPONSE 1 (ETR1), ETHYLENE RESPONSE SENSOR 1 (ERS1), ETHYLENE RESPONSE 2 (ETR2), ETHYLENE RESPONSE SENSOR 2 (ERS2), and ETHYLENE INSENSITIVE 4 (EIN4) (Chang *et al.*, 1993; Resnick *et al.*, 2006). The ethylene receptors are transmembrane proteins located in the endoplasmic reticulum (ER) and Golgi membranes that function as homo- and hetero-dimers (Rodriguez *et al.*, 1999; Chen *et al.*, 2002; Ma *et al.*, 2006; Dong *et al.*, 2008; Gao *et al.*, 2008). Ethylene signaling is conserved in higher plants, and is negatively regulated (Hua and Meyerowitz, 1998).

In the absence of ethylene, the receptors actively suppress downstream responses through direct binding of CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), a serine threonine MAPKKK 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 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 which cannot be inactivated by ethylene (Wang et al., 2006). *ETHYLENE-INSENSITIVE 2* (*EIN2*) acts genetically

downstream of the ethylene receptors and *CTR1* and encodes a protein with homology to the NRAMP family of metal ion transporters (Roman et al., 1995; Alonso et al., 1999). The biochemical function of EIN2 remains unknown although mutations at the *ein2* locus lead to strong ethylene-insensitive phenotypes and recent research has indicated that EIN2 is localized in the ER where it interacts with the kinase domain of each ethylene 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 SENSITIVITY 1 (RTE1), a protein thought to facilitate the conformational change in the receptor following ethylene binding (Resnick et al., 2008). *RTE1* 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 *RTE1* function in *Arabidopsis* leads to enhanced ethylene responsiveness whereas over-expression results in reduced sensitivity (Resnick et al., 2006). RTE1 co-localizes 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; Dong et al., 2010). Interestingly, mutations at the *rte1* locus suppress ethylene-insensitivity mediated by a subset, but not all, *Etr1* mutant alleles (Resnick et al., 2006; Resnick et al., 2008). The significance of the allele specificity of RTE1 function is unclear but is suggestive of a role for RTE1 in the conformational changes in the receptor following ethylene binding (Resnick et al., 2008).

Furthermore, the effect of *RTE1* appears specific for the *ETR1* receptor as introduction of *RTE1*-dependent alleles of *ETR1* into additional *Arabidopsis* ethylene receptors fails to confer dominant ethylene insensitivity in seedling assays, even though RTE1 is able to physically interact with ERS1 (Rivarola et al., 2009; Dong et al., 2010).

Several other plant species also serve as model systems for investigating ethylene responses including rice, petunia and tomato. Although the ethylene signaling pathway is generally conserved between plant species, there are differences in the sizes of gene families and the receptor complement in different species. For example, the *GREEN RIPE/RTE1* family in tomato and other members of the Solanaceae family is larger than in other eudicot species, including *Arabidopsis*. There are three *GR/RTE1* genes in tomato; *GREEN RIPE* (*SlGR*), *GREEN RIPE LIKE 1* (*SlGRL1*) and *GREEN RIPE LIKE 2* (*SlGRL2*) (Barry and Giovannoni, 2006). The *Gr* mutant displays ethylene insensitivity leading to inhibition of fruit ripening, flower senescence and abscission together with reduced ethylene inhibition of root elongation (Barry et al., 2005; Barry and Giovannoni, 2006). However ethylene responses associated with hypocotyl elongation during the seedling triple response and petiole epinasty are normal, suggesting that GR modulates tissue specific ethylene responses in tomato (Barry et al., 2005; Barry and Giovannoni, 2006). *Sl*GR and *At*RTE1 belong to a family of conserved proteins, but their function is unclear (Barry and Giovannoni, 2006). In the current study, the function of *SlGRL1* and *SlGRL2* in ethylene signaling, and the relationship of *SlGR*, *SlGRL1* to *AtRTE1* were investigated.

A combination of over-expression in tomato and *Arabidopsis* reveals that *Sl*GR and *Sl*GRL1 function in ethylene signaling, although they function differently, while analysis of *Sl*GRL2

over-expression lines suggested this gene is not involved in ethylene signaling. Furthermore, the function of *At*RTE1 more closely resembles that of *Sl*GRL1 rather than *Sl*GR. These data provide new insight into the function of *Sl*GR, *Sl*GRL1, and *Sl*GRL2 in ethylene signaling.

Results

Over-expression 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* that leads to ectopic expression of *SlGR* and over-expression of *SlGR* recreates the *Gr* mutant phenotype (Barry and Giovannoni, 2006). Similarly, over-expression of *RTE1* in *Arabidopsis* leads to reduced ethylene responsiveness (Resnick et al., 2006). These data suggest that over-expression of *SlGRL1* in tomato may confer reduced ethylene responsiveness. To examine this possibility, transgenic lines over-expressing either *SlGRL1* under the control of the *CaMV35S* promoter were generated.

Three homozygous independent transgenic *CaMV35S::SlGRL1* lines were developed that possess elevated *SlGRL1* transcript levels in both fruit and seedlings. 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 AC control fruit indicating normal ethylene responsiveness (Figure 2.1 A-D). Analysis of the seedling triple response in the *CaMV35S::SlGRL1* lines indicated that hypocotyl responses to increasing concentrations of 1-

aminocyclopropane-1-carboxylic acid (ACC) are similar to those of wild type Ailsa Craig (AC), whereas hypocotyls of the *Never-ripe* (*Nr*) mutant (Lanahan et al., 1994) display the expected characteristic reduction in ethylene responsiveness (Figure 2.1E, Table 2.1). In contrast, root lengths of the *CaMV35S::SlGRL1* lines displayed a partial ethylene-insensitive phenotype with root lengths intermediate between those observed in AC and *Nr* seedlings that are similar to those observed in the *Gr* mutant (Figure 2.1F). Together, these data indicate that the *CaMV35S::SlGRL1* lines possess a triple response phenotype which is identical to that observed in the *Gr* mutant and *CaMV35S::SlGR* over-expression lines (Figure 2.1E, F) (Barry and Giovannoni, 2006).

The *Gr* mutant and *CaMV35S::SlGR* over-expression lines display a reduced response to ethylene-induced floral abscission that lies between that observed in AC and *Nr* (Barry et al., 2005; Barry and Giovannoni, 2006). Similarly, the *CaMV35S::SlGRL1* lines have reduced rates of ethylene-induced floral abscission, comparable to those of the *Gr* mutant (Figure 2.1G). Petiole angle in response to ethylene treatment was also determined in the *CaMV35S::SlGRL1* lines indicating a partial ethylene-insensitive phenotype, intermediate between AC and *Nr* (Figure 2.1H). In contrast, the petiole angle in the *Gr* mutant and *CaMV35S::SlGR* lines in response to ethylene treatment, is similar to that observed in wild type seedlings (Figure 2.1H; Figure 2.2). Together, these data indicate that over-expression of *SlGRL1* in tomato does not phenocopy the *Gr* mutant but rather influences a distinct subset of ethylene responses that are at the same time similar to the *Gr* mutant (triple response, floral abscission), yet also distinct (fruit ripening, petiole epinasty). Together these data indicate over-expression of *SlGRL1* in tomato does not recreate the *Gr* mutant phenotype.

Combining *Gr* with *CaMV35S::SlGRL1* enhances ethylene insensitivity in a subset of responses

The data described above indicates that *SlGR* and *SlGRL1* each influence a subset of ethylene responses when over-expressed in tomato (Figure 2.1). However, individually neither is able to confer reduced ethylene responsiveness in all plant tissues and in particular the response of dark-grown hypocotyls to the ethylene precursor ACC is similar to that observed in AC seedlings with only a very mild reduction in ethylene-insensitivity observed (Figure 2.1E). In contrast, over-expression of *RTE1* in *Arabidopsis* leads to a more pronounced, albeit weak, ethylene-insensitive phenotype in hypocotyls (Resnick et al., 2006). To determine whether ethylene responses can be influenced through combining *SlGR* and *SlGRL1*, the *CaMV35S::SlGRL1* transgene was introduced into the *Gr* mutant background through a cross and homozygous lines were recovered through genotyping. In some tissues, the double over-expression line displays a phenotype similar to that observed in either *Gr* or the single *CaMV35S::GRL1* over-expression line. For example, fruit of the *Gr* X *CaMV35S::GRL1* line resembles that of the single *Gr* mutant showing greatly inhibited fruit ripening (Figure 2.3A). Similarly, upon exposure to ethylene, petiole epinasty in the *Gr* X *CaMV35S::GRL1* line is identical to that observed in the single *CaMV35S::GRL1* transgenic line (Figure 2.3E). However, in response to ethylene-induced floral abscission and during the seedling triple response, the *Gr* X *CaMV35S::GRL1* line has an additive phenotype that is stronger than that observed in either *Gr* or the single *CaMV35S::GRL1* over-expression line (Figure 2.3B, C, and D, Table 2.2). In particular, the roots of dark-grown *Gr* X *CaMV35S::GRL1* seedlings grown in the presence of ACC also possess an additive phenotype

over *Gr* or the single *CaMV35S::GRL1* over-expression line with levels of ethylene-insensitivity comparable to that observed in the *Nr* mutant (Figure 2.3C).

Over-expression of *SlGRL2* in tomato does not confer reduced ethylene responsiveness

The previous results and data above indicate that both *SlGR* and *SlGRL1* function in ethylene signaling (Barry and Giovannoni, 2006) (Figure 2.1). The *Arabidopsis* genome carries a second homolog of *RTE1*, known as *RTE1-HOMOLOG* (*RTH*), which is the ortholog of *Sl*GRL2 but the function of this gene is unknown. To detect whether *Sl*GRL2 plays a role in ethylene signaling, transgenic lines over-expressing *SlGRL2* under the control of the *CaMV35S* promoter were generated and characterized using the same phenotypic responses as previously described above for the *CaMV35S::GRL1* over-expression lines.

Four homozygous independent transgenic *CaMV35S::SlGRL2* lines were developed. These lines with different *SlGRL2* transcript levels did not show reduced ethylene responsiveness in the phenotypes that were examined (Figure 2.4A-F). Fruits of *CaMV35S::SlGRL2* lines ripened normally, seedlings possessed a typical triple response phenotype when grown in the presence of ACC (Figure 2.4A, C, D, Table 2.3). Furthermore, ethylene-induced flower abscission and petiole epinasty were also unaltered in the *CaMV35S::SlGRL2* lines (Figure 2.4E-F). Together, these data suggest that *Sl*GRL2 probably does not play a role in ethylene signaling.

Over-expression of *AtRTE1* in tomato does not confer reduced ethylene responsiveness in all plant tissues

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. Two independent homozygous transgenic lines were developed and both *AtRTE1* independent transgenic lines showed a normal fruit ripening phenotype (Figure 2.5A). Although the transgenic lines had elevated relative expression level of *AtRTE1* in fruits, the ethylene- and ripening-related *E4* gene was expressed without any statistically significant difference with wild type fruits in the same development stage suggesting that *AtRTE1* is unable to influence ethylene responses in tomato fruit (Figure 2.5B, C). However, the *CaMV35S::AtRTE1* transgenic lines display a partial ethylene-insensitive phenotype in both hypocotyls and roots displaying a phenotype that is intermediate between that of wild type AC and *Nr* seedlings (Figure 2.5D, E, Table 2.4). Similarly, the *CaMV35S::AtRTE1* lines also possess reduced rates of ethylene-induced flower abscission and petiole epinasty compared to that observed in AC (Figure 2.5F, G). Together, these data indicate over-expression of *AtRTE1* in tomato influences ethylene responsiveness in a number of tissues, sharing characteristics of both *SlGR* and *SlGRL1* over-expression lines, but not recreating the *Gr* mutant phenotype, or leading to a whole plant reduction in ethylene responsiveness.

***SlGR* is unable to fully complement the *rte1* mutant phenotype**

The previous data show *SlGR*, *SlGRL1*, and *AtRTE1* influence distinct subsets of ethylene responses when over-expressed in tomato, but over-expression of these genes in tomato does not recreate the same ethylene-related phenotypes (Barry and Giovannoni, 2006)(Figure 2.1, 2.2 and

2.5). To examine the relationship of *SlGR*, *SlGRL1* and *AtRTE1* in more detail, the ability of *SlGR* and *SlGRL1* to complement the *rte1-3* allele was examined. *N*-terminal epitope-tagged versions of *SlGR* and *SlGRL1* under the control of the *CaMV35S* promoter were transformed in the *etr1-2/rte1-3* double mutant in which ethylene insensitivity conferred by the *etr1-2* mutant allele is suppressed by the *rte1-3* allele (Resnick et al., 2006). Two homozygous *CaMV35S::MYC-SlGRL1* 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 comparable to those of the *etr1-2* allele (Figure 2.6A, C, D, E).

In contrast, three independent lines expressing the *CaMV35S::MYC-SlGR* transgene did not fully complement the *rte1-3* allele (Figure 2.6A, B, D, E). However, the *CaMV35S::MYC-SlGR* 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-SlGR* lines are longer than those of the *etr1-2/rte1-3* double mutant and identical to those observed in Col-0 seedlings (Figure 2.6D, Table 2.5). However, at higher concentrations of ACC this difference was eliminated (Table 2.5). Similarly, root lengths of the *CaMV35S::MYC-SlGR* 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 the roots of Col-0 seedlings (Figure 2.6E). Similar phenotypes were also observed in transgenic lines expressing untagged versions of each gene (Figure 2.7). These data further illustrate functional divergence of *SlGR* and *SlGRL1* and indicate that *SlGR* is not functionally equivalent to *RTE1*.

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). Such plasticity is important during the synthesis and perception of plant hormones, which generally control multiple aspects of plant growth and development, together with responses to environmental perturbation and allows plants to appropriately regulate hormone responses. Several components within the ethylene response pathway are encoded by multigene families and subfunctionalization is observed, particularly at the level of transcriptional control. For example, *Arabidopsis* EIN3 and EIL1 proteins act semi-redundantly influence separate ethylene responses associated with seedling growth and stem and leaf expansion, respectively (An et al., 2010). Similarly, individual members of the large *ETHYLENE RESPONSE FACTOR (ERF)* gene family are known to act downstream of EIN3 to mediate distinct ethylene responses, particularly in response to environmental stress (Hattori et al., 2009; Zhang et al., 2011). However, evidence for subfunctionalization in the upstream components of the ethylene signaling pathway, including the receptors, is less well defined. Furthermore, the role of gene family complexity and diversity between species and how this influences ethylene responsiveness is not understood. For example, when compared to *Arabidopsis*, tomato possesses an extra subfamily-1 ethylene receptor, *LeETR2*, but lacks a copy of the subfamily 2 receptor *ERS2* (Bleecker, 1999; Klee,

2004). Similarly, while rice and maize possesses a subfamily-1 receptor that is comparable in structure to *Arabidopsis ERS1*, both lack *ETR1*-like subfamily-1 receptors (Rzewuski and Sauter, 2008; Chen and Gallie, 2010). Furthermore, while the subfamily-1 ethylene receptors are the primary receptors regulating ethylene responsiveness in *Arabidopsis*, the subfamily-2 receptors appear to have an important role in tomato and rice (Tieman et al., 2000; Qu et al., 2007; Wuriyanghan et al., 2009). In addition, copy number variation is also present in *CTR1*-like genes between *Arabidopsis* and other species, including tomato and rice with complementation studies revealing that the tomato *CTR1*-like genes are not functionally equivalent (Adams-Phillips et al., 2004; Rzewuski and Sauter, 2008). For example, *LeCTR3* is able to fully complement the *ctr1-8* allele of Arabidospsis whereas *LeCTR1* and *LeCTR4* can only partially complement loss of *CTR1* function (Adams-Phillips et al., 2004).

Variation in copy number between species is also evident for the *GR/RTE1* family. *Arabidopsis* have two *GR/RTE1* family genes, defined by *AtRTE1* and *AtRTH*, while there are three *GR/RTE1* family genes in tomato, defined by *SlGR, SlGRL1* and *SlGRL2*. *AtRTE1* is closely related to *SlGRL1*, and *AtRTH* is closely related to *SlGRL2*. The significance of gene copy number variability between species is not known but infers the potential to form distinct ethylene signaling networks.

Characterization of the *Gr* mutant and over-expression of *SlGR* in tomato indicated that this gene has the ability to influence a subset of ethylene responses in tomato (Barry et al., 2005; Barry and Giovannoni, 2006). Furthermore, over-expression of *SlGRL1* in tomato causes inhibition of a subset of ethylene responses that were distinct from those observed in the *Gr* mutant and

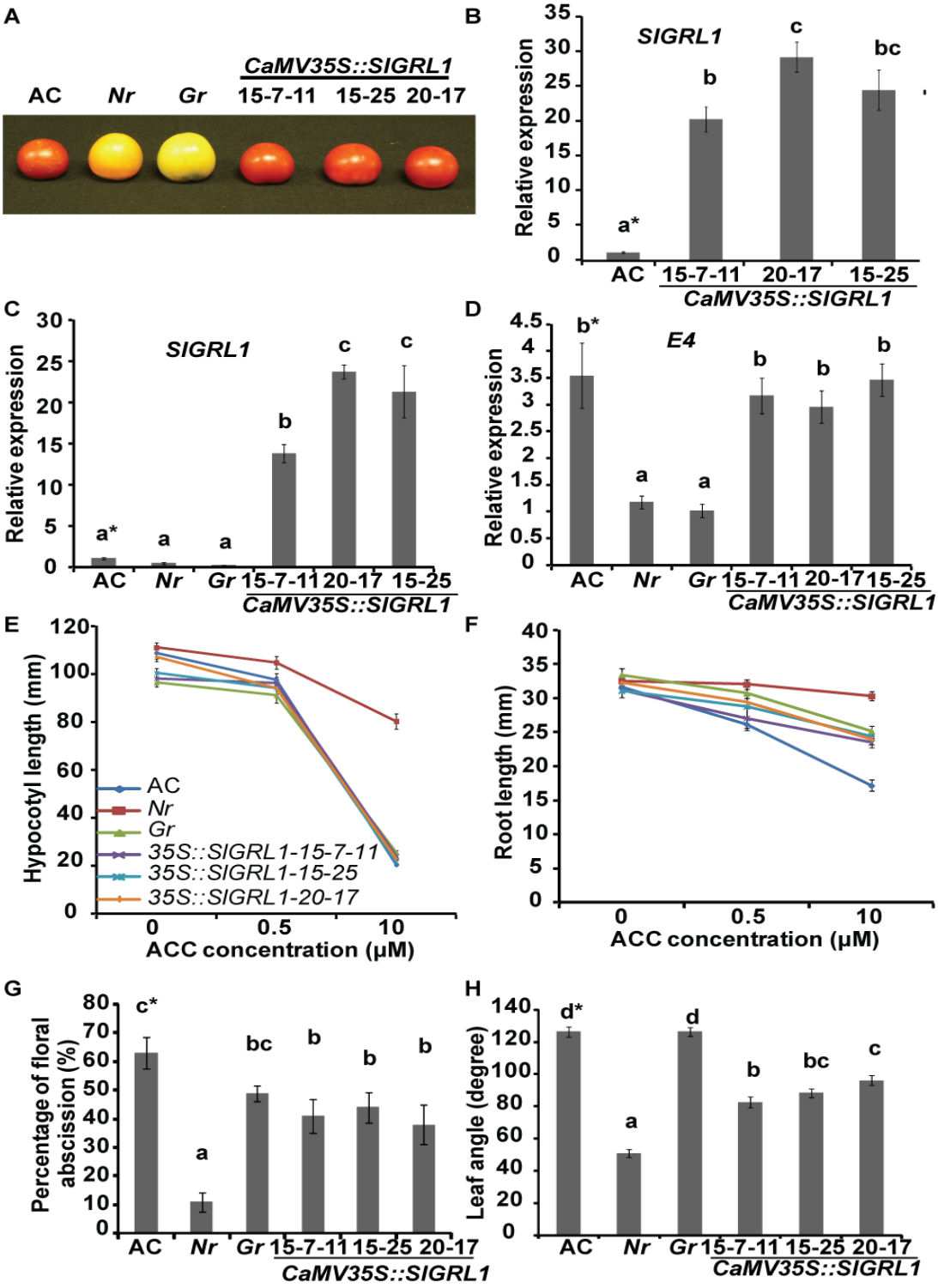

Figure 2.1 Phenotypic evaluation of *SlGRL1* over-expression lines of tomato. A, Fruit phenotypes of three independent homozygous *CaMV35S::SlGRL1* transgenic lines in

comparison to wild-type (AC) and the ethylene-insensitive *Nr* and *Gr* mutants. B, Relative expression level 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 the mean 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 eight days. Data presented are the means ±SE of at least 17 seedlings (Statistical analysis is presented in Table 2.1). G, Percentage floral abscission in *CaMV35S::SlGRL1* lines in comparison to AC, *Nr* and *Gr*. Detached flower trusses were immersed in water in conical flasks and treated with 2 µl l^{-1} of ethylene for 72 hours. Data presented are the mean ±SE of three independent experiments collected from at least 417 flowers per genotype. H, Petiole epinasty in *CaMV35S::SlGRL1* lines in comparison to AC, *Nr* and *Gr*. The adaxial leaf angle of four week old plants treated with 20 µl l^{-1} of ethylene for 16 hours was measured. Three leaves for each plant were examined and at least 11 plants were used for each genotype and the data presented as the mean ±SE. *In all experiments, means followed by the same letter are not significantly different at α=0.05 level.

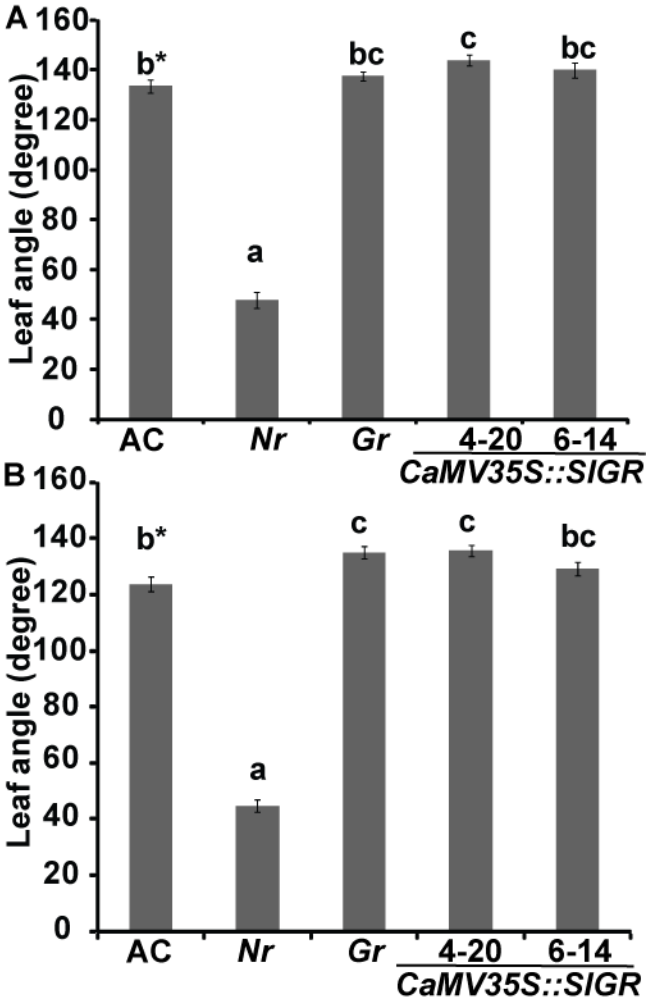

Figure 2.2 Petiole epinasty in response to ethylene in *CaMV35S::SlGR* lines. Petiole epinasty in four week old *CaMV35S::SlGR* lines treated for 16 hours with either 20 µl l^{-1} (A) or 1 µl l^{-1} (B) ethylene in comparison to wild type (AC) and the ethylene-insensitive *Nr* and *Gr* mutants. The adaxial angle was measured in three petioles for each plant in at least 11 plants of each genotype and the data are presented as the mean ± SE. * Means followed by the same letter are not significantly different at α=0.05 level.

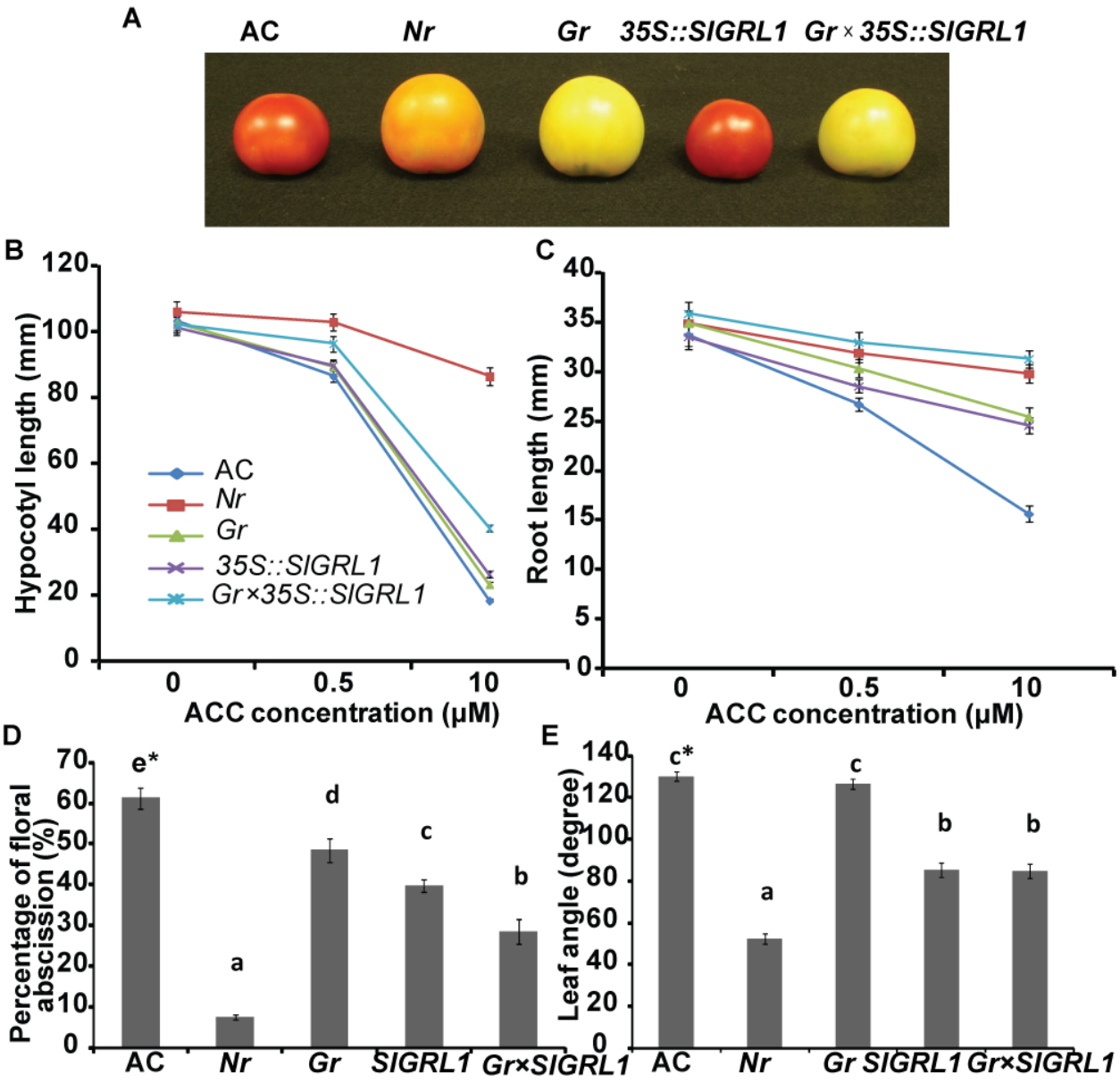

Figure 2.3 Phenotypic analysis of *SlGRL1* over-expression in the *Gr* mutant background. A-E Phenotypes of *Gr* X *CaMV35S::SlGRL1* in comparison to AC, *Nr* and *Gr*, and the *CaMV35S::SlGRL1* line (15-7-11). A, the phenotype of ripe fruits of each genotype. B and C, hypocotyl and root length, respectively of dark-grown seedlings germinated and grown in the presence of ACC for eight days. Data presented are the means ±SE of at least 30 seedlings (Statistical analysis is presented in Table 2.2). D, percentage of ethylene induced floral

abscission. Data presented are the mean ±SE 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 the mean ±SE. Experimental details are available in the Materials and Methods section. * Means followed by the same letter are not significantly different at α=0.05 level.

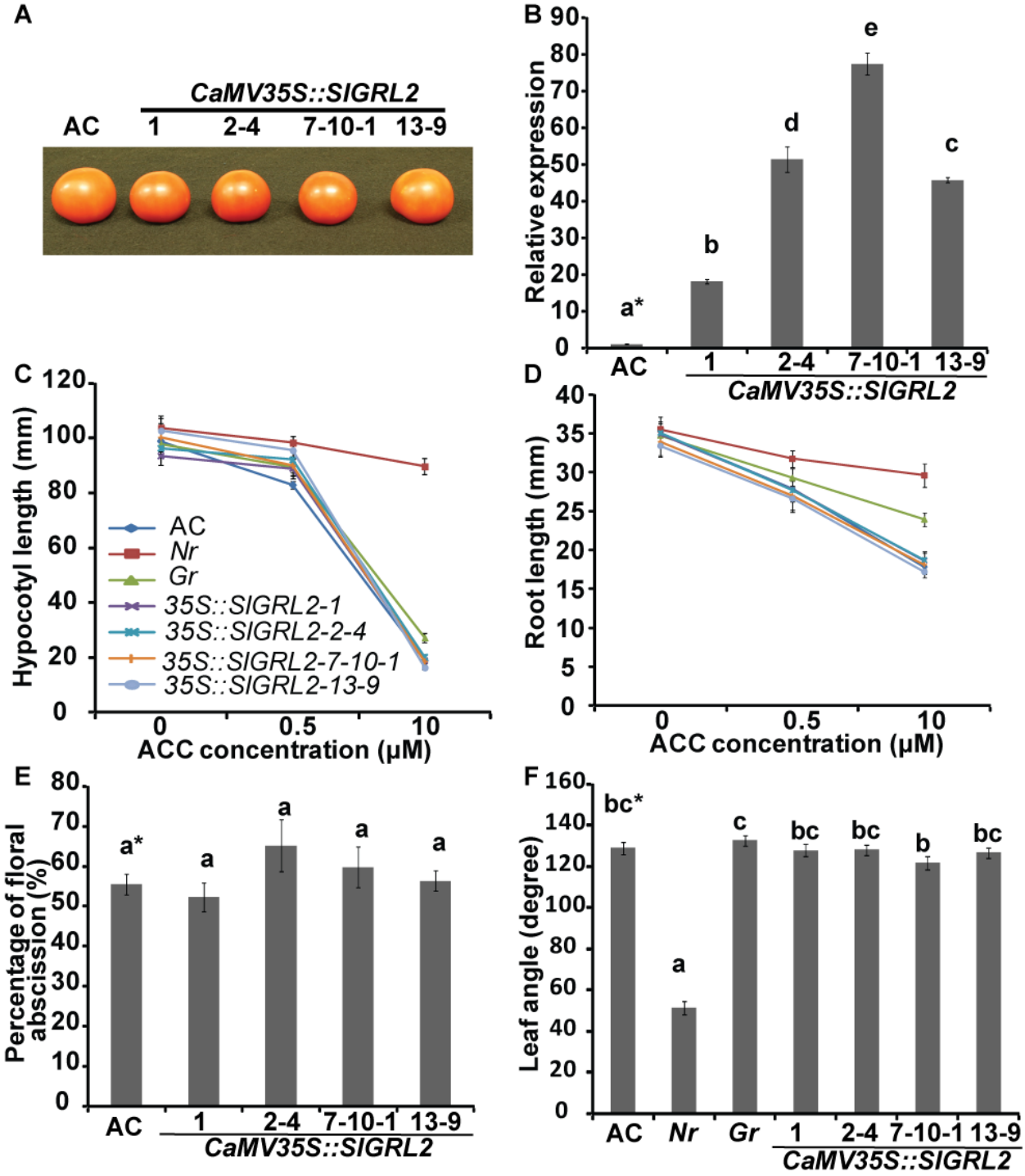

Figure 2.4 Analysis of ethylene responses in *CaMV35S::SlGRL2* lines. A, Ripe fruit phenotype of four independent homozygous *CaMV35S::SlGRL2* transgenic lines in comparison to wild-type

(AC). B, Relative expression level of *SlGRL2* in the young leaves of *CaMV35S::SlGRL2* transgenic lines as detected by qRT-PCR. Data are presented as the mean of three biological and three technical replicates for each sample. C and D, hypocotyl and root lengths, respectively of dark-grown seedlings of AC, *Nr*, *Gr* and *CaMV35S::SlGRL2* seedlings germinated and grown in the presence of ACC for eight days. Data presented are the means ±SE of at least 15 seedlings (Statistical analysis is presented in Table 2.3). E, Percent floral abscission in *CaMV35S::SlGRL2* lines in comparison to AC. Detached flower trusses were immersed in water in conical flasks and treated with 2 µl l^{-1} of ethylene for 72 hours. Data presented are the mean ± SE collected from at least 307 flowers per genotype. F, Petiole epinasty in *CaMV35S::SlGRL2* lines in comparison to AC, *Nr* and *Gr*. The adaxial leaf angle of four week old plants treated with 20 µl l^{-1} of ethylene for 16 hours was measured. Three leaves for each plant were examined and at least 13 plants were used for each genotype. Data are presented as the mean ±SE. * Means followed by the same letter are not significantly different at α=0.05 level.

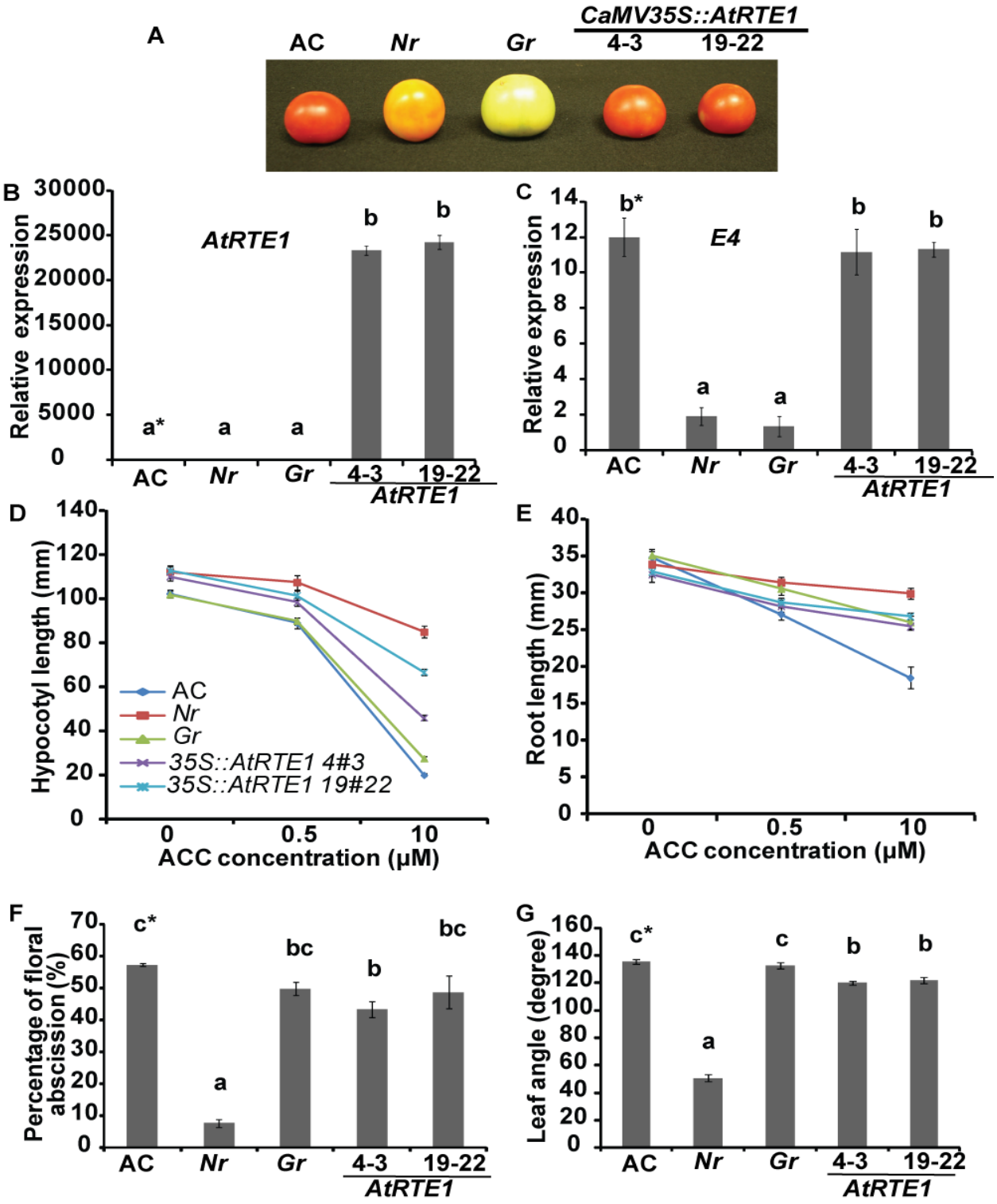

Figure 2.5 Phenotypic evaluation of *AtRTE1* over-expression lines of tomato. A-G, Phenotypes of *CaMV35S::AtRTE1* lines in comparison to AC and the ethylene-insensitive *Nr* and *Gr* mutants.

A, The phenotypes of ripe fruit of *CaMV35S::AtRTE1* lines. B and C, Relative expression levels of *RTE1* (B) and the ethylene- and ripening-related gene *E4* (C) in fruit of two independent homozygous *CaMV35S::AtRTE1* transgenic lines at the breaker stage of development as determined by qRT-PCR. Data are presented as the mean ±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 the means ±SE of at least 18 seedlings (Statistical analysis is presented in Table 2.4). F, Percentage of ethylene-induced floral abscission. Data presented are the mean ± SE collected from at least 352 flowers per genotype. G, Ethylene-induced petiole epinasty in *CaMV35S::AtRTE1* lines. Three leaves for each plant were examined and at least 12 plants were used for each genotype. Data presented are the mean ± SE.
* Means followed by the same letter are not significantly different at α=0.05 level.

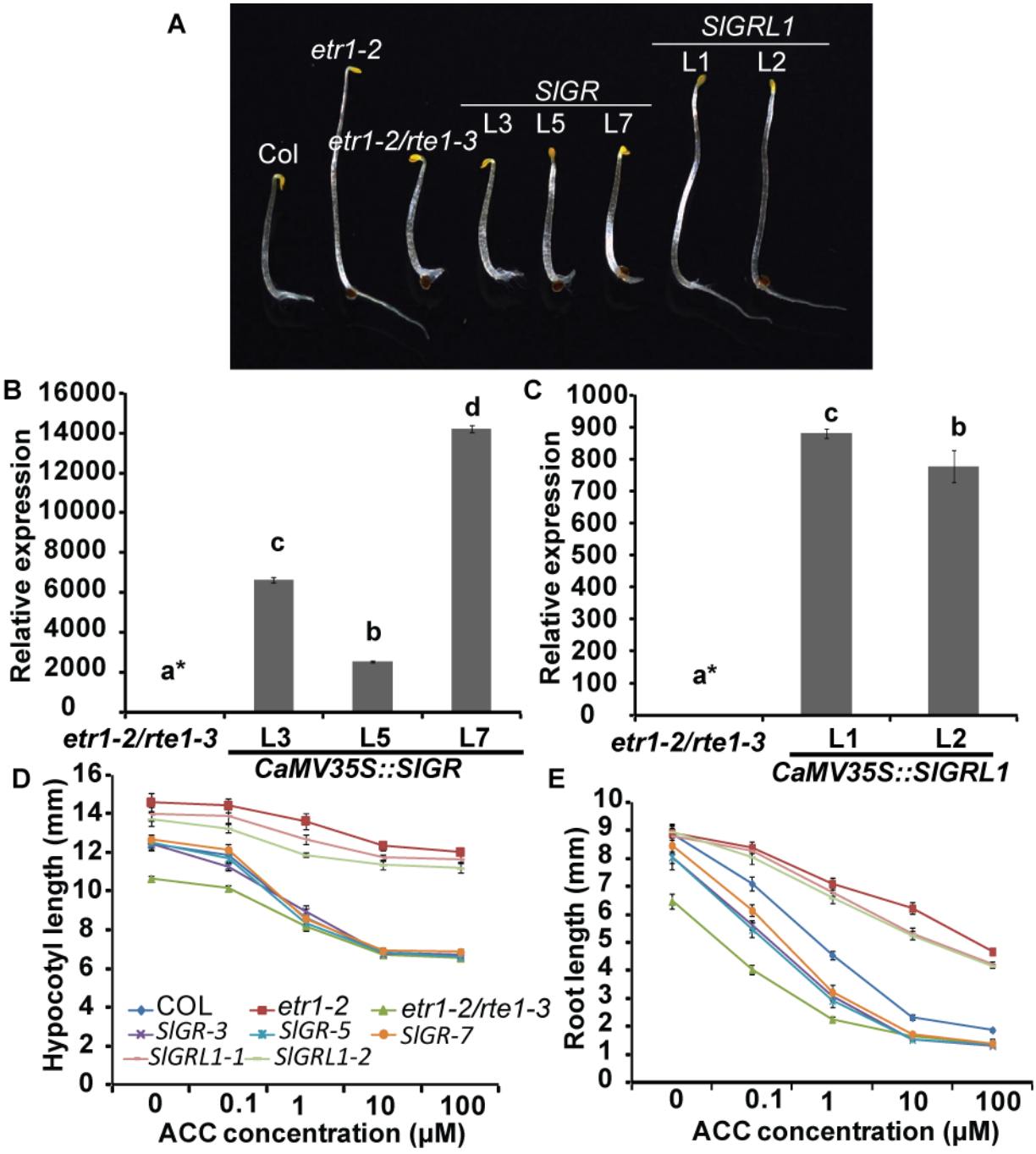

Figure 2.6 Differential complementation of the *Arabidopsis rte1-3* allele by *SlGR* and *SlGRL1*. A, The triple response phenotype of *Arabidopsis* seedlings from Col-0, *etr1-2*, *etr1-2/rte1-3*, together with three independent *CaMV35S::MYC-SlGR* lines and two independent

CaMV35S::MYC-SlGRL1 lines transformed into the *etr1-2/rte1-3* mutant background. Seedlings were grown in the dark at room temperature for six days on 100 μM ACC. B and C, Relative expression levels of *SlGR* (B) and *SlGRL1* (C) in seedlings of *CaMV35S::MYC-SlGR* and *CaMV35S::MYC-SlGRL1* lines, respectively as determined by qRT-PCR analysis. Data are presented as the mean ±SE of three biological and three technical replicates. D and E, dose response curve of hypocotyl (D) and root (E) lengths of *Arabidopsis* seedlings grown in the presence of ACC. Each data point represents the mean ±SE of 15 seedlings (Statistical analysis is presented in Table 2.5). * Means followed by the same letter are not significantly different at α=0.05 level.

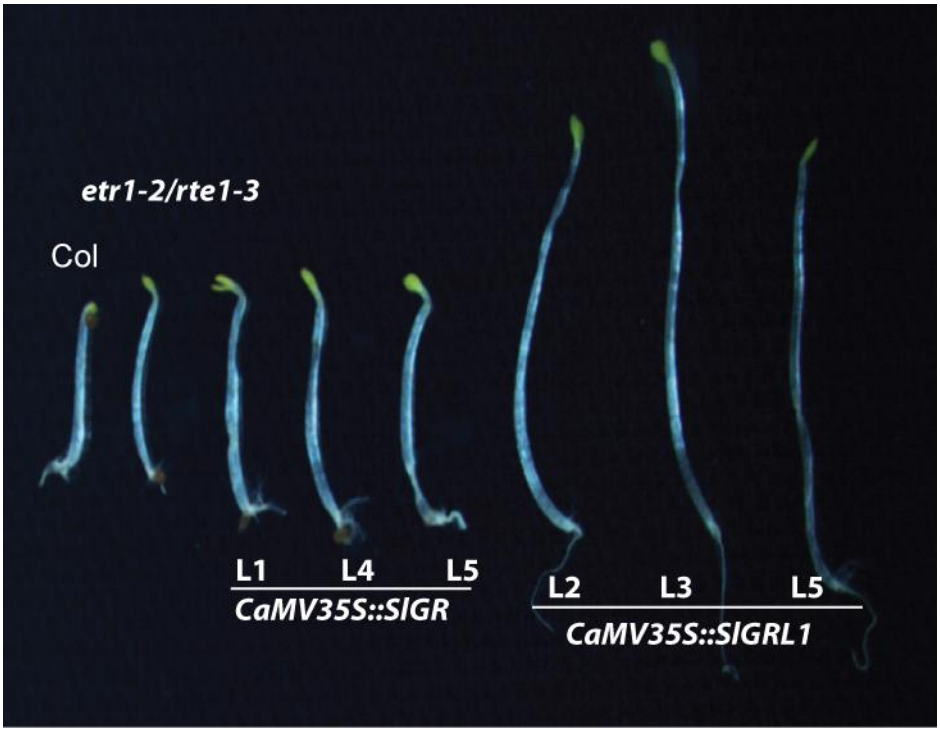

Figure 2.7 Differential complementation of *rte1-3* by untagged versions of *SlGR* and *SlGRL1*. The triple response phenotype of *Arabidopsis* seedlings from Col-0, *etr1-2/rte1-3*, together with three independent *CaMV35S::SlGR* lines and three independent *CaMV35S::SlGRL1* lines transformed into *etr1-2/rte1-3* background. Seedlings were grown in the dark at room temperature for six days on 100 μM ACC. Note, the elongated phenotype of the *CaMV35S::SlGRL1* lines demonstrating complementation of the *rte1-3* mutant allele whereas the *CaMV35S::SlGR* lines do not complement the *rte1-3* mutant allele. See Figure 2.6 for comparison with MYC-tagged versions.

Table 2.1 Statistical analysis of the seedling triple response assay in *CaMV35S::SlGRL1* lines of tomato

Group	0 μM ACC		0.5 μM ACC		10 μM ACC	
	Hypocotyl	Root	Hypocotyl	Root	Hypocotyl	Root
AC	108.8b*	31.67a	97.64ab	26.2a	20.57**a**	17.17a
Nr	111.03b	32.53a	104.83b	32.03d	80.31c	30.31c
Gr	96.44a	33.44a	91.16a	30.72cd	25.6**b**	25.17b
35S::SlGRL1-15-7-11	98.13a	31.17a	96.28a	27.07ab	24.04**ab**	23.48b
35S::SlGRL1-15-25	100.43a	31.04a	94.05a	28.75abc	21.76**ab**	24.35b
35S::SlGRL1-20-17	107.26b	32.3a	94.16a	29.44bcd	23.05**ab**	23.95b

*Means followed by the same letters are not significantly different at α=0.05 level.

Note statistically significant hypocotyl lengths in *Gr* and *CaMV35S::SlGRL1* transgenic lines in comparison to AC in seedlings grown in the presence of 10 μM ACC (shown in bold). Graphical representation of this data is provided in Figure 2.1.

Table 2.2 Statistical analysis of the seedling triple response assay in *Gr ×35S::SlGRL1* line of tomato

Group	0 μM ACC		0.5 μM ACC		10 μM ACC	
	Hypocotyl	Root	Hypocotyl	Root	Hypocotyl	Root
AC	103.27a	33.7a	86.47a	26.7a	18.43a	15.63a
Nr	106.03a	34.9a	102.87c	31.9cd	86.43d	29.8c
Gr	102.6a	34.9a	89.4a	30.33bc	23.4**b**	25.43**b**
35S::SlGRL1	101.2a	33.53a	89.73a	28.53ab	26.5**b**	24.57**b**
Gr ×35S::SlGRL1	102.3a	35.87a	96.33b	32.97d	40.43**c**	31.3**c**

*Means followed by the same letters are not significantly different at α=0.05 level.

Note statistically significant hypocotyl and root lengths in *Gr ×35S::SlGRL1* line in comparison to *Gr* and *35S::SlGRL1* in seedlings grown in the presence of 10 μM ACC (shown in bold). Graphical representation of this data is provided in Figure 2.3.

Table 2.3 Statistical analysis of the seedling triple response assay in *SlGRL2* over-expression lines of tomato

Group	0 μM ACC		0.5 μM ACC		10 μM ACC	
	Hypocotyl	Root	Hypocotyl	Root	Hypocotyl	Root
AC	98.87ab	34.93a	82.87a	27.87ab	18.8**a**	17.87**a**
Nr	103.73b	35.53a	98.33b	31.73b	89.67c	29.6c
Gr	97.93ab	34.73a	89.33ab	29.27ab	27.27b	23.93b
35S::SlGRL2-1	93.47a	34.87a	88.93ab	27.8a	18.8**a**	18.67**a**
35S::SlGRL2-2-4	96.27ab	35.07a	92.07ab	27.67a	20.13**a**	18.73**a**
35S::SlGRL2-7-10-1	100.2ab	33.93a	89.93ab	27a	18.53**a**	18.13**a**
35S::SlGRL2-13-9	102.73ab	33.4a	95.53b	26.6a	16.33**a**	17.2**a**

*Means followed by the same letters are not significantly different at α=0.05 level.

No any statistically significant differences in the hypocotyl and root lengths between *CaMV35S::SlGRL2* transgenic lines and AC in seedlings grown in the presence of 10 μM ACC (shown in bold). Graphical representation of this data is provided in Figure 2.4.

Table 2.4 Statistical analysis of the seedling triple response assay in *CaMV35S::AtRTE1* lines of tomato

Group	0 μM ACC		0.5 μM ACC		10 μM ACC	
	Hypocotyl	Root	Hypocotyl	Root	Hypocotyl	Root
AC	102.35a	34.84ab	89.11a	27.14a	20.11**a**	18.5**a**
Nr	112.19b	33.89ab	107.5c	31.43c	84.97e	29.91c
Gr	101.96a	35.11b	89.96a	30.6bc	27.67b	26.06b
35S::AtRTE1-4-3	110b	32.5a	98.41b	28.21a	46.12**c**	25.42**b**
35S::AtRTE1-19-22	112.74b	32.88ab	101.57bc	28.73ab	66.77**d**	26.83**b**

*Means followed by the same letters are not significantly different at α=0.05 level.

Note statistically significant hypocotyl and root lengths in *CaMV35S::AtRTE1* transgenic lines in comparison to AC in seedlings grown in the presence of 10 μM ACC (shown in bold). Graphical representation of this data is provided in Figure 2.5.

Table 2.5 Statistical analysis of the seeding triple response assay in *etr1-2/rte1-3* lines of *Arabidopsis* transformed with either *CaMV35S::MYC-SlGR* or *CaMV35S::MYC-SlGRL1*

Group	0 μM ACC		0.1 μM ACC		1 μM ACC		10 μM ACC		100 μM ACC	
	Hypocotyl	Root	Hypocotyl	Root	Hypocotyl	Root	Hypocotyl	Root	Hypocotyl	Root
COL	12.47**b***	8.88bc	11.87**bc**	7.1d	8.36ab	4.55c	6.84**a**	2.33b	6.7**a**	1.88b
etr1-2	14.59**c**	8.91c	14.42**e**	8.38e	13.63e	7.1d	12.35**c**	6.22d	12.02**c**	4.68d
etr1-2/rte1-3	10.66**a**	6.48a	10.15**a**	4.05a	8.21a	2.27a	6.72**a**	1.67a	6.56**a**	1.41a
35S::SlGR-3	12.45**b**	8.04b	11.28**b**	5.61bc	8.97b	3.09b	6.76**a**	1.54a	6.69**a**	1.32a
35S::SlGR-5	12.5**b**	8.06b	11.67**bc**	5.46b	8.37ab	2.93b	6.84**a**	1.55a	6.6**a**	1.37a
35S::SlGR-7	12.67**b**	8.47bc	12.14**c**	6.14c	8.6ab	3.23b	6.92**a**	1.73a	6.87**a**	1.41a
35S::SlGRL1-1	13.98c	8.79bc	13.89de	8.27e	12.69d	6.78d	11.73b	5.32c	11.65bc	4.21c
35S::SlGRL1-2	13.73c	8.96c	13.25d	8.05e	11.86c	6.59d	11.36b	5.26c	11.19b	4.16c

*Means followed by the same letters are not significantly different at α=0.05 level. Note statistically significant hypocotyl lengths in *CaMV35S::MYC-SlGR* transgenic lines in comparison to the *etr1-2/rte1-3* in seedlings grown in the absence (0 μM) or presence of 0.1

μM ACC (shown in bold). In comparison, hypocotyl lengths in seedlings grown on comparatively high concentrations of ACC (10 and 100 μM) are not statistically significant. Graphical representation of this data is provided in Figure 2.6.

Table 2.6 Oligonucleotide primers used in this study

Primer	**Sequence**	**Use**
GRL1OE-F	5´-TTGGATCCGATTGCTTTCTTGTGTGCTTCATC-3´	Construct assembly
GRL1OE-R	5´-TTGAGCTCGGTAACTTGATATTGTCCAAATTC-3´	Construct assembly
RTE1OE-F	5´-TTGGATCCATGTCACGTGGAAGAGGAGTTC-3´	Construct assembly
RTE1OE-R	5´-TTGTCGACCTGCTTCAAGTAATTATGTTC-3´	Construct assembly
GRL2OE-F	5´-TTGGATCCGTGCCAACGCACAATTTTATTAGC-3´	Construct assembly
GRL2OE-R	5´-TTGAGCTCCCATGGACAAATAAAACTTCATGTC-3´	Construct assembly
GRENT-F	5´-CACCATGCTGCCAAGAAGATATCCTCA-3´	Construct assembly
GRENTSTOP-R	5´-CTAATTGTCATCCTCAATCTTGC-3´	Construct assembly
GRL1ENT-F	5´-CACCATGCCATCAGGAAGACGTTCTT-3´	Construct assembly
GRL1ENTSTOP-R	5´-CTAGGAATCCAACAGATTTTTGA-3´	Construct assembly
GRL1Q-F	5´- TGCAAAATTTCATGTCGCCATA-3´	qRT-PCR(tomato)
GRL1Q-R	5´- AGGCAGGTTCCAAATCCATTAA-3´	qRT-PCR(tomato)
GRL2Q-F	5´- GTGCTGCTGCCTTTCTCCTT-3´	qRT-PCR(tomato)

Table 2.6 (cont'd)

GRL2Q-R	5´- AGATTCATCATGGTTCTCGACATATT-3´	qRT-PCR(tomato)
RTE1Q-F	5´- TCATCAGTAGTCCGCTCGTTTCT-3´	qRT-PCR(tomato)
RTE1Q-R	5´- AAGCACCACCCCCAAAGAC -3´	qRT-PCR(tomato)
E4Q-F	5´- AGGGTAATGATGTGGGAAAG-3´	qRT-PCR(tomato)
E4Q-R	5´- CTTCTAACGACTCCCTTGCC-3´	qRT-PCR(tomato)
GADPHQ-F	5´- ATGCTCCCATGTTTGTTGTGGGTG-3´	qRT-PCR(tomato)
GADPHQ-R	5´- TTAGCCAAAGGTGCAAGGCAGTTC-3´	qRT-PCR(tomato)
CACQ-F	5´- CCTCCGTTGTGATGTAACTGG-3´	qRT-PCR(tomato)
CACQ-R	5´- ATTGGTGGAAAGTAACATCATCG-3´	qRT-PCR(tomato)
GRCDSQ-F	5´- TTGCAACGGCCACTCATTC-3´	qRT-PCR(*Arabidopsis*)
GRCDSQ-R	5´- CGCATTGATCCTCTAAATGATAGC-3´	qRT-PCR(*Arabidopsis*)
GRL1CDSQ-F	5´- GGCCAAATACCTTCAACTAGACAGA -3´	qRT-PCR(*Arabidopsis*)
GRL1CDSQ-R	5´- TGTGTGCAGCAAGGTTTCGT-3´	qRT-PCR(*Arabidopsis*)
AtUBQ10Q-F	5´- AAAGAGATAACAGGAACGGAAACATAGT-3´	qRT-PCR(*Arabidopsis*)
AtUBQ10Q-R	5´- GGCCTTGTATAATCCCTGATGAATAAG-3´	qRT-PCR(*Arabidopsis*)

CaMV35S::SlGR lines (Figure 2.1). Notably, over-expression of *SlGRL1* did not lead to inhibition of fruit ripening, which is a feature of the *Gr* mutant and *CaMV35S::SlGR* lines (Barry and Giovannoni, 2006). However, the *CaMV35S::SlGRL1* lines displayed inhibition of ethylene-induced petiole epinasty, which is not a feature of the *Gr* mutant or the *CaMV35S::SlGR* lines (Figure 2.1 & Figure 2.2). These data suggest that *SlGR* and *SlGRL1* are not truly functionally equivalent but have evolved to influence different subsets of ethylene responses. Additional evidence for functional divergence of *SlGR* and *SlGRL1* is derived from their differential ability to complement the *rte1-3* mutant phenotype. *SlGRL1* is able to almost fully restore ethylene-insensitivity to the *etr1-2/rte1-3* double mutant, indicating complementation of the *rte1-3* allele, whereas *SlGR* can only recover the ethylene-hypersensitive phenotype of *rte1-3* at low concentrations of ACC (Figure 2.6). Similarly, while over-expression of *AtRTE1* in tomato leads to reduced ethylene responsiveness in multiple tissues (Figure 2.5), it does not result in a whole plant reduction in ethylene responsiveness and *CaMV35S::AtRTE1* lines do not mimic the *Gr* mutant phenotype. In addition, a recent study on members of the rice *GR/RTE1* family indicated that *OsGRL1a*/*OsRTH1* was able to complement loss of *AtRTE1* function in *Arabidopsis* and confer reduced ethylene sensitivity in *Arabidopsis* and rice when over-expressed (Zhang et al., 2012). In contrast, *OsGRL1b*/*OsRTH2* and *OsGRL2*/*OsRTH3* could not complement the *rte1-2* mutant allele and over-expression lines did not display reduced ethylene responsiveness. Together, these data point to considerable heterogeneity among the members of the *GR/RTE1* family suggesting that they likely exert their influence on ethylene signaling through specific components of ethylene pathway and have diverged to the point where no single protein can fully substitute for another.

Evidence for the existence of distinct ethylene signaling modules in tomato

The ethylene receptors are known to form large heteromeric complexes and interaction experiments with between the ethylene receptors and additional signaling components suggest that these complexes likely contain RTE1, EIN2 and CTR1 (Clark et al., 1998; Gao et al., 2008; Bisson et al., 2009; Chen et al., 2010; Dong et al., 2010). The presence of different receptor isoforms and the expansion of gene families in tomato and other species, coupled with differential expression and / or accumulation of individual signaling components are likely to contribute to heterogeneity within these complexes that may result in the formation of distinct signaling modules that produce diverse outputs. A model has been proposed in which RTE1 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). Genetic analysis indicates that the effect of RTE1 is highly specific for the ETR1 receptor although RTE1 also weakly interacts with the ERS1 receptor (Resnick et al., 2006; Resnick et al., 2008; Rivarola et al., 2009; Dong et al., 2010).

The ability of *SlGR* and *SlGRL1* to influence separate subsets of ethylene responses, when over-expressed 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-SlGR* transgene (Figure 2.6). Furthermore, in tissues where over-expression of either *SlGR* or *SlGRL1* fails to give rise to an ethylene-insensitive phenotype, it is possible that

the appropriate target receptor may be absent, present at reduced levels compared to other receptors, or that the response is mediated by receptors that do not require GR/RTE1 family proteins. The latter hypothesis is supported by the observation that the *etr1-9/ers1-3* double null mutant of *Arabidopsis* remains responsive to ethylene suggesting that certain ethylene responses are therefore likely to be independent of RTE1 given that RTE1 acts specifically with the ETR1 receptor (Resnick et al., 2006; Qu et al., 2007; Rivarola et al., 2009). Similarly, over-expression of *GR/RTE1* family members in tomato does not always lead to ethylene-insensitivity of the magnitude observed in the dominant *Nr* ethylene receptor mutant. For example, compare hypocotyl lengths in *Gr* X *CaMV35S::SlGRL1* and *CaMV35S::AtRTE1* seedlings grown on ACC with those of the *Nr* mutant (Figure 2.3B and 2.5D) suggesting either a failure of the GR/RTE1 proteins to completely maintain the ethylene receptors in an "on signaling state" (Resnick et al., 2008) or that GR/RTE1 independent receptors maintain some tissue responsiveness to ethylene.

Analysis of *SlGRL1* over-expression in the *Gr* mutant (Figure 2.3) supports the hypothesis that distinct ethylene signaling modules are formed in different tomato tissues. For example, expression of *CaMV35S::SlGRL1* in the *Gr* mutant background led to three distinct classes of phenotype; those characteristic of either the *Gr* mutant phenotype (inhibition of fruit ripening) or *SlGRL1* over-expression (inhibition of petiole epinasty) and a set of responses that were additive in the double over-expression line (inhibition of floral abscission and the seedling triple response). Therefore, the failure of *SlGR* to reduce the petiole response to ethylene may be caused by lack of the appropriate target receptor complex, whereas a complex that is a target for the *Sl*GRL1 protein is present. Together, these data support a model in which at least two signaling modules comprised of distinct components control ethylene responses in tomato. In

some tissues or responses a module susceptible to the action of either *Sl*GR or *Sl*GRL1 operates while in other situations both modules operate to cooperatively influence ethylene responses.

SlGRL2 is unlikely to play a role in ethylene signaling

SlGRL2 is closely related to *AtRTH*, an *Arabidopsis* gene of unknown function (Barry and Giovannoni, 2006). Although the function of *At*RTH is still unknown, it was reported that no bimolecular fluorescence complementation (BiFC) signal was detected from co-infiltration of *cYFP-RTH* and *ETR1-nYFP*, suggesting that RTH does not play the same role as RTE1 in ethylene signaling (Dong et al., 2010). Similarly the putative rice ortholog of *AtRTH*, *OsGRL2/OsRTH3*, cannot complement the *rte1-2* mutant allele and over-expression in *Arabidopsis* does not confer reduced ethylene responsiveness (Zhang et al., 2012). Our data from over-expression analysis of *SlGRL2* also suggest that this gene is not involved in regulating ethylene responsiveness and the *CaMV353::GRL2* lines possessed phenotypes that were identical to wild type plants. The function of *SlGRL2*, *AtRTH* and *OsRTH3* remain unknown and will require additional experimentation to resolve. Interestingly, sequence and phylogenetic analysis indicates that animals and protist genomes contain a single copy of a protein closely related to GR/RTE1 (Barry and Giovannoni, 2006; Klee, 2006). These organisms have not been reported to signal using ethylene and do not contain other components of the ethylene signaling pathway. Therefore, it is possible that *SlGRL2* and its putative orthologs from other plant species are involved in processes unrelated to ethylene signaling.

Materials and Methods

Plant Growth and Treatments

The parental cultivar Ailsa Craig (AC), the ethylene-insensitive mutants *Never-ripe* (*Nr*) and *Green-ripe* (*Gr*), and *CaMV35S:SlGR* transgenic tomato lines have been described in the published paper (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 at Michigan State University, East Lansing, MI. Experiments to evaluate the triple response phenotype in dark grown tomato seedlings and floral abscission were performed as previously described (Barry et al., 2005) with the exception that seedlings were measured at 8 days after sowing and flowers were induced to abscise by treatment with ethylene at a concentration of 2 μl l^{-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-hour dark at 28^{o}C and 65% relative humidity. 4-week old plants were treated with 20 μl l^{-1} ethylene for 16 hours and the adaxial leaf angle determined.

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) together with the ethylene-signaling mutants *etr1-2* and *etr1-2/rte1-3* were sown in 1:1:1 Sure mix: Medium vermiculite: Perlite (Michigan Grower Products Inc., www.suremix.com) and exposed to a three day cold treatment at 4^{o}C. Seed trays were transferred to a growth chamber at 22^{o}C under 16-h light/ 8-hour dark at 145 μmol m^{-2} s^{-1} and 65% relative humidity. Plants were supplemented with fertilizer 0.25 X Hoaglands solution pH 5.5. The *Arabidopsis* triple response screen was performed using a

slightly modified version of a previously published protocol (Alonso et al., 2003). *Arabidopsis* seeds were sterilized with 70% ethanol for 10 minutes, followed by three washes with 100% ethanol. Surface sterilized seeds were dried on sterile filter paper in a laminar flow hood and dried seeds were sprinkled onto 0.8% phytagar containing 1 ×Murashige and Skoog salts, pH 6.0 and 1% sucrose supplemented with 1-aminocyclopropane-1-carboxylic acid (ACC) at 0, 0.1, 1, 10, and 100 μM. Plates were placed at 4^{o}C for 3 days, exposed to light for 12 h, and then incubated at room temperature in the dark for 6 days. Hypocotyl and root lengths were measured using ImageJ (http://rsbweb.nih.gov/ij/).

qRT-PCR analysis

Total RNA was extracted using the RNeasy® Mini Kit and subjected to on column DNase treatment (Qiagen, http://www.qiagen.com). 1 μg of RNA was used for reverse transcription using SuperScript^{TM} III First-Strand Synthesis System (Invitrogen, http://www.lifetechnologies.com/). *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* was used as the endogenous control for tomato, except the experiment in Figure 2.1 C&D, which used the *clathrin adaptor complexes medium subunit (CAC)*. The GAPDH and CAC primers for qRT-PCR were as previously described (Balaji et al., 2008; Exposito-Rodriguez et al., 2008). Primers for the ripening- and ethylene-related *E4* gene were as previously described (Gimenez et al., 2010). *Polyubiquitin 10 (AtUBQ10)* was used as the endogenous control for *Arabidopsis*, and the primers sequences were designed according to (De Vos and Jander, 2009). Gene-specific primers for *SlGR*, *SlGRL1*, *SlGRL2*, and *AtRTE1* were designed by Primer Express 3.0 (Applied Biosystems, http://www.lifetechnologies.com), and all pairs of primers used in this research are

listed in the Table 2.6. The PCR reactions were performed with FAST SYBR® Master Mix, 2x (Applied Biosystems) in a 25 µL volume using an Applied Biosystems StepOnePlus™ Real-Time PCR System (ABI) with the following cycling program: 10 min at 95 ℃, followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min. The primer efficiency was tested by generating standard curves and results were analyzed by comparative ΔΔCT method (Livak and Schmittgen, 2001).

DNA constructs and plant transformation

The *CaMV35S::SlGRL1* construct was assembled as follows: The full-length coding sequence of *SlGRL1* was re-amplified from the EST clone cLEG37H01 using the primers GRL1OE-F and GRL1OE-R, which contain a *Bam*HI and *Sac*I 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 *CaMV*35S promoter in the binary vector pBI121, previously modified by removal of the *UidA* coding region by digestion with *Bam*HI and *Sac*I. The *CaMV35S::SlGRL2* construct was assembled in the same way except that the *SlGRL2* coding was re-amplified from the EST clone cTOD-5-M16 using the primers GRL2OE-F and GRL2OE-R. The *CaMV35S::RTE1* construct was assembled as follows: The full-length coding sequence of *RTE1* was amplified by RT-PCR from *Arabidopsis* leaf cDNA using the primers RTE1OE-F and RTE1OE-R, which contain a *Bam*HI and *Sal*I 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 *Sal*I, the overhang rendered blunt by incubation with the Klenow fragment of

DNA polymerase I and the insert released from the vector by digestion with *Bam*HI. The insert ligated downstream of the *CaMV*35S promoter in the binary vector pBI121, previously modified by removal of the *UidA* coding region by digestion with *Sac*I followed by polishing with T4 DNA polymerase and subsequent *Bam*HI 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 *via Agrobacterium tumefaciens* mediated transformation of the AC cultivar using strain LBA4404 (Fillatti et al., 1987). The presence of transgenes in tomato and the subsequent development of homozygous lines, was achieved using a combination of PCR screening and Southern blot hybridization using probes or markers designed to transgenes or selection markers as previously described (Barry et al., 2005; Barry and Giovannoni, 2006). All primers used for assembling constructs were listed in Table 2.6.

Epitope-tagged versions of *SlGR* and *SlGRL1* were assembled using Gateway cloning technology. Briefly, Gateway Entry clones were developed for each gene using primers listed in Table 2.6 to amplify the corresponding gene fragments and insert them into the pENTR-D-TOPO vector (Invitrogen). The resultant clones were digested with *Mlu*I which cuts in the vector backbone but not the insert and the digestion mix was used together with the binary vector pEarleyGate 203 (*CaMV35S::MYC*-Gateway-OCS) to create two constructs: *CaMV35S::MYC-SlGR* and *CaMV35S::MYC-SlGRL1*. All PCR fragments used for construct assembly were amplified using *Pfu* Ultra™ DNA polymerase (Agilent Technologies) and the fidelity of the constructs confirmed by DNA sequencing. The constructs were transferred into *Agrobacterium*

tumefaciens strain GV3101 and transformed into *Arabidopsis etr1-2/rte1-3* double mutant 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.

DNA sequence analysis

DNA sequences were assembled using Sequencher™ version 4.7 (Genecodes Corporation, Ann Arbor, MI http://genecodes.com).

Statistical analysis

Statistical analyses were performed using SAS (SAS Institute, www.sas.com). The genotypic constituents were evaluated by Student's *t-test* and LS Means.

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Chapter 3 Exploring putative functional domains in GR/RTE1 proteins

Abstract

The gaseous plant hormone ethylene influences many aspects of plant development and mediates responses to biotic and abiotic stresses. GREEN RIPE (*Sl*GR) and REVERSION TO ETHYLENE SENSITIVITY 1 (*At*RTE1) are the founder members of the GR/RTE1 protein family and function in ethylene signaling in tomato (*Solanum lycopersicum*) and *Arabidopsis* (*Arabidopsis thaliana*), respectively. In tomato, *SlGR* and its paralog GREEN RIPE LIKE1 (*Sl*GRL1) exhibit distinct functional characteristics when over-expressed in tomato and while *SlGRL1* is able to fully complement loss of *rte1* function, *Sl*GR is only able to recover the ethylene hypersensitivity observed in the *rte1-3* mutant allele. Putative Solanaceae orthologs of *SlGR* and *SlGRL1* were isolated through a combination of library screening and *in silico* analysis. Phylogenetic and sequence alignments revealed that putative *SlGR* orthologs within the eudicot lineage are restricted to the Solanaceae family and are more divergent than the putative *SlGRL1* orthologs. A combination of over-expression in tomato and complementation of the *rte1-3* mutant allele of *Arabidopsis* revealed that *Ph*GR from *Petunia hybrida* and *Sm*GR from eggplant (*Solanum melongena*) share functional characteristics of both *SlGR* and *SlGRL1*. Utilizing a comparative approach, a series of amino acid residues were identified that correlate with the ability of the Solanaceae GR and GRL1 proteins to complement the *rte1* mutant phenotype. Expression of synthetic constructs in which amino acids present in *Sl*GR were converted to those present in either *Ph*GR or *Sm*GR led to the identification of a set of 10 amino acids that are important for the ability of Solanaceae GR and GRL1 orthologs to complement the *rte1* mutant phenotype. Furthermore, previous mutant analysis demonstrated the importance of the C-

terminus of GR/RTE1 for function. In this study, the roles of two highly conserved amino acids, Y235 and K238, that reside in the C-terminus of GR/RTE1 proteins was investigated through a combination of site-directed mutagenesis and deletion analysis. Three *Sl*GRL1 mutant variants, *Sl*GRL1Y235A, *Sl*GRL1K238A, and *Sl*GRL1Δ9, complemented the *rte1-3* mutant allele with the same efficiency observed using the wild-type *Sl*GRL1 protein suggesting that although these residues are highly conserved, Y235 and K238 are not essential for function.

Introduction

Hormone signaling pathways are generally conserved in higher plants and conservation of ethylene perception and signaling between diverse species is probably best illustrated by transgenic expression of a dominant mutant form of the *Arabidopsis etr1-1* receptor in many plant species including petunia, tomato, carnation, *Campanula carpatica* and tobacco (Wilkinson et al., 1997; Knoester et al., 1998; Bovy et al., 1999; Sriskandarajah et al., 2004). These experiments demonstrate that multiple ethylene responses in diverse species can be controlled by a single heterologous transgene. The conservation of ethylene signaling mechanisms is also supported by research demonstrating the isolation and functional characterization of ethylene signaling components from several plant species (Sato-Nara et al., 1999; Takahashi et al., 2002; Guo and Ecker, 2004; Kendrick and Chang, 2008; Ma et al., 2010; Tatsuki, 2010). However, outside of *Arabidopsis*, a deep understanding of the role of many of these signaling components is lacking particularly in species where ethylene influences developmental processes that are not part of the *Arabidopsis* life cycle including fruit ripening, internode elongation during

submergence tolerance in rice, andromonoecy, and nodule formation (Oeller et al., 1991; Xu et al., 2006; Boualem et al., 2008; Penmetsa et al., 2008; Hattori et al., 2009).

Furthermore, increasing evidence suggests that there is considerable variation in the composition of ethylene signaling components between species. For example, compared with *Arabidopsis*, tomato has an extra subfamily-1 ethylene receptor *LeETR2*, but lacks a copy of the subfamily-2 receptor *ERS2* (Bleecker, 1999; Klee, 2004). Similarly, there is no ETR1-type ethylene receptor in rice, wheat, and maize (Cao et al., 2003; Ma and Wang, 2003; Gallie and Young, 2004; Watanabe et al., 2004; Yau et al., 2004). In general, gene silencing and analysis of knockout mutants has revealed a more pronounced role for subfamily-2 ethylene receptors in tomato, rice, and petunia compared to *Arabidopsis* (Tieman et al., 2000; Qu et al., 2007; Wang and Kumar, 2007; Wuriyanghan et al., 2009). In addition to variation in the ethylene receptors, variation is also present in *CTR1*-like genes. While a single CTR1 protein is encoded by the *Arabidopsis* genome; four *CTR1*-like genes are present in tomato, *LeCTR1*, *LeCTR2*, *LeCTR3*, and *LeCTR4* (Lin, 1998; Leclercq et al., 2002; Adams-Phillips et al., 2004). In *Arabidopsis*, CTR1 interacts with all *Arabidopsis* ethylene receptors, although the affinity between CTR1 and ETR1 is the stronger than with the other receptors (Clark et al., 1998; Wang et al., 2003; Qu et al., 2007). In tomato, *Le*CTR1, *Le*CTR3, and *Le*CTR4 proteins interact with the tomato subfamily-1 receptors, whereas *Le*CTR2 only interacts with *Le*ETR1 and *Le*ETR2 (Lin et al., 2008; Zhong et al., 2008). Although not fully understood, this variation in gene copy number and functional characteristics between species suggests that subtle differences may exist in ethylene signaling cascades of diverse species that may contribute to distinct developmental processes, or environmental responses leading to phenotypic variation.

In tomato, copy number variation is also evident within the *GREEN-RIPE/REVERSION TO ETHYLENE SENSITIVITY 1* (*GR/RTE1*) gene family with tomato containing two paralogous genes designated *GREEN-RIPE* (*SlGR*) and *GREEN-RIPE LIKE 1*(*SlGRL1*) whereas *Arabidopsis* and other eudicot species contain a single copy gene that has higher sequence similarity to *SlGRL1* than to *SlGR* (Barry and Giovannoni, 2006). In *Arabidopsis,* RTE1 is required for the function of the ETR1 ethylene receptor (Resnick et al., 2006; Zhou et al., 2007) and the presence of the extra gene copy in tomato creates the potential for subfunctionalization leading to increased complexity within the ethylene signaling pathway. This hypothesis was supported by a combination of characterization of the *Gr* mutant and over-expression analysis of *SlGRL1*. The *Green-ripe* (*Gr*) mutant of tomato 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 and results from a promoter deletion that causes over-expression of *GR* (Barry et al., 2005; Barry and Giovannoni, 2006). Over-expression of *SlGR* under the control of the *CaMV35S* promoter recreates the *Gr* mutant phenotype but does not lead to a whole plant reduction in ethylene responsiveness suggesting that *Sl*GR modulates a subset of ethylene responses possibly at the post-transcriptional level (Barry and Giovannoni, 2006). Similarly, over-expression of *SlGRL1* in tomato also leads to reduced ethylene responsiveness but affects distinct yet overlapping responses compared to *SlGR* suggesting that these genes are not completely functionally equivalent, a hypothesis supported by their differential ability to complement loss of *rte1* function in *Arabidopsis* (Chapter 2). Furthermore, over-expression of *SlGRL1* in a *Gr* mutant background supports a model in which

separate ethylene responses are influenced by either *SlGR* or *SlGRL1* or a combination of both, suggesting the existence of distinct signaling modules in tomato (Chapter 2).

Functional analysis of ethylene signaling components in *Arabidopsis* is facilitated by the availability of multiple mutant alleles in genes of interest that carry amino acid substitutions resulting in altered phenotypic responses (Chang et al., 1993; Kieber et al., 1993; Hua et al., 1995; Schaller and Bleecker, 1995; Hua et al., 1998; Hall et al., 1999; Rodriguez et al., 1999; Resnick et al., 2006; Wang et al., 2006). In other species, a range of mutant alleles are typically unavailable, limiting detailed structure-function analysis of proteins. We hypothesized that the differences in functional properties between *SlGR* and *SlGRL1* are the result of differences in their primary amino acid sequences. Using a comparative approach, putative orthologs of *SlGR* and *SlGRL1* were isolated from several Solanaceous species. Sequence analysis revealed that putative *GRL1* orthologs share a high level of sequence conservation whereas putative *GR* orthologs are more divergent. A combination of over-expression analysis in tomato together with complementation of the *rte1-3* mutant allele of *Arabidopsis* suggests considerable heterogeneity in the functional characteristics of the putative *GR* orthologs. Sequence analysis identified amino acid residues within select GR and GRL1orthologs that correlate with the ability of each protein to complement the *rte1-3* mutant allele of *Arabidopsis*. Custom gene synthesis and site-directed mutagenesis was utilized to attempt to identify amino acids required for functional characteristics of GR and GRL1 proteins. This analysis identified 10 amino acids that are important for the ability of Solanaceae GR and GRL1 orthologs to complement the *rte1-3* mutant allele. In addition, highly conserved amino acids present within the C-terminus of *GR/RTE1* family members were found not to be required for functional characteristics of *SlGRL1*.

Results

Putative *SlGR* orthologs within the eudicot lineage are restricted to the Solanaceae family

Previously, *SlGR* and two additional *GR* homologs were identified from tomato designated *SlGRL1* and *SlGRL2* with phylogenetic analysis revealing that *SlGR* and *SlGRL1* are more closely related to one another and to *Arabidopsis AtRTE1* whereas *SlGRL2* is more divergent and similar to *Arabidopsis RTE1-HOMOLOG* (*AtRTH*) (Barry and Giovannoni, 2006; Resnick et al., 2006). Three way sequence comparisons between *SlGR* and *SlGRL1* with available sequences consistently revealed that *SlGRL1* displayed higher sequence homology to genes from other eudicot species than did *SlGR* (data not shown). Furthermore, all eudicot species examined, even those with available genome sequences, contain only two genes that are either highly similar to *SlGRL1* or *SlGRL2* (Figure 3.1, Table 3.1). Together, these data suggest that *SlGR* is more divergent and may represent a gene that is not widely distributed in eudicot families. In an attempt to identify putative *GR* orthologs, bacterial artificial chromosome (BAC) libraries of three Solanaceae species, eggplant (*Solanum melongena*), pepper (*Capsicum annuum*), and petunia (*Petunia inflata*) were screened with probes to both *SlGR* and *SlGRL1*. Several clones were identified from each library and restriction mapping coupled with hybridization was used to group clones into families (Barry, unpublished data). DNA sequencing of representative BAC clones using primers designed to conserved regions of *SlGR* and *SlGRL1* followed by successive rounds of primer walking were utilized to complete the sequence of the BAC clone covering the predicted *SlGR* and *SlGRL1* genomic regions. Utilizing this approach putative *GR* orthologs

from pepper, eggplant and petunia and putative *GRL1* orthologs from eggplant and petunia were identified (Table 3.1, Barry unpublished data). The subsequent release of the draft sequence of the potato genome also revealed the presence of putative *GR*, *GRL1* and *GRL2* orthologs (Xu et al., 2011) (Table 3.1). Phylogenetic analysis of the predicted proteins revealed that the putative *GR* orthologs formed a separate subclade that only contained sequences from other Solanaceae species whereas the putative Solanaceae GRL1 proteins clustered with RTE1 and related proteins from other eudicot species (Figure 3.1). Furthermore, pairwise sequence comparisons and branch lengths on the phylogenetic tree indicated that the GRL1 related proteins are highly similar to one another, ranging between 89-95 percent identical, whereas the putative GR proteins are more divergent, ranging between 60–89 percent identity at the amino acid level (Figure 3.1, Table 3.2). The phylogenetic relationship of *SlGR* and *SlGRL1* support the hypothesis that these genes possess distinct roles within ethylene signaling.

Over-expression of *PhGR* in tomato phenocopies the *Never-ripe* mutant

Previous research has indicated that *SlGR* and *SlGRL1* are able to influence distinct and overlapping ethylene responses when over-expressed in tomato and also exhibit a differential ability to complement loss of *rte1* function in *Arabidopsis* (Barry and Giovannoni, 2006) (Chapter 2) (Figure 2.1, 2.2, 2.6, 2.7). The amino acid identities of the putative Solanaceae GRL1 orthologs ranges between 89-95 percent whereas the putative Solanaceae GR orthologs, are only between 60-89 percent identical (Table 3.2). As the putative GR orthologs are considerably more divergent, we hypothesized that they have been subject to subfunctionalization resulting in an altered capacity to influence ethylene responsiveness. To test

this hypothesis, *Ph*GR over-expression lines under the control of the *CaMV35S* promoter were generated in tomato and analyzed for altered ethylene responsiveness. Two homozygous independent transgenic *CaMV35S::PhGR* lines were developed that possess elevated *PhGR* transcript levels (Figure 3.2B). Transgenic lines displayed the typical non-ripening phenotype observed by the tomato *Gr* mutant and *CaMV35S::GR* lines (Figure 3.2A). However, unlike the *Gr* mutant and the *CaMV35S::GR* transgenic lines, *CaMV35S::PhGR* etiolated seedlings grown on the ethylene precursor ACC displayed reduced ethylene responsiveness as indicated by significantly increased hypocotyl and root lengths (Figure 3.2C, D, Table 3.4). Similarly, *CaMV35S::PhGR* over-expression lines displayed reduced rates of ethylene-induced floral abscission and petiole epinasty (Figure 3.2E, F). Together, in all of the phenotypes and responses examined, the *PhGR* over-expression lines displayed reduced ethylene responsiveness that resembles the dominant ethylene-insensitive receptor mutant, *Never-ripe* (Lanahan et al., 1994). These data contrast with previous studies on *SlGR* and *SlGRL1* over-expression which indicate that these genes influence only subsets of ethylene responses in tomato (Barry and Giovannoni, 2006) (Chapter 2), revealing that considerable heterogeneity in the control of ethylene responses mediated by members of the *GR/RTE* family.

Ph*GR and *Sm*GR can complement the *rte1-3* loss of function in *Arabidopsis

Our previous research indicates that *SlGR* and *SlGRL1* influence distinct subsets of ethylene responses in tomato (Chapter 2). Furthermore, while *SlGRL1* was able to complement loss of *rte1* function, restoring ethylene insensitivity to the *etr1-2/rte1-3* double mutant, *SlGR* was only

able to recover the ethylene hypersensitivity observed in the *rte1-3* mutant allele (Chapter 2) (Figure 2.6). Given the sequence divergence of the putative *GR* orthologs relative to the high sequence conservation experienced by the putative *GRL1* orthologs, the putative *GR* orthologs may have different functional properties and influence diverse ethylene responses. To test this hypothesis, the ability of *PhGR* and *SmGR* to complement the *rte1-3* allele was examined. *N*-terminal epitope-tagged versions of *PhGR* and *SmGR* 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 loss of *RTE1* function (Resnick et al., 2006). Three homozygous *CaMV35S::MYC-PhGR* lines and two homozygous *CaMV35S::MYC-SmGR* lines were recovered. Analysis of the seedling triple response in these lines indicate that both *PhGR* and *SmGR* are able to complement loss of *rte1* function although *PhGR* has a more pronounced affect than *SmGR* as highlighted by the almost complete restoration of the ethylene-insensitivity to the *etr1-2/rte1-3* seedlings (Figure 3.3, Table 3.3). These data suggest that *PhGR* and *SmGR* possess functional characteristics more similar to *SlGRL1* than *SlGR*. Furthermore, the relatively weak complementation phenotype of *Sm*GR compared to *PhGR* is likely caused by the closer phylogenetic relationship to *SlGR* than *PhGR*. These data indicate that the ability to complement loss of *rte1* function is not restricted to the *GRL1* orthologs and suggest that sequence differences between *SlGR* and other the other putative *GR* orthologs are likely responsible for determining these functional differences.

Identification of amino acids within the GR proteins required for complementation of the *rte1-3* mutant allele

*Ph*GR and *Sm*GR are able to almost fully complement loss of *rte1* function in *Arabidopsis* (Figure 3.3). In contrast, *Sl*GR can only restore the ethylene-hypersensitive phenotype of the *rte1-3* mutant allele to the level of sensitivity observed in wild-type Columbia seedlings grown in either the absence of ACC or in the presence of 0.1 μM ACC (Chapter 2). The differential ability of GR homologs to complement the *rte1* mutant coupled with their moderately high amino acid sequence identity suggest that it might be possible to utilize a comparative approach to identify amino acid residues or motifs that may confer the ability to complement the *rte1-3* mutant allele of *Arabidopsis*. Amino acid alignments were developed comparing all of the putative Solanaceae GR and GRL1 orthologs. Given that the putative GRL1 orthologs share high amino acid sequence identity (89-95 percent) an assumption was made that, like *SlGRL1*, these proteins would be able to complement loss of *rte1* function. Therefore, it was hypothesized that amino acids that are fully conserved within the GRL1 orthologs together with *Sm*GR and *Ph*GR but were divergent in *Sl*GR would facilitate complementation of the *rte1-3* mutant allele. Utilizing this approach, 19 amino acids, which are conserved in putative GRL1 orthologs and *Ph*GR but differ in *Sl*GR, were identified together with 10 amino acids that are conserved in putative GRL1 orthologs and *Sm*GR but differ in *Sl*GR (Figure 3.4). Utilizing custom gene synthesis, two synthetic genes were developed that converted either the 10 or 19 amino acids in *Sl*GR to those present in *Sm*GR and *Ph*GR, respectively. These synthetic genes, referred to as *SlGR-SmGR* and *SlGR-PhGR*, respectively, were cloned into binary vectors downstream of the *CaMV35S* promoter as N-terminally tagged YFP fusions. The *CaMV35S::YFP-(SlGR-PhGR)* and *CaMV35S::YFP-(SlGR-SmGR)* constructs were introduced into the *etr1-2/rte1-3* double mutant which is ethylene hypersensitive due to the suppression of the *etr1-2* phenotype (Resnick et al.,

2006) and the ability of each construct to complement *rte1-3* loss of function was assessed. Three transgenic *CaMV35S::YFP-(SlGR-PhGR)* lines and two transgenic *CaMV35S::YFP-(SlGR-SmGR)* lines restored ethylene insensitivity in both hypocotyls and roots to similar levels observed in the *CaMV35S::MYC-PhGR* and *CaMV35S::MYC-SmGR* lines, when grown in media containing ACC (Figure 3.5, 3.6). These data indicate that the 19 amino acids changed in *Sl*GR-*Ph*GR, and 10 amino acids changed in *Sl*GR-*Sm*GR are functional amino acids, and these amino acids are required for complementation of the *rte1-3* mutant allele.

The last 9 amino acids of *SlGRL1* including the highly conserved tyrosine and lysine residues are not required for the ability of *Sl*GRL1 to complement the *rte1-3* allele

The C-terminus of *At*RTE1 contains a transmembrane domain, and frame shift mutations or deletions that change the C-terminal of *At*RTE1 render the corresponding proteins nonfunctional (Resnick et al., 2006; Dong et al., 2008; Dong et al., 2010). For example, the *rte1-2* mutation, which lacks a single nucleotide in the C-terminus of coding sequence, results in a frame shift that replaces the last 27 residues with 15 incorrect residues and a premature termination codon leading to plants that display phenotypes indicative of enhanced ethylene responsiveness, including shorter hypocotyl and root lengths during the seedling triple response (Resnick et al., 2006). Furthermore a C-terminal tagged version of *At*RTE1 (*At*RTE1-RFP) under the control of native RTE1 promoter region does not complement the *rte1-3* mutant phenotype, whereas N-terminally tagged versions of RTE1 are fully functional (Dong et al., 2008). Similarly, a deletion of the last 12 amino acids of *Sl*GR, renders the truncated protein unable to confer ripening inhibition in transgenic tomato plants (Figure 3.7A) (Barry, unpublished data). Together, these

data indicate that the C-terminus of GR/RTE1 proteins is important for their functional properties.

An alignment of several GR/RTE1 family proteins revealed the presence of conserved tyrosine and lysine motifs corresponding to residues 235 and 238 in *Sl*GR (Figure 3.7B). Due to the highly conserved nature of these amino acids, it was hypothesized that Y235 and K238 are important for the function of the GR/RTE1 family. To test this hypothesis, we utilized the ability of *SlGRL1* to complement the *rte1* mutant phenotype of *Arabidopsis* as a functional screen and generated mutant variants of this gene in which Y235 and K238 were individually converted to alanine residues. In addition, a third construct in which the last nine amino acids of *Sl*GRL1 were deleted to remove these conserved amino acids was created. *N*-terminal YFP-tagged versions of these constructs were assembled transformed in to the *Arabidopsis etr1-2/rte1-3* double mutant and their ability to restore ethylene-insensitivity in the seedling triple response was assessed. All three mutant variant constructs complemented the *rte1-3* mutant allele with the similar efficiency to that observed for *Sl*GRL1 (Figure 3.8, 3.9, 3.10). Together, these data suggest that the last 9 amino acids of *SlGRL1* including the highly conserved tyrosine and lysine residues are not required for the ability of *Sl*GRL1 to complement the *rte1-3* allele.

Discussion

Variation in gene copy number and functional heterogeneity of the *GR/RTE1* family in plants

The *GR/RTE1* family is conserved in plants, animals and protozoa (Barry and Giovannoni, 2006; Klee, 2006) however, gene copy number varies between species. For example, animal and protozoan genomes contain a single *GR/RTE1* homolog of unknown function whereas most plant species contain two or three gene copies. For example, most eudicot species contain a single copy of a gene that is more similar to *AtRTE1* and *SlGRL1* together with a single copy of the distantly related gene, defined by *AtRTH* and *SlGRL2* (Figure 3.1), the latter of which does not appear to influence ethylene responses in tomato (Figure 2.4). However, in addition, tomato and other members of the Solanaceae family possess a single copy of a gene defined by *SlGR* which is a phylogenetically distinct paralog of *SlGRL1* (Figure 3.1; Table 3.1, 3.2). The origins of *SlGR* and *SlGRL1* are unclear but it is possible that they may have arisen as a result of a recent triplication event that is present in the *Solanum* lineage (Sato et al., 2012) although they are fairly divergent at the amino acid level (Table 3.2).

Similarly, most monocot species also contain two *GRL1* homologs together with a copy of a gene that is more similar to *AtRTH* and *SlGRL2*. With respect to ethylene signaling, these homologs appear to possess distinct yet sometimes overlapping functions. For example, both *SlGR* and *SlGRL1* are able to influence distinct subsets of ethylene responses when over-expressed in tomato and each differentially complements the *rte1-3* mutant allele (Chapter 2). Similarly, over-expression of *AtRTE1* in *Arabidopsis* and tomato reduces ethylene responsiveness although *AtRTE1* over-expression lines of tomato more closely resemble *SlGRL1* over-expression lines (Chapter 2). In contrast, our data suggests that *SlGRL2* is not involved in mediating ethylene responses (Chapter 2). In rice, *OsGRL1a/OsRTH1*a influences ethylene responsiveness when over-expressed in rice and *Arabidopsis* and complements the *rte1-2* mutant

allele (Zhang et al., 2012). In contrast, in the responses examined, both *OsGRL1b/OsRTH2* and *OsGRL2/OsRTH3* failed to influence ethylene responsiveness (Zhang et al., 2012).

Putative Solanaceae GR orthologs are more divergent in comparison to the putative Solanaceae GRL1 orthologs suggesting that the putative GR orthologs may possess different functional properties. This hypothesis was confirmed through over-expression analysis in tomato and through complementation of the *rte1-3* mutant allele. Both *PhGR* and *SmGR* are able to complement loss of *rte1* function although *PhGR* has a more pronounced affect (Figure 3.3). Similarly, over-expression of *PhGR*, which displays considerable sequence divergence to either *SlGR* or *SlGRL1*, in tomato led to a broad spectrum reduction in ethylene responsiveness that closely resembles that of the *Nr* ethylene receptor mutant (Lanahan et al., 1994; Wilkinson et al., 1995). These data suggest that *PhGR* and *SmGR* are functionally more similar to the putative *GRL1* orthologs than *SlGR*. However, when over-expressed in tomato, *PhGR* shared characteristics of both the *Gr* mutant and *SlGRL1* over-expression lines (Figure 3.2). The mechanisms through which this functional heterogeneity occurs is not understood but based on analysis of the functional properties of *At*RTE1 (Chapter 2), may involve specific interactions with ethylene receptors. Such specificity is also apparent with interactions between the CTR1-like proteins and the ethylene receptors of tomato. For example, *Le*CTR1, *Le*CTR3, and *Le*CTR4 proteins interact with the tomato subfamily-1 receptors, whereas *Le*CTR2 only interacts with *Le*ETR1 and *Le*ETR2 (Lin et al., 2008; Zhong et al., 2008).

Utilizing a comparative approach to identify functional amino acids in GR/RTE1 proteins

In *Arabidopsis*, mutagenesis is a powerful approach to identify multiple alleles in genes of interest either through traditional forward genetic screens or by reverse genetic screens using approaches such as TILLING (McCallum et al., 2000; Colbert et al., 2001; Henikoff et al., 2004). For example, dissection of ethylene receptor function has been facilitated through the identification and characterization of multiple mutant alleles either derived from EMS populations or through targeted site-directed mutagenesis (Chang et al., 1993; Hua et al., 1995; Schaller and Bleecker, 1995; Hua et al., 1998; Hall et al., 1999; Rodriguez et al., 1999; Wang et al., 2006). Similarly, multiple mutant alleles have been identified for other ethylene signaling components of *Arabidopsis* including *CTR1*, *EIN2* and *RTE1* (Guzman and Ecker, 1990; Kieber et al., 1993; Roman et al., 1995; Alonso et al., 1999; Resnick et al., 2006). In contrast, there are only a small number of mutant alleles available for ethylene signaling components in tomato which limits detailed functional analysis of ethylene signal transduction in species beyond *Arabidopsis*. The tomato ripening mutant, *Never-ripe* (*Nr*) carries a single amino acid substitution within the N-terminal region of an ERS1 type ethylene receptor leading to dominant ethylene-insensitivity (Lanahan et al., 1994; Wilkinson et al., 1995) and recently two mutant alleles within the *SlETR1* receptor, designated *SlEtr1-1* and *SlEtr1-2*, were isolated using a TILLING approach (Okabe et al., 2011). Similar to other dominant ethylene receptor mutants, the *SlEtr1-1* and *SlEtr1-2* mutants exhibited reduced ethylene responses during the seedling triple response leaf epinasty, delayed petal abscission and reduced rates of fruit ripening leading to prolonged fruit shelf life (Okabe et al., 2011).

In the absence of availability of mutant alleles of *SlGR* and *SlGRL1* we considered adopting a comparative approach to identify determinants of ethylene signaling specificity by focusing on a

phenotypic screen based on the differential ability of each gene to complement the *rte1* mutant phenotype. We hypothesized that the inability of *SlGR* to complement *rte1* loss of function in *Arabidopsis*, when compared to the ability of *SlGRL1*, *PhGR*, and *SmGR* to each complement the *rte1-3* mutant allele (Figure 2.6, 2.7, and 3.3) could be utilized as a functional screen for identifying amino acid residues and domains important for protein function. Aligning the amino acid sequences of *Sl*GR, *Ph*GR, and Solanaceae GRL1 orthologs, 19 amino acids were identified that are conserved in putative GRL1 orthologs and *Ph*GR, but not in *Sl*GR (Figure 3.4A). It was hypothesized that within these 19 amino acids, some are responsible for the ability to complement the *rte1-3* mutant allele. Similarly, 10 amino acids were identified within the *Sm*GR protein that correlated with the ability to complement *rte1* loss of function (Figure 3.4B). The 10 amino acids identified as potentially important for activity in *Sm*GR overlap with the 19 amino acids selected from the sequence of *Ph*GR (Figure 3.4). To test the hypothesis, *Sl*GR-*Ph*GR construct in which the selected 19 amino acids of *Sl*GR were changed to the corresponding amino acids of *Ph*GR, and *Sl*GR-*Sm*GR construct in which the selected 10 amino acids of *Sl*GR were changed to the corresponding amino acids of *Sm*GR, were assembled and over-expressed in the *etr1-2/rte1-3* double mutant. *PhGR* appears to possess an enhanced capability to complement loss of *rte1* function compared to *SmGR* (Figure 3.3). This was confirmed by the ability of *SlGR-PhGR* and *SlGR-SmGR* constructs to complement the *rte1-3* allele (Figure 3.5, Figure 3.6). In total, these results reveal that the overlapping 10 amino acids may be important for the ability of the Solanaceae GR and GRL1 proteins to complement the *rte1* mutant phenotype, and the other 9 amino acids probably enhance that function, considering the *PhGR* have a more pronounced affect to complement loss of *rte1* function than the *SmGR*. The specific functions of each amino acid identified in this research, are still unclear, and additional

mutagenesis will be needed to refine important amino acid residues. However, this analysis provides a starting point for more in depth analysis of the specific role of domains and amino acids of *Sl*GR and related proteins that are involved in regulating ethylene responsiveness.

The functional importance of membrane-spanning domains within GR/RTE1 proteins

Several components of the *Arabidopsis* ethylene signaling pathway are located within the endomembrane system including the ethylene receptors, GR/RTE1, and EIN2 (Chen et al., 2002; Ma et al., 2006; Zhou et al., 2007; Dong et al., 2008; Bisson et al., 2009; Dong et al., 2010). Furthermore, the membrane location of the ethylene binding domain within receptor dimmers highlights the importance of localization of the ethylene signaling pathway. Similarly, *RTE1* encodes a membrane bound protein with an N-terminus that lies in the cytoplasm and a C-terminus that is either located in the Golgi, or both the ER and Golgi (Zhou et al., 2007; Dong et al., 2008; Dong et al., 2010). Disruption of coding sequence in C-terminus of *At*RTE1 in the *rte1-2* mutant allele as a result of a frame shift mutation inhibits protein activity and C-terminally tagged versions of *At*RTE1 cannot complement the *rte1* mutant phenotype (Resnick et al., 2006; Dong et al., 2008). These data demonstrate the importance of the C-terminus for the correct functional properties of *At*RTE1. Similarly, we have shown that deletion of the last 12 amino acids of *Sl*GR in tomato disrupts the ability of the GR protein to modulate ethylene responsiveness although it is presently unknown whether this mutation disrupts membrane localization (Figure 3.7A) (Barry, unpublished data). In the present study, we investigated the potential role of highly conserved tyrosine and lysine residues that lie within the C-terminus of members of the *GR/RTE1* family. The ability of *SlGRL1* to complement the *rte1* mutant

phenotype was utilized together with site-directed mutagenesis and deletion of the last 9 amino acids of *SlGRL1* to assess the potential importance of these amino acids for function. In each case, the mutated or deleted version of *Sl*GRL1 complemented the *rte1-3* mutant allele (Figure 3.8, 3.9, 3.10) suggesting that these residues, although highly conserved are not required for the function of *SlGRL1*. The basis for conflicting data from our previous analysis of the *SlGR*Δ12 construct and the data obtained in this study using the *SlGRL1*Δ9 construct is currently unknown. It is possible that the Δ12 construct does not insert into the membrane correctly whereas the Δ9 likely exhibits correct membrane localization based on its ability to complement the *rte1* mutant. Alternatively, it is possible that amino acid residues important for *Sl*GR-mediated modulation of ethylene responsiveness in fruit are different from those modulating *Sl*GRL1 mediated complementation of *rte1*. Additional experimentation will be required to address these possibilities.

Materials and Methods

Plant materials and growth conditions

The parental tomato cultivar Ailsa Craig (AC), the ethylene-insensitive tomato mutants *Never-ripe* (*Nr*) and *Green-ripe* (*Gr*) were previously described (Lanahan et al., 1994; Barry et al., 2005; Barry and Giovannoni, 2006). Plants were grown in peat-based compost supplemented with fertilizer in greenhouses at Michigan State University, East Lansing, MI. Experiments to evaluate the triple response phenotype in dark grown tomato seedlings and floral abscission were performed as previously described (Barry et al., 2005) with the exception that seedlings were

measured at 8 days after sowing and flowers were induced to abscise by treatment with ethylene at a concentration of 2 μl l^{-1}. Tomato plants for investigating ethylene responses during petiole epinasty were grown in growth chamber for 4 weeks at 28^{o}C at Michigan State University, East Lansing, MI. 4-week old plants were treated with 20 μl l^{-1} ethylene for 16 hours and the adaxial leaf angle determined.

Arabidopsis thaliana Columbia (Col-0) ecotype, *etr1-2* (Hall et al., 1999), and *etr1-2/rte1-3* (Resnick et al., 2006) transgenic plants with Col-0 background were sown in sown in 1:1:1 Sure mix: Medium vermiculite: Perlite (Michigan Grower Products Inc., www.suremix.com) and exposed to a three day cold treatment at 4^{o}C. Seed trays were transferred to a growth chamber at 22^{o}C under 16-h light/ 8-hour dark at 145 μmol m^{-2} s^{-1} and 65% relative humidity. Plants were supplemented with fertilizer 0.25 X Hoaglands solution pH 5.5. The *Arabidopsis* triple response screen was performed using a slightly modified version of a previously published protocol (Alonso et al., 2003). *Arabidopsis* seeds were sterilized with 70% ethanol for 10 minutes, followed by three washes with 100% ethanol. Surface sterilized seeds were dried on sterile filter paper in a laminar flow hood and dried seeds were sprinkled onto 0.8% phytagar containing 1×Murashige and Skoog salts, pH 6.0 and 1% sucrose supplemented with 1-aminocyclopropane-1-carboxylic acid (ACC) at 0, 0.1, 1, 10, and 100 μM. Plates were placed at 4^{o}C for 3 days, exposed to light for 12 h, and then incubated at room temperature in the dark for 6 days. Hypocotyl and root lengths were measured using ImageJ (http://rsbweb.nih.gov/ij/).

Bacterial artificial chromosome library screening and isolation of genomic and cDNA clones

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 ^{32}P-radiolabelled and DNA probes derived from tomato *SlGR* and *SlGRL1* as previously described (Barry et al., 2005; Barry and Giovannoni, 2006). Clones were grouped based on restriction digest fragments following digestion with *Hin*DIII, hybridization patterns with *SlGR* and *SlGRL1* and, in the case of BAC clones harboring putative *GR* orthologs, the presence of synteny with genes previously shown to flank the *Gr* locus (data not shown). Based on these data, the following individual BAC clones were selected for sequence analysis of putative *SlGR* orthologs (eggplant (72L01), pepper (501K23), petunia (21M10)) and *SlGRL1* orthologs (eggplant (150M15), petunia (16J10)), respectively. Sequencing of the genomic regions covering either *SlGR* or *SlGRL1* orthologs was accomplished by successive rounds of primer walking. Putative open reading frames were deduced based on alignment with tomato and potato cDNA clones. cDNA clones corresponding to *PhGR* and *PhGRL1* were amplified by RT-PCR from cDNA isolated from *Petunia hybrida* ovary. cDNA clones corresponding to *SmGR* was amplified by RT-PCR from cDNA isolated from *Solanum melongena* whole fruits. Sequences are deposited in Genbank under the following accession numbers (JQ659027-JQ659031).

qRT-PCR analysis

Total RNA was extracted using the RNeasy® Mini Kit and subjected to on column DNase treatment (Qiagen, http://www.qiagen.com). 1 µg of RNA was used for reverse transcription

using SuperScript™ III First-Strand Synthesis System (Invitrogen, http://www.lifetechnologies.com/). *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* was used as the endogenous control. The GAPDH primers were as previously describe (Balaji et al., 2008). The qRT-PCR analysis for *CaMV35S::PhGR* transgenic lines were performed with FAST SYBR® Master Mix, 2x (Applied Biosystems) in a 25 µl volume using an Applied Biosystems StepOnePlus™ Real-Time PCR System (ABI) with the following cycling program: 10 min at 95 ℃, followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min. The primer efficiency was tested by generating standard curves and results were analyzed by comparative ΔΔCT method (Livak and Schmittgen, 2001). The relative expression level of *PhGR* transgene was detected using primers of PETGRQ-F and PETGRQ-R, which were designed by Primer Express 3.0 (Applied Biosystems, http://www.lifetechnologies.com). Primer sequences are listed in the Table 3.5.

DNA constructs and plant transformation

The *CaMV35S::PhGR* construct was assembled as follows: The full-length coding sequence of *PhGR* was re-amplified from a full-length cDNA clone using the primers PHGROE-F and PHGROE-R, which contain a *Bam*HI and *Sac*I linker sites on the forward and reverse primer, respectively (Table 3.5). 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 *CaMV*35S promoter in the binary vector pBI121, previously modified by removal of the *UidA* coding region by digestion with *Bam*HI and *Sac*I. The PCR fragment used for construct assembly was amplified using *Pfu* Ultra™ DNA polymerase (Agilent

Technologies, www.agilent.com) and the fidelity of the construct was confirmed by DNA sequencing. Transgenic tomato plants were generated through cotyledon-derived explants *via Agrobacterium tumefaciens* mediated transformation of the AC cultivar 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 selection marker NPTII as previously described (Barry et al., 2005; Barry and Giovannoni, 2006).

MYC-tagged versions of *PhGR* and *SmGR* were assembled using Gateway cloning technology. Briefly, the corresponding gene fragments were amplified using primers PETGRENT-F/ PETGRENTSTOP-R and SMGRENT-F/ SMGRENTSTOP-R respectively, and the amplified fragments were inserted into the pENTR-D-TOPO vector (Invitrogen). The resultant clones were digested with *Mlu*I which cuts in the vector backbone but not the insert and the digestion mix was used together with the binary vector pEarleyGate 203 (*CaMV35S::MYC*-Gateway-OCS 3') to create the constructs: *CaMV35S::MYC-PhGR*, and *CaMV35S::MYC-SmGR*. PCR fragments used for construct assembly were amplified using *Pfu* Ultra™ DNA polymerase (Agilent Technologies) and the fidelity of the constructs confirmed by DNA sequencing. YFP-tagged versions of *SlGR-PhGR* and *SlGR-SmGR* were also assembled using Gateway cloning. Briefly, the mutated *SlGR-PhGR* and *SlGR-SmGR* synthetic genes were synthesized in GenScript (http://www.genscript.com/) and the fragments were amplified using primers GRENT-F and GRENTSTOP-R. The fragments were inserted into the pENTR-D-TOPO vector (Invitrogen), and then inserted into the binary vector pEarleyGate 104 (*CaMV35S::YFP*-Gateway-OCS 3') to create *CaMV35S::YFP-(SlGR-PhGR)* and *CaMV35S::YFP-(SlGR-SmGR).* The fragments were

amplified in PCR using KOD polymerase (Novagen), and the fidelity of the constructs was confirmed by DNA sequencing.

Mutant versions of *SlGRL1* carrying amino acid substitutions at the C-terminus of the protein were assembled as follows. The mutated *SlGRL1Y235A* and *SlGRL1K238A* sequences were amplified from the *SlGRL1* ORF clone in PCR-Blunt II vector using the primers GRL1Y235A-F/GRL1Y235A-R and GRL1K238A-F/ GRL1K238A-R, respectively. The fragments were amplified using *Pfu* Ultra™ DNA polymerase (Agilent Technologies). Epitope-tagged *SlGRL1Y235A* and *SlGRL1K238A* were assembled using Gateway cloning technology. Firstly, amplified *SlGRL1Y235A* and *SlGRL1K238A* were inserted into the pENTR-D-TOPO vector (Invitrogen), and digested with *Mlu*I which cuts in the entry vector backbone to linearize each entry clone. Products in the digestion mixes were inserted into the binary vector pEarleyGate 104 (*CaMV35S::YFP*-Gateway-OCS 3') to create *CaMV35S::YFP-SlGRL1Y235A* and *CaMV35S::YFP-SlGRL1K238A* constructs using the LR reaction (Invitrogen). The fidelity of the constructs was confirmed by DNA sequencing. The *SlGRL1Δ9* sequence was amplified using the primers GRL1ENT-F and GRL1Δ9-R, and assembled into pEarleyGate 104 (*CaMV35S::YFP*-Gateway-OCS 3') using Gateway cloning technology as *SlGRL1Y235A* and *SlGRL1K238A*. The fidelity of all constructs was confirmed by DNA sequencing and primer sequences used for construct assembly are summarized in Table 3.5. Constructs were transferred into *Agrobacterium tumefaciens* strain GV3101 and transformed into *Arabidopsis etr1-2/rte1-3* (Resnick et al., 2006) double mutant using the floral dip method (Clough and Bent, 1998).

DNA sequence, phylogenetic analysis, and multiple sequence alignments

DNA sequences were assembled using Sequencher™ version 4.7 (Genecodes Corporation, Ann Arbor, MI 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/) (Table 3.1). 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 V5.0 software and bootstrap values were calculated from 1000 replicates (Tamura et al., 2011). Multiple sequence alignments were constructed using MULTALIN (http://multalin.toulouse.inra.fr/multalin/).

Statistical analysis

Statistical analyses were performed using SAS and the differences among genotypic constituents were evaluated by Student's *t-test* and LS Means.

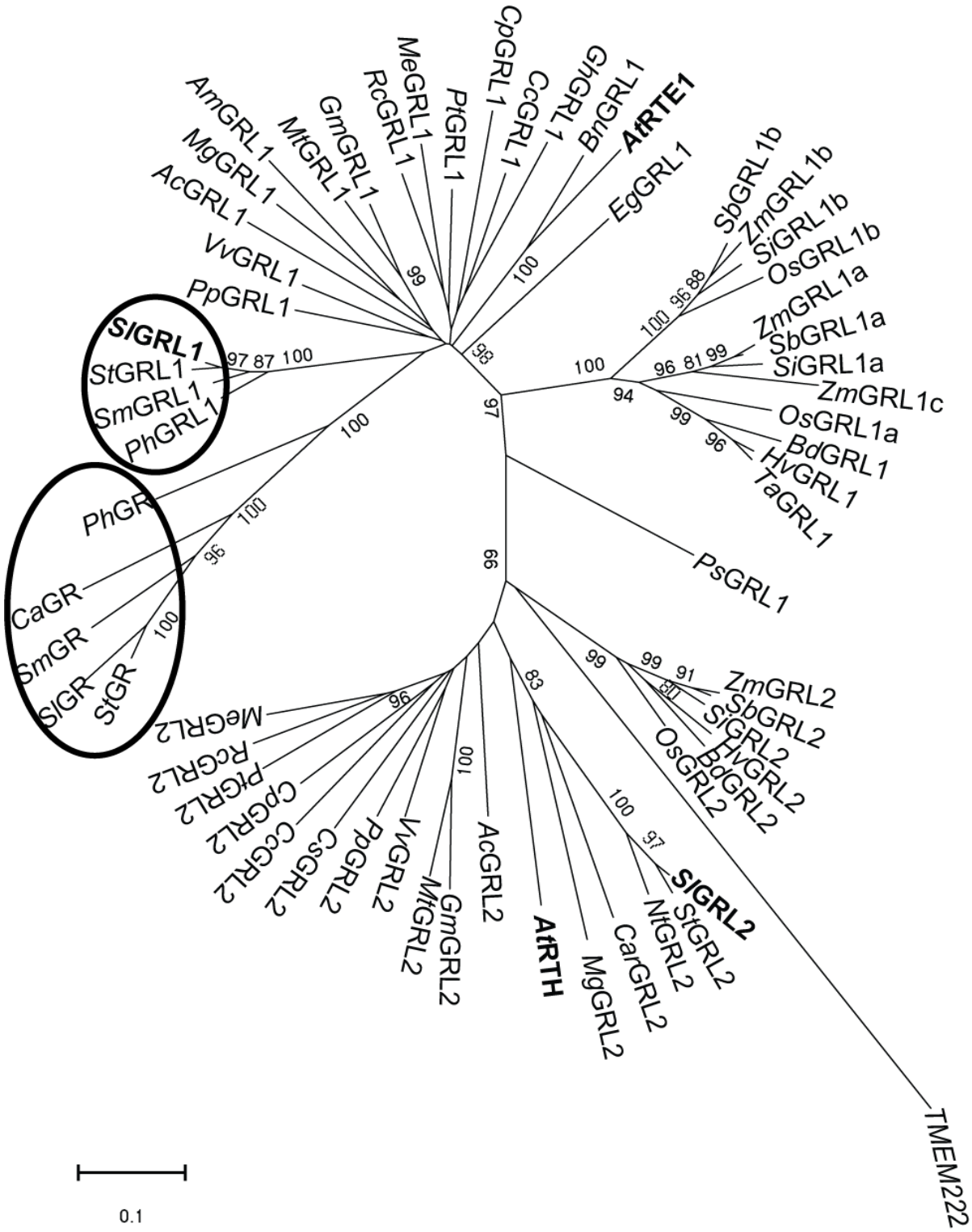

Figure 3.1 Phylogenetic analysis of the GR/RTE1 family of proteins. A neighbor-joining phylogenetic tree derived from a multiple sequence alignment of the deduced full-length amino

acid sequences of GR/RTE1-related proteins of plants was constructed using MEGA V5.0 (Tamura et al., 2011). TMEM222, the human homolog of GR/RTE1 is included as an out-group. Bootstrap values greater than 80 percent, derived from 1000 replicates are indicated above the nodes. Circled regions indicate putative Solanaceae GR and GRL1 orthologous groups. Details of the proteins used to construct the phylogeny are provided in Table 3.1.

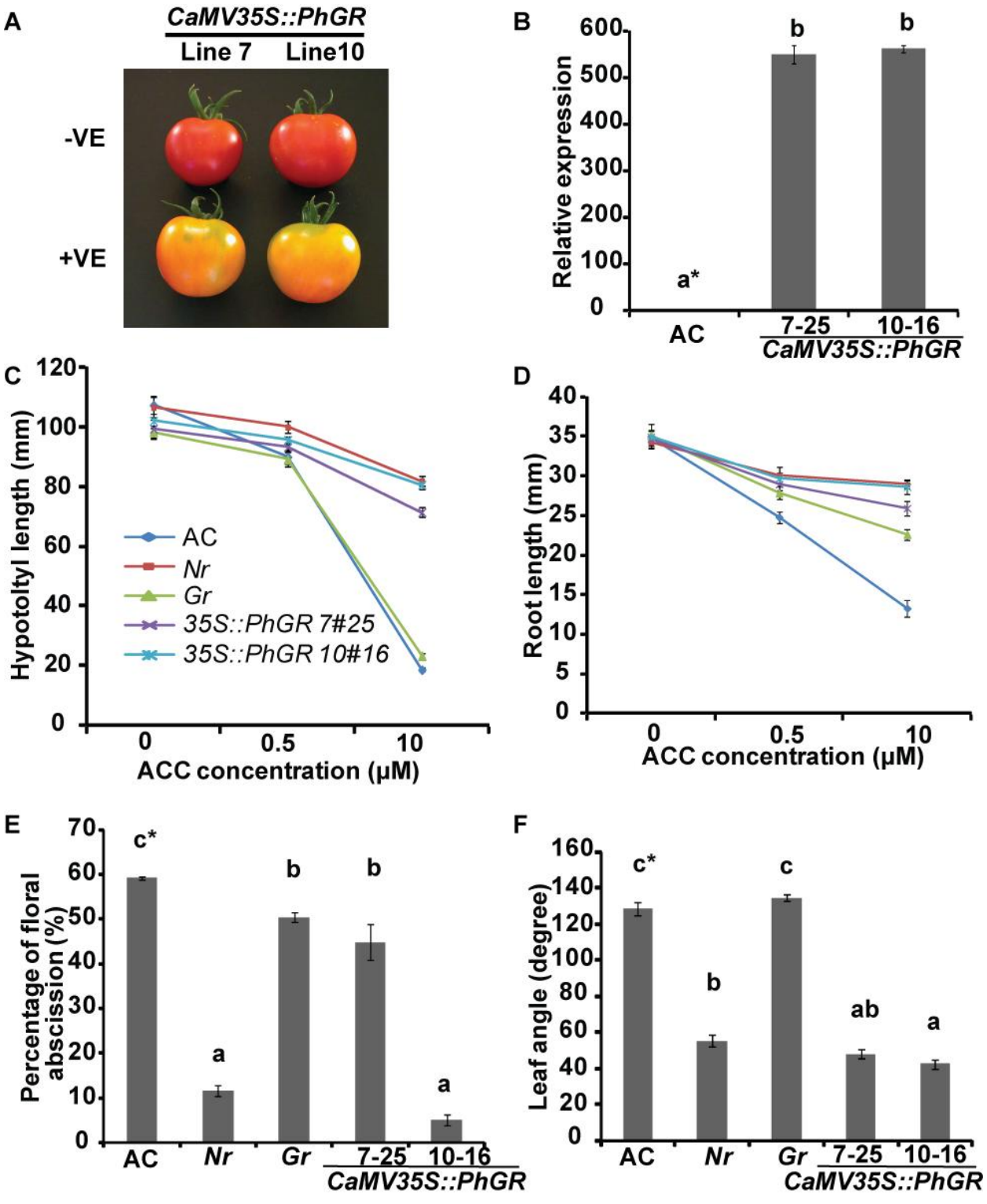

Figure 3.2 Phenotypic analysis of *PhGR* over-expression lines of tomato. A, fruit ripening phenotypes of two T1 segregating lines expressing the *CaMV35S::PhGR* transgene. Ripening

inhibition is shown in lines that contain the transgene (+ve) and normal ripening is observed in sibling lines that have segregated away the transgene (-ve). B, Relative expression levels of *PhGR* in the hypocotyl of two independent homozygous *CaMV35S::PhGR* transgenic lines as determined by qRT-PCR. C, and D, hypocotyl and root lengths, respectively of dark-grown AC, *Nr*, *Gr*, and *CaMV35S::PhGR* seedlings germinated and *grown* in the presence of ACC for eight days at room temperature. Data presented are the means ±SE of at least 17 seedlings (Statistical analysis is presented in Table 3.4). E, percent floral abscission in *CaMV35S::PhGR* lines in comparison to AC, *Nr* and *Gr*. Detached flower trusses were immersed in water in conical flasks and treated with 2 µl l^{-1} of ethylene for 72 hours. Data presented are the mean ±SE of three independent experiments collected from at least 370 flowers per genotype. H, Petiole epinasty in *CaMV35S::PhGR* lines in comparison to AC, *Nr* and *Gr*. The adaxial leaf angle of four week old plants treated with 20 µl l^{-1} of ethylene for 16 hours was measured. Three leaves for each plant were examined and at least 10 plants were used for each genotype and the data presented as the mean ±SE. * Means followed by the same letter are not significantly different at α=0.05 level.

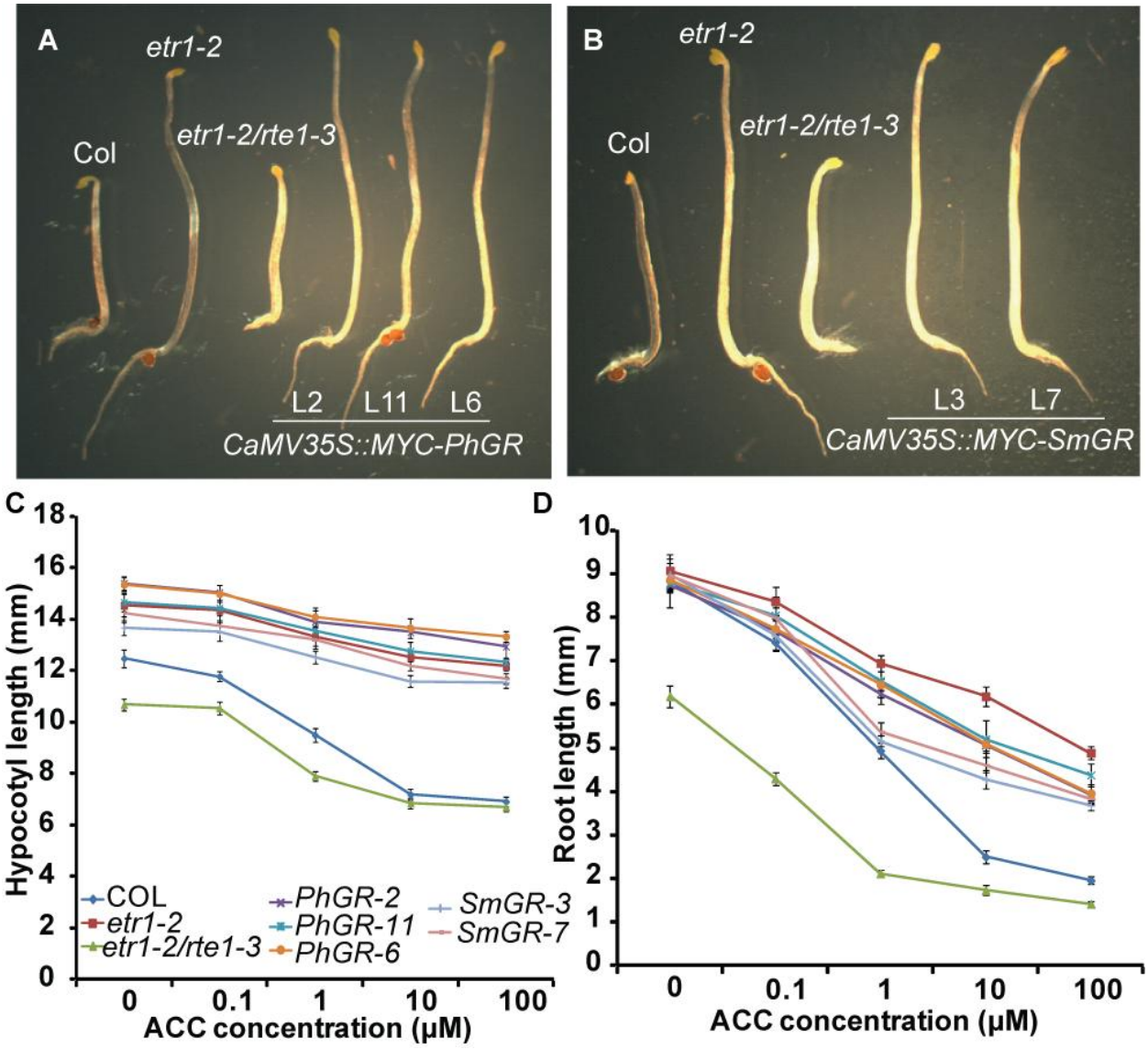

Figure 3.3 Complementation of the *Arabidopsis rte1-3* allele by *PhGR* and *SmGR.* A and B, The triple response phenotype of *Arabidopsis* seedlings from Col-0, *etr1-2*, *etr1-2/rte1-3*, together with three independent *CaMV35S::MYC-PhGR* lines (A) or two independent *CaMV35S::MYC-SmGR* lines (B) transformed into *etr1-2/rte1-3* background. Seedlings were grown in the dark at room temperature for six days on 100 μM ACC. C and D, dose response curve of hypocotyl (C) and root (D) lengths of *Arabidopsis* seedlings grown in the presence of ACC. Each data point

represents the mean ±SE of 15 seedlings. Genotypes are the same as described in (A) and (B). Statistical analyses of the dose response data is provided in Table 3.3.

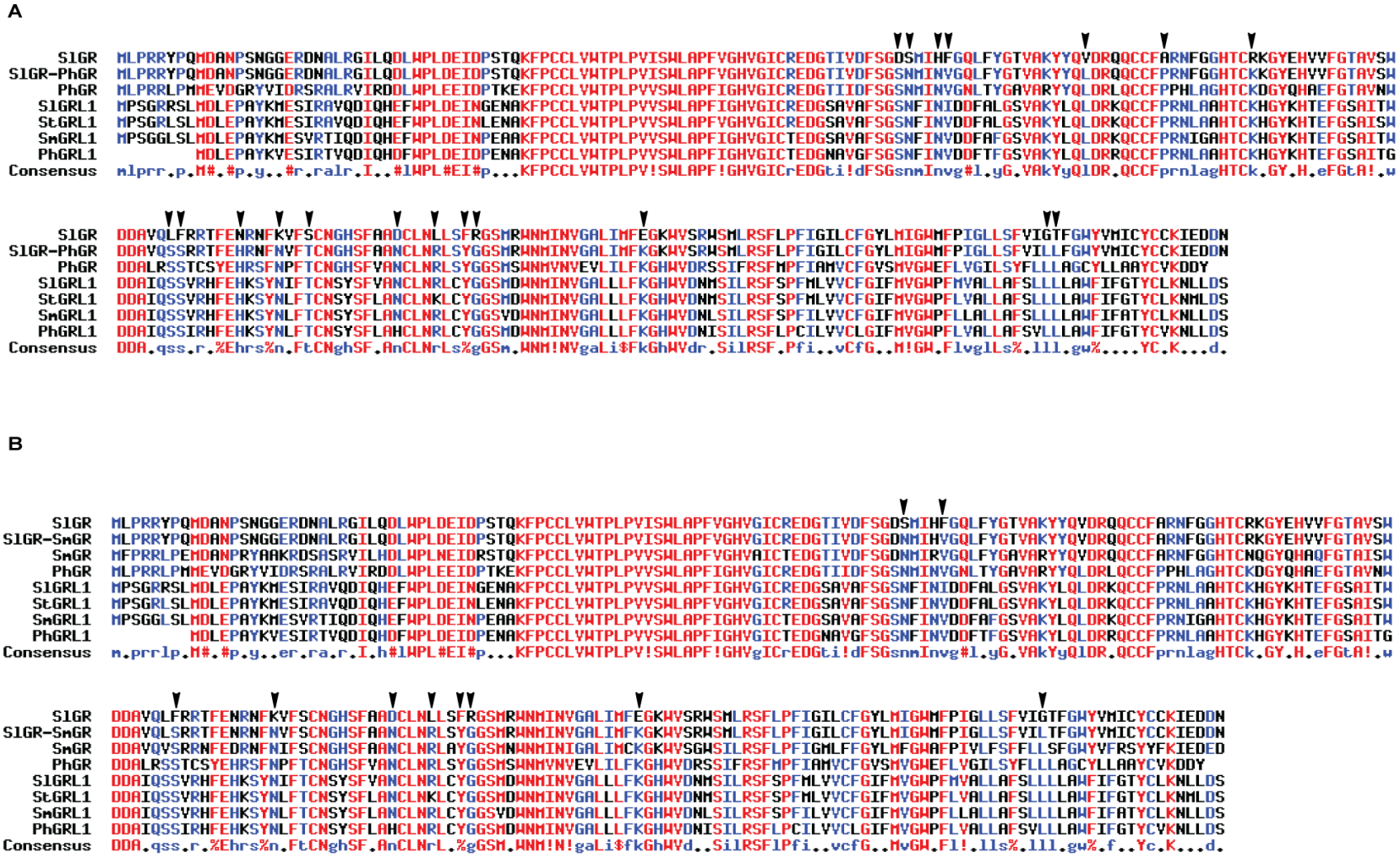

Figure 3.4 Amino acid alignments highlighting conserved amino acids in Solanaceae orthologs of *Sl*GR and *Sl*GRL1 that correlate with the ability to complement the *rte1* mutant phenotype. (A) Amino acids that are conserved in *Ph*GR and putative Solanaceae

GRL1 orthologs in comparison to the divergent amino acids observed in *Sl*GR. (B) Amino acids that are conserved in *Ph*GR, *Sm*GR and putative Solanaceae GRL1 orthologs in comparison to the divergent amino acids observed in *Sl*GR. In each alignment the sequence of a synthetic gene designated either *Sl*GR-*Ph*GR or *Sl*GR-*Sm*GR is shown. Alignments were constructed using MULTALIN (http://multalin.toulouse.inra.fr/multalin/). Sequence details are provided in Table 3.1. Arrows highlight amino acids that are divergent in *Sl*GR that are changed to the corresponding residues, present in the remaining sequences, in the synthetic genes.

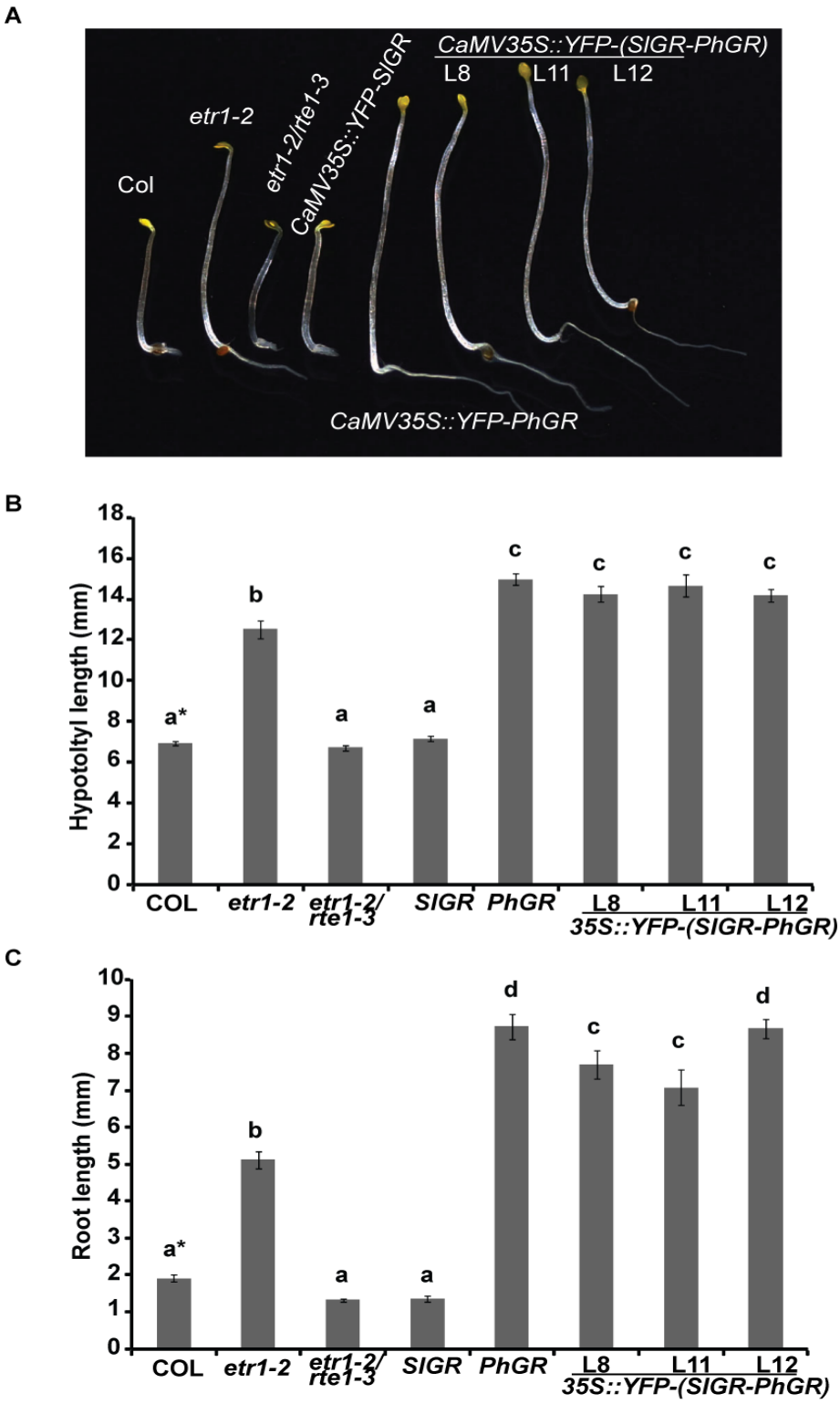

Figure 3.5 Complementation of the *Arabidopsis rte1-3* allele by *SlGR-PhGR*. A, Triple response phenotype of *Arabidopsis* seedlings from Col-0, *etr1-2*, *etr1-2/rte1-3*, together with three

independent *CaMV35S::YFP-(SlGR-PhGR)* lines transformed into *etr1-2/rte1-3* background. Seedlings were grown in the dark at room temperature for six days on 100 μM ACC. B and C, The hypocotyl (B) and root (C) lengths of *Arabidopsis* seedlings grown in the presence of 100 μM ACC. Each data point represents the mean ±SE of 15 seedlings. Genotypes are the same as described in (A). * Means followed by the same letter are not significantly different at α=0.05 level.

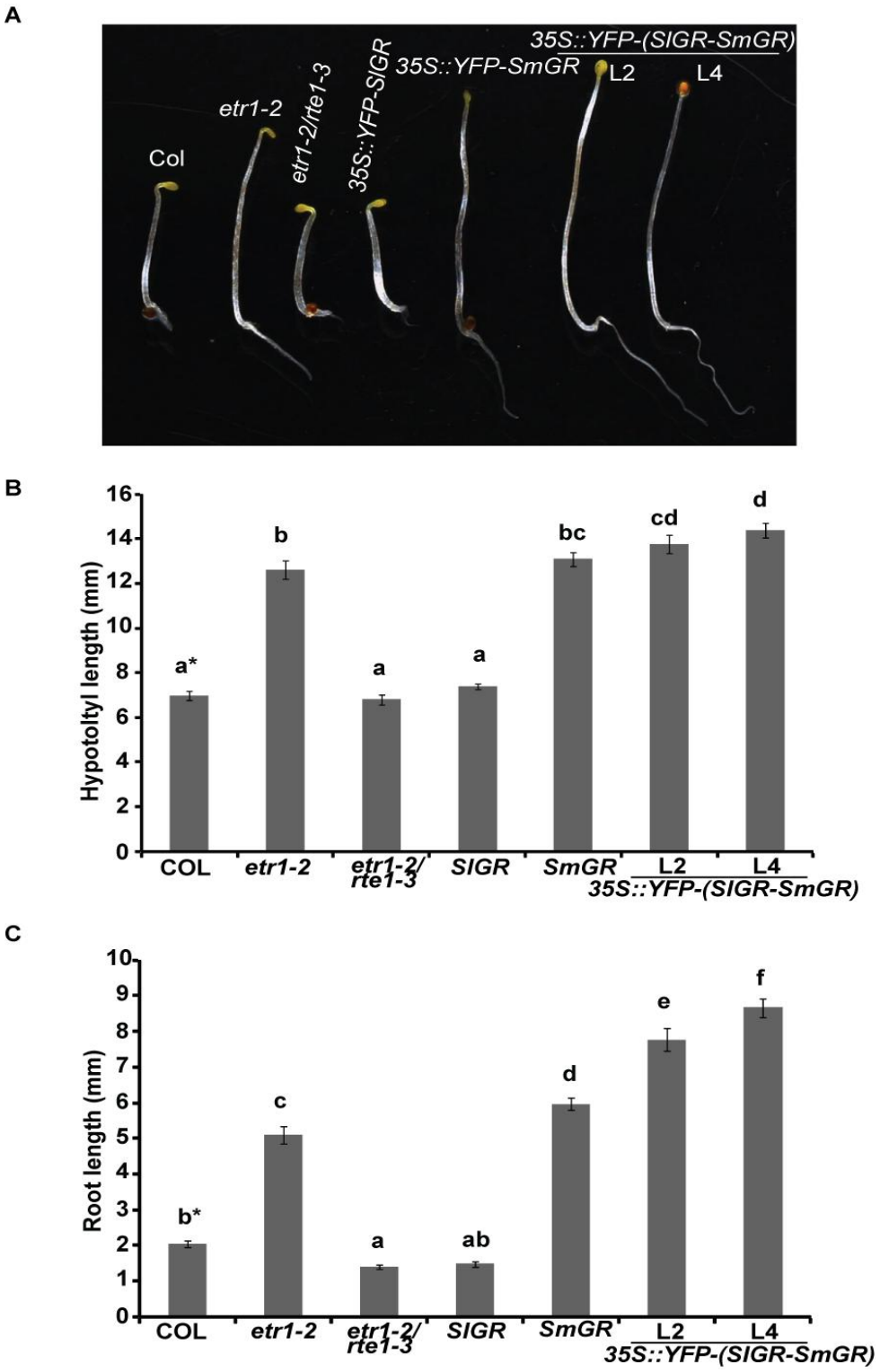

Figure 3.6 Complementation of the *Arabidopsis rte1-3* allele by *SlGR-SmGR*. A, Triple response phenotype of *Arabidopsis* seedlings from Col-0, *etr1-2*, *etr1-2/rte1-3*, together with two

independent *CaMV35S::YFP-(SlGR-SmGR)* lines transformed into *etr1-2/rte1-3* background. Seedlings were grown in the dark at room temperature for six days on 100 μM ACC. B and C, The hypocotyl (B) and root (C) lengths of *Arabidopsis* seedlings grown in the presence of 100 μM ACC. Each data point represents the mean ±SE of 15 seedlings. Genotypes are the same as described in (A). *Means followed by the same letter are not significantly different at α=0.05 level.

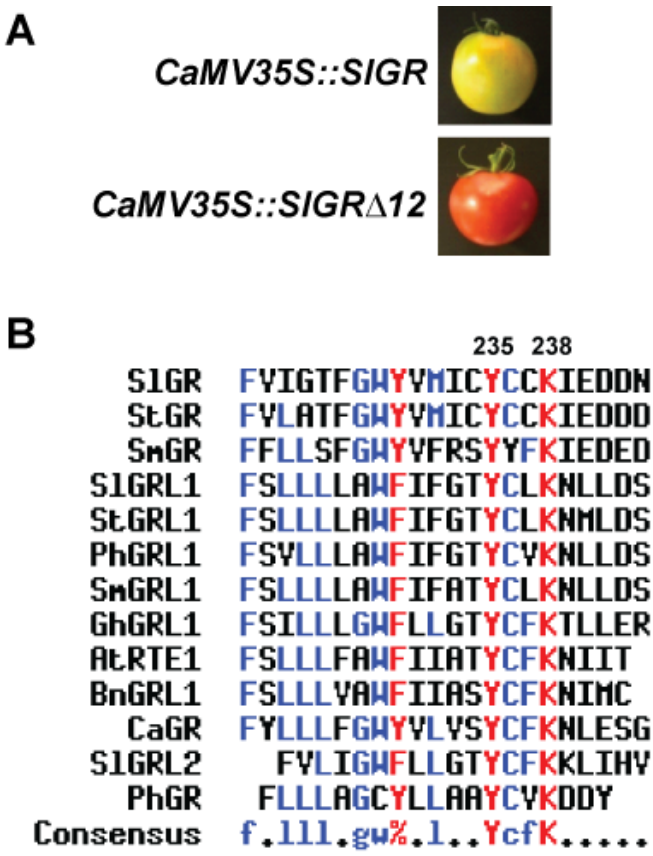

Figure 3.7 The C-terminus of *SlGR* is important for conferring ethylene-insensitivity. A, Fruit phenotypes of *CaMV35S:SlGRΔ12* transgenic line of tomato in comparison to *CaMV35S:SlGR*. B, alignment of last 20 amino acids of *Sl*GR and its homologs, using MULTALIN (http://multalin.toulouse.inra.fr/multalin/). Sequence details are provided in Table 3.1. Tyrosine 235 and lysine 238 are invariant amino acids within the C-terminal domain of GR-relayed proteins.

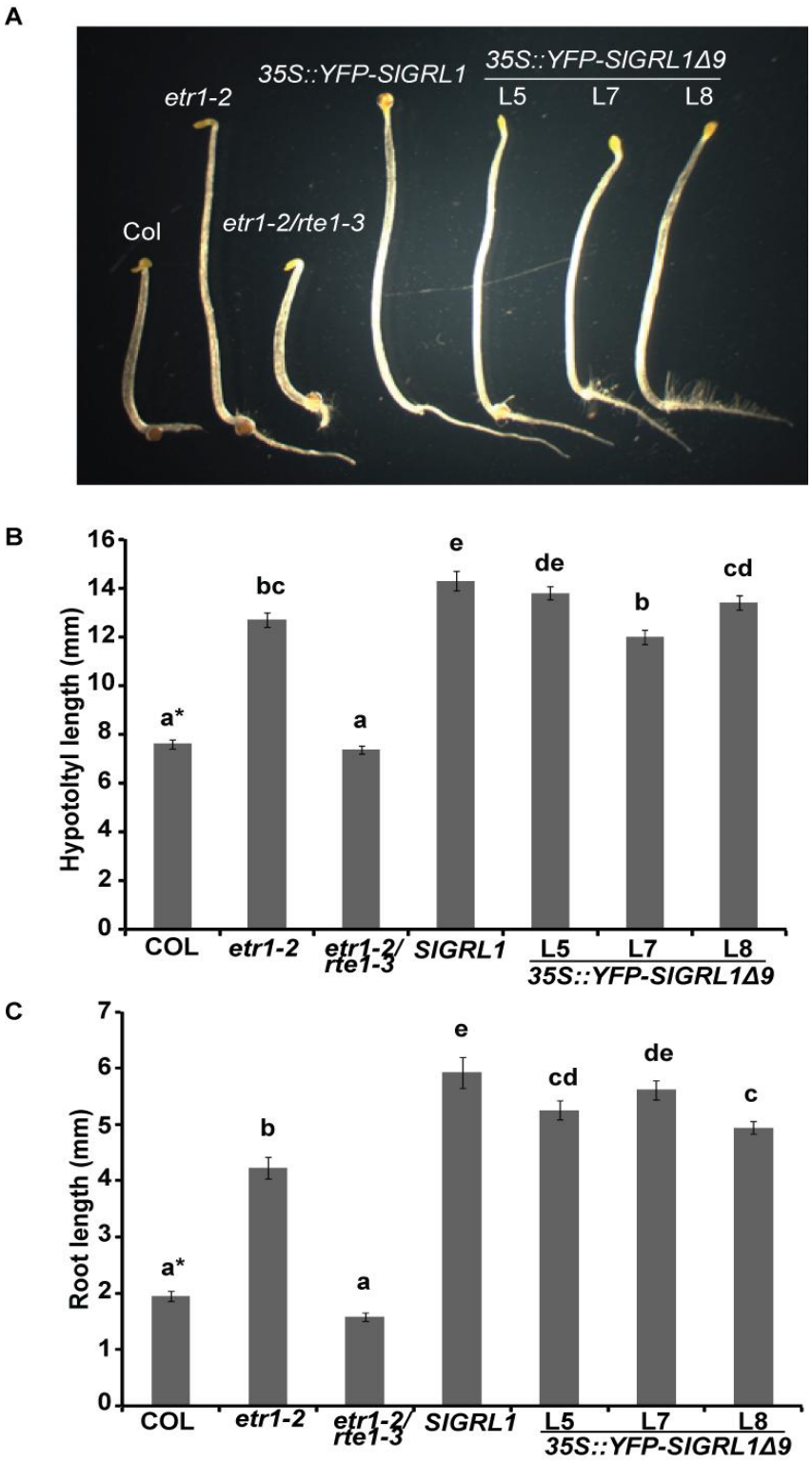

Figure 3.8 Complementation of the *Arabidopsis rte1-3* allele by *SlGRL1Δ9*. A, Triple response phenotype of *Arabidopsis* seedlings from Col-0, *etr1-2*, *etr1-2/rte1-3*, together with one

CaMV35S::YFP-SlGRL1 homozygous line and three independent *CaMV35S::YFP-SlGRL1Δ9* lines transformed into *etr1-2/rte1-3* background. Seedlings were grown in the dark at room temperature for six days on 100 μM ACC. B and C, The hypocotyl (B) and root (C) lengths of *Arabidopsis* seedlings grown in the presence of 100 μM ACC. Each data point represents the mean ±SE of 12 seedlings. Genotypes are the same as described in (A). * Means followed by the same letter are not significantly different at α=0.05 level.

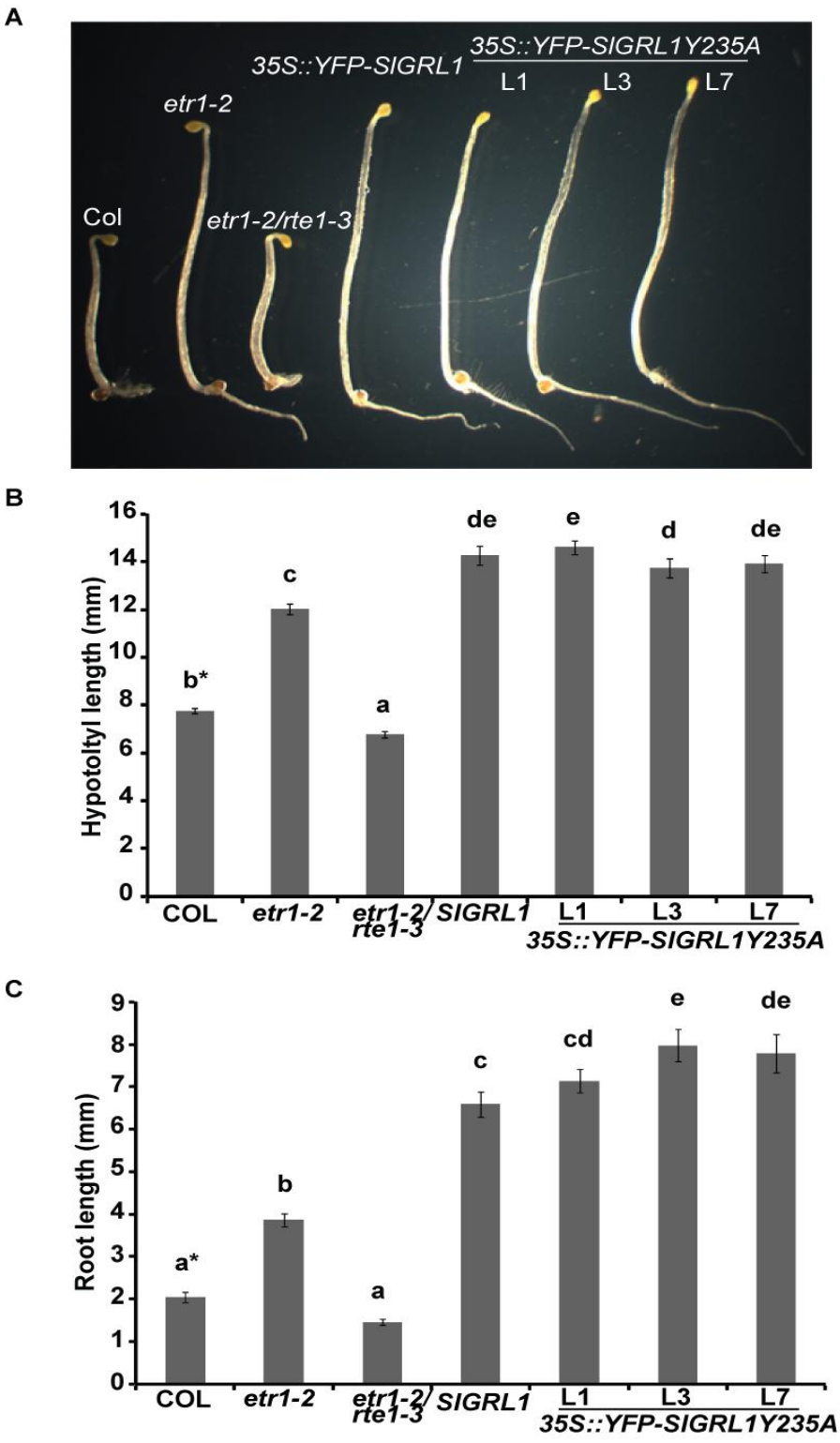

Figure 3.9 Complementation of the *Arabidopsis rte1-3* allele by *SlGRL1Y235A*. A, Triple response phenotype of *Arabidopsis* seedlings from Col-0, *etr1-2*, *etr1-2/rte1-3*, together with one

CaMV35S::YFP-SlGRL1 homozygous line and three independent *CaMV35S::YFP-SlGRL1Y235A* lines transformed into *etr1-2/rte1-3* background. Seedlings were grown in the dark at room temperature for six days on 100 μM ACC. B and C, The hypocotyl (B) and root (C) lengths of *Arabidopsis* seedlings grown in the presence of 100 μM ACC. Each data point represents the mean ±SE of 12 seedlings. Genotypes are the same as described in (A). * Means followed by the same letter are not significantly different at α=0.05 level.

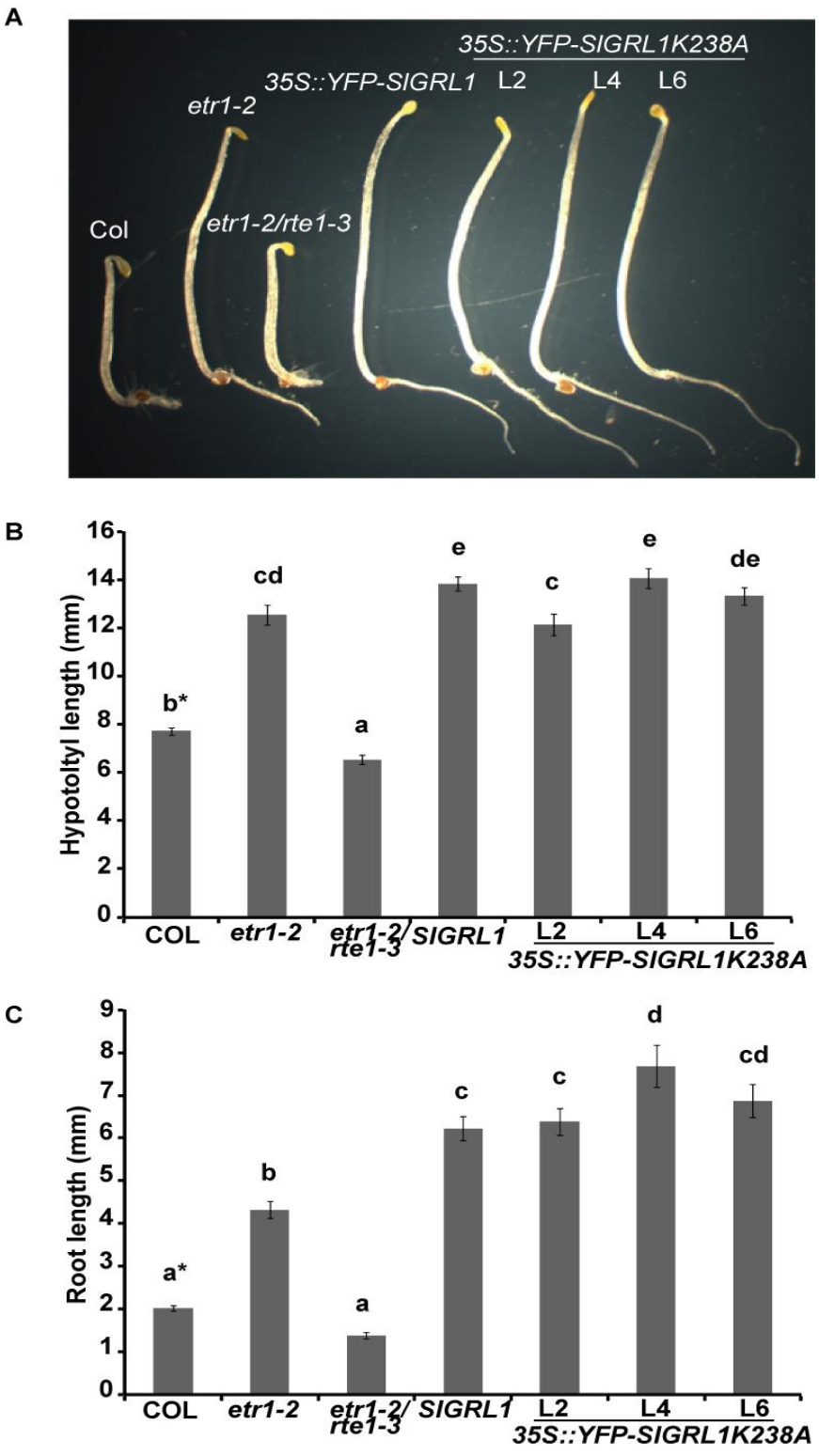

Figure 3.10 Complementation of the *Arabidopsis rte1-3* allele by *SlGRL1K238A*. A, Triple response phenotype of *Arabidopsis* seedlings from Col-0, *etr1-2*, *etr1-2/rte1-3*, together with one

CaMV35S::YFP-SlGRL1 homozygous line and three independent *CaMV35S::YFP-SlGRL1K238A* lines transformed into *etr1-2/rte1-3* background. Seedlings were grown in the dark at room temperature for six days on 100 μM ACC. B and C, The hypocotyl (B) and root (C) lengths of *Arabidopsis* seedlings grown in the presence of 100 μM ACC. Each data point represents the mean ±SE of 12 seedlings. Genotypes are the same as described in (A). * Means followed by the same letter are not significantly different at α=0.05 level.

Table 3.1 GR/RTE1 family proteins utilized for phylogenetic analysis.

Species name	Protein	Identifier #	Source
Arabidopsis thaliana	*At*RTE1	NM_128166	www.ncbi.nlm.nih.gov
	*At*RTH	AY045821	www.ncbi.nlm.nih.gov
Solanum lycopersicum	*Sl*GR	DQ372895	www.ncbi.nlm.nih.gov
	*Sl*GRL1	DQ372898	www.ncbi.nlm.nih.gov
	*Sl*GRL2	DQ372899	www.ncbi.nlm.nih.gov
Solanum tuberosum	*St*GR	NM_001246966	www.ncbi.nlm.nih.gov
	*St*GRL1	DQ372900	www.ncbi.nlm.nih.gov
	*St*GRL2	NM_001246980	www.ncbi.nlm.nih.gov
Petunia×hybrida	*Ph*GR	JQ659030	www.ncbi.nlm.nih.gov
	*Ph*GRL1	JQ659031	www.ncbi.nlm.nih.gov
Solanum melongena	*Sm*GR	JQ659028	www.ncbi.nlm.nih.gov
	*Sm*GRL1	JQ659027	www.ncbi.nlm.nih.gov
Capsicum annuum	*Ca*GR	JQ659029	www.ncbi.nlm.nih.gov
Brassica napus	*Bn*GRL1	Bra007769	www.phytozome.org
Gossypium hirsutum	*Gh*GRL1	TC34712	www.tigr.org/tdb/tgi
Triticum aestivum	*Ta*GRL1	TC268045	www.tigr.org/tdb/tgi
Vitis vinifera	*Vv*GRL1	XM_002279795	www.ncbi.nlm.nih.gov
	*Vv*GRL2	XM_002274070	www.ncbi.nlm.nih.gov
Hordeum vulgare	*Hv*GRL1	AK249698	www.ncbi.nlm.nih.gov
subsp. vulgare	*Hv*GRL2	AK370319	www.ncbi.nlm.nih.gov

Table 3.1 (cont'd)

Sorghum bicolor	*Sb*GRL1a	XM_002456196	www.ncbi.nlm.nih.gov
	*Sb*GRL1b	Sb09g026920	www.phytozome.org
	*Sb*GRL2	XM_002466235	www.ncbi.nlm.nih.gov
Setaria italica	*Si*GRL1a	Si002730m	www.phytozome.org
	*Si*GRL1b	Si023131m	www.phytozome.org
	*Si*GRL2	Si037556m	www.phytozome.org
Oryza sativa	*Os*GRL1a	AK099559	www.ncbi.nlm.nih.gov
	*Os*GRL1b	AK102316	www.ncbi.nlm.nih.gov
	*Os*GRL2	AK071709	www.ncbi.nlm.nih.gov
Manihot esculenta	*Me*GRL1	cassava4.1_027499m	www.phytozome.org
	*Me*GRL2	cassava4.1_015544m	www.phytozome.org
Ricinus communis	*RC*GRL1	XM_002516403	www.ncbi.nlm.nih.gov
	*RC*GRL2	XM_002534086	www.ncbi.nlm.nih.gov
Medicago truncatula	*Mt*GRL1	BT051930	www.ncbi.nlm.nih.gov
	*Mt*GRL2	BT052742	www.ncbi.nlm.nih.gov
Glycine max	*Gm*GRL1	NM_001254223	www.ncbi.nlm.nih.gov
	*Gm*GRL2	NM_001252770	www.ncbi.nlm.nih.gov
Prunus persica	*Pp*GRL1	ppa010598m	www.phytozome.org
	*Pp*GRL2	ppa010911m	www.phytozome.org
Carica papaya	*Cp*GRL1	evm.model.supercontig_7.177	www.phytozome.org
	*Cp*GRL2	evm.model.supercontig_6.192	www.phytozome.org

Table 3.1 (cont'd)

Citrus clementina	*Cc*GRL1	clementine0.9_029097m	www.phytozome.org
	*Cc*GRL2	clementine0.9_020766m	www.phytozome.org
Zea mays	*Zm*GRL1a	NM_001138974	www.ncbi.nlm.nih.gov
	*Zm*GRL1b	BT070200	www.ncbi.nlm.nih.gov
	*Zm*GRL1c	NM_001151994	www.ncbi.nlm.nih.gov
	*Zm*GRL2	NM_001150599	www.ncbi.nlm.nih.gov
Brachypodium	*Bd*GRL1	XM_003569625	www.ncbi.nlm.nih.gov
distachyon	*Bd*GRL2	XM_003557214	www.ncbi.nlm.nih.gov
Populus trichocarpa	*Pt*GRL1	XM_002308529	www.ncbi.nlm.nih.gov
	*Pt*GRL2	XM_002310795	www.ncbi.nlm.nih.gov
Eucalyptus grandis	*Eg*GRL1	Eucgr.C03045.1	www.phytozome.org
Antirrhinum majus	*Am*GRL1	SGN-U391536	www.solgenomics.net
Mimulus guttatus	*Mg*GRL1	mgv1a012670m	www.phytozome.org
	*Mg*GRL2	mgv1a013138m	www.phytozome.org
Aquilegia coerulea	*Ac*GRL1	Aquca_001_00087	www.phytozome.org
	*Ac*GRL2	Aquca_010_00600	www.phytozome.org
Picea sitchensis	*Ps*GRL1	BT122840	www.ncbi.nlm.nih.gov
Nicotiana tabacum	*Nt*GRL2	SGN-U468505	www.solgenomics.net
Coffea arabica	*Car*GRL2	SGN-E1318947	www.solgenomics.net
Cucumis sativus	*Cs*GRL2	Cucsa.201110	www.phytozome.org
Homo sapiens	TMEM222	AAL99388	www.ncbi.nlm.nih.gov

Table 3.2 Percent amino acids identity between GR and GRL1 related proteins from selected Solanaceae species.

Proteins	*Sl*GR	*St*GR	*Sm*GR	*Ca*GR	*Ph*GR	*Sl*GRL1	*St*GRL1	*Sm*GRL1	*Ph*GRL1	*At*RTE1	*At*RTH	*Sl*GRL2
*Sl*GR	100											
*St*GR	89.3	100										
*Sm*GR	74.5	80.2	100									
*Ca*GR	68.1	72.7	75.2	100								
*Ph*GR	59.5	62.8	63.4	64.0	100							
*Sl*GRL1	53.4	56.4	57.0	58.2	60.4	100						
*St*GRL1	54.3	57.3	57.3	57.6	60.4	95.9	100					
*Sm*GRL1	53.9	57.4	57.1	57.8	59.8	91.8	92.2	100				
*Ph*GRL1	53.9	56.5	56.5	58.2	61.0	89.4	89.4	89.8	100			
*At*RTE1	51.9	55.2	57.1	52.9	57.6	59.4	58.2	59.8	61.3	100		
*At*RTH	37.1	38.6	38.3	41.9	43.7	44.6	42.3	44.7	44.6	50.5	100	
*Sl*GRL2	37.4	41.3	38.0	37.8	44.2	44.5	42.6	44.3	46.4	50.4	59.5	100

Shaded boxes highlight comparisons between putative GR and GRL1 orthologs. Identity of the proteins is provided in Table 3.1.

Table 3.3 Statistical analysis of the seeding triple response assay in *etr1-2/rte1-3* lines of *Arabidopsis* transformed with either *CaMV35S::MYC-PhGR* or *CaMV35S::MYC-SmGR*

	0 μM ACC		0.1 μM ACC		1 μM ACC		10 μM ACC		100 μM ACC	
Group	Hypocotyl	Root	Hypocotyl	Root	Hypocotyl	Root	Hypocotyl	Root	Hypocotyl	Root
COL	12.48b*	8.83b	11.78b	7.43b	9.5b	4.94b	7.19a	2.5b	6.91b	1.96b
etr1-2	14.54cde	9.07b	14.35cde	8.37c	13.32cd	6.95d	12.53c	6.19e	12.18cd	4.89e
etr1-2/rte1-3	10.68a	6.19a	10.55a	4.3a	7.9a	2.12a	6.86a	1.73a	6.7a	1.42a
35S::PhGR-2	15.4e	8.74b	15.04e	7.69bc	13.91d	6.25c	13.5d	5.07d	12.94ef	3.92cd
35S::PhGR-11	14.66de	8.8b	14.44de	8.05bc	13.56d	6.55d	12.77c	5.21d	12.32de	4.38d
35S::PhGR-6	15.36e	8.87b	14.99e	7.74bc	14.09d	6.46d	13.65d	5.09d	13.33f	3.96cd
35S::SmGR-3	13.67c	8.98b	13.52c	7.58b	12.53c	5.16b	11.59b	4.27c	11.54c	3.68c
35S::SmGR-7	14.25cd	8.96b	13.74cd	7.99bc	13.2cd	5.38b	12.19bc	4.61cd	11.69cd	3.84c

*Means followed by the same letters are not significantly different at α=0.05 level. Graphical representation of this data is provided in Figure 3.3.

Table 3.4 Statistical analysis of the seedling triple response assay in *CaMV35S::PhGR* lines of tomato

Group	0 μM ACC		0.5 μM ACC		10 μM ACC	
	Hypocotyl	Root	Hypocotyl	Root	Hypocotyl	Root
AC	107.22b	34.93a	89.89ab	24.79a	18.68**a**	13.26**a**
Nr	106.48ab	34.24a	99.9c	30.07b	81.84d	28.92d
Gr	98.25a	35.18a	89.11a	27.84b	23.37b	22.6b
35S::PhGR-7-25	99.3ab	34.7a	93.14ab	28.95b	71.38**c**	25.92**c**
35S::PhGR-10-16	101.94ab	34.94a	95.52bc	29.67b	80.56**d**	28.6**d**

*Means followed by the same letters are not significantly different at α=0.05 level.

Note statistically significant hypocotyl and root lengths in *CaMV35S::PhGR* transgenic lines in comparison to AC in seedlings grown in the presence of 10 μM ACC (shown in bold). Graphical representation of this data is provided in Figure 3.2.

Table 3.5 Oligonucleotide primers used in this study

Primer	**Sequence**	**Use**
PETGROE-F	5 ´-TTTCTAGATGCTTCCAAGAAGACTTCCTATGA-3 ´	Construct assembly
PETGROE-R	5 ´-TTGAGCTCCTAATAGTCATCCTTCACACAGT-3 ´	Construct assembly
PETGRENT-F	5 ´-CACCATGCTTCCAAGAAGACTTCCTA-3 ´	Construct assembly
PETGRENTSTOP-R	5 ´-CTAATAGTCATCCTTCACACAGTA-3 ´	Construct assembly
SMGRENT-F	5 ´- CACCATGTTTCCAAGAAGATTACCTGA-3 ´	Construct assembly
SMGRENTSTOP-R	5 ´-CTAGTCTTCATCCTCAATCTTGAAATA-3 ´	Construct assembly
PETGRQ-F	5 ´- GGGATGATGCTCTTCGCTCTAGT-3 ´	qRT-PCR
PETGRQ-R	5 ´- GCAAGTGAAAGGGTTGAAGGAT -3 ´	qRT-PCR
GRENT-F	5 ´-CACCATGCTGCCAAGAAGATATCCTCA-3 ´	Construct assembly
GRENTSTOP-R	5 ´-CTAATTGTCATCCTCAATCTTGC-3 ´	Construct assembly
GRL1K238A-F	5 ´- GGTACTTACTGTCTCGCAAATCTGTTGGATTCCTAG -3 ´	Construct assembly
GRL1K238A-R	5 ´- CTAGGAATCCAACAGATTTGCGAGACAGTAAGTACC -3 ´	Construct assembly
GRL1Y235A-F	5 ´- GGTACTGCCTGTCTCAAAAATCTGTTGGATTCCTAG -3 ´	Construct assembly
GRL1Y235A-R	5 ´- CTAGGAATCCAACAGATTTTTGAGACAGGCAGTACC -3 ´	Construct assembly

Table 3.5 (cont'd)

GRL1ENT-F	5 ´-CACCATGCCATCAGGAAGACGTTCTT-3 ´	Construct assembly
GRL1Δ9-R	5 ´-TTAGCATGGTTTATTTTTGGTACTTAGGTCGACTT-3 ´	Construct assembly

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Chapter 4 Gene expression analysis and subcellular localization of GR/RTE1 proteins

Abstract

Regulation of plant hormone biosynthesis, turnover, transport, perception and signaling pathways must be tightly controlled to ensure normal growth and development as well as appropriate responses to environmental stimuli. Genes involved in plant hormone biosynthesis and responses are frequently encoded by multigene families, creating the potential for subfunctionalization of individual family members and generating flexibility in hormonal outputs and their protein products must be efficiently and accurately transported to the correct subcellular compartments to function appropriately. In particular, several steps of the ethylene synthesis and perception machinery are encoded by multigene families that are differentially expressed and studies have highlighted the importance of the endomembrane system in mediating ethylene responses. In this study, the expression of *SlGR*, *SlGRL1* and *SlGRL2*, tomato homologs of a family of genes that influence ethylene responses, was determined during tomato development, in response to ethylene treatment and following wounding, revealing that these genes are differentially expressed. In addition, using a combination of transient expression in tobacco and stable expression in *Arabidopsis*, fluorescently tagged versions of *Sl*GR, *Sl*GRL1 and *Sl*GRL2 are predominantly localized to the Golgi. These data suggest that the differential expression of *SlGR*, *SlGRL1* and *SlGRL2* may influence the ethylene responsiveness of individual tissues during tomato development but that differential ethylene responsiveness is unlikely to be associated with the differential subcellular localization of this protein family.

Introduction

Ethylene regulates multiple aspects plant growth and development together with a variety of responses to biotic and abiotic stresses, and ethylene signaling is conserved in higher plants, including rice, *Arabidopsis*, tomato, and petunia (Bleecker and Kende, 2000; Guo and Ecker, 2004; Kendrick and Chang, 2008). In all higher plants analyzed, genes involved in both ethylene biosynthesis and signaling are encoded by multigene families, allowing the plant flexibility to differentially regulate ethylene biosynthesis/signaling in different tissues at various stages of development and in response to environmental stimuli (Sato and Theologis, 1989; Yip et al., 1992; Zarembinski and Theologis, 1994; Wilkinson et al., 1995; Payton et al., 1996; Zhou et al., 1996; Whittaker et al., 1997; Hua and Meyerowitz, 1998; Lashbrook et al., 1998; Tieman and Klee, 1999; Bleecker and Kende, 2000; Klee, 2004; Barry and Giovannoni, 2006; Wang and Kumar, 2007).

The presence of gene families encoding multiple steps of the ethylene signaling pathway facilitates subfunctionalization and in particular creates the potential for differential expression of individual family members. This, in turn, could lead to the formation of signaling complexes that contain different stoichiometry in individual cell types, during developmental processes or in response to external stimuli. Differential expression of gene families encoding the ethylene receptors has been reported for several species. For example, each of the six tomato ethylene receptors, *LeETR1*, *LeETR2*, *NR*, *LeETR4*, *LeETR5*, and *LeETR6* have unique but often overlapping expression patterns. *LeETR1* and *LeETR2* are ubiquitously expressed, although *LeETR1* is expressed at approximately a 5-fold higher level than *LeETR2* (Zhou et al., 1996;

Lashbrook et al., 1998). *NR* is detected in floral ovaries and ripening fruit (Lashbrook et al., 1998). *LeETR4* and *LeETR5* have a higher expression level in flowers and during fruit development than in vegetative tissues (Tieman and Klee, 1999). The expression of ethylene receptor genes can also be influenced by wounding, ozone, or other treatments. For example, in tomato *LeETR2* expression is transiently suppressed by ozone treatment (Moeder et al., 2002). In rice, one of ethylene receptors *OsETR2* is induced by flooding, IAA, ethylene, and GA and in petunia *PhETR2* expression increases following wounding and salt treatment (Watanabe et al., 2004; Yau et al., 2004; Wang and Kumar, 2007). Similarly, downstream ethylene signaling components are also differentially expressed during development and in response to the same sets of treatments that modulate ethylene receptor expression (Leclercq et al., 2002; Adams-Phillips et al., 2004). For example, four *CTR1*-like genes are present in tomato, *LeCTR1*, *LeCTR2*, *LeCTR3*, and *LeCTR4* (Leclercq et al., 2002; Adams-Phillips et al., 2004; Lin et al., 2008). *LeCTR1* expression is increased in response to ethylene treatment, and during fruit ripening, pedicel abscission, and petal senescence, but no similar increase in *LeCTR3* and *LeCTR4* transcript accumulation was observed (Leclercq et al., 2002; Adams-Phillips et al., 2004). Together, these data suggest that the ethylene signaling state of plants can be modified during development and in response to environmental perturbation at the level of gene expression changes that may alter the composition of signaling networks.

Several characterized members of the *GR/RTE1* family influence ethylene responsiveness in plants possibly through direct interactions with ethylene receptors (Barry and Giovannoni, 2006; Resnick et al., 2006; Dong et al., 2010; Zhang et al., 2012). The tomato genome contains three *GR/RTE1* family members designated *GREEN RIPE* (*SlGR*), *GREEN RIPE LIKE 1* (*SlGRL1*)

and *GREEN RIPE LIKE 2* (*SlGRL2*) (Barry and Giovannoni, 2006). The *Gr* mutant displays ethylene insensitivity leading to inhibition of fruit ripening, flower senescence and abscission together with reduced ethylene inhibition of root elongation, but ethylene responses associated with petiole epinasty are normal (Barry et al., 2005; Barry and Giovannoni, 2006). The *Gr* mutant is caused by a 334 bp deletion in the promoter region of *GR* that leads to ectopic expression of the gene and altered ethylene responsiveness (Barry and Giovannoni, 2006). Therefore, appropriate control of *GR* expression during plant development is important to maintain an appropriate response to ethylene. Previously, *GR* expression was found to be at a low level in most tissues with transcript abundance peaking in developing seed (Barry and Giovannoni, 2006). However, the expression pattern of *SlGR*, *SlGRL1*, and *SlGRL2* during tomato development remains undefined, and aside from the finding that ectopic expression of *GR/RTE1* genes causes altered ethylene responsiveness there is no information of how the natural expression pattern of these genes and how this could potentially influence tomato development.

Differential sub-cellular localization may also influence the functional properties of GR/RTE1 proteins. Components in the upstream section of the ethylene signaling pathway are localized to the endomembrane system. The *Arabidopsis* ethylene receptors localize to the endoplasmic reticulum (ER) and Golgi membranes (Chen et al., 2002; Ma et al., 2006; Dong et al., 2008). The CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) is connected to the ER through binding to the ethylene receptors (Clark et al., 1998; Cancel and Larsen, 2002; Gao et al., 2003). ETHYLENE INSENSITIVE 2 (EIN2) is also located at the ER membrane where it interacts with all five ethylene receptors in *Arabidopsis* (Bisson et al., 2009). The presence of ethylene leads to

proteolytic cleavage at the carboxyl-terminal of EIN2 (EIN2-C'), and as a result, EIN2-C' is released and rapidly translocates to the nucleus (Qiao et al., 2012).

Separate studies have investigated the subcellular localization of the *At*RTE1 protein. Transient expression in onion epidermal cells suggested that *At*RTE1 is localized to the Golgi whereas transient expression in *Arabidopsis* protoplasts and stable transformation in *Arabidopsis* suggested dual localization to both the ER and the Golgi (Zhou et al., 2007; Dong et al., 2008). The distant homolog of AtRTE1, *At*RTH, was recently reported to be localized to the ER and nucleus by transient expression in onion epidermal cells as was its putative rice ortholog, *Os*GRL2/*Os*RTH3 (Zhang et al., 2012). In contrast, transient expression in onion epidermal cells suggested dual localization of *Os*GRL1a/*Os*RTH1 and *Os*GRL1b/*Os*RTH2 to the Golgi and ER membranes (Zhang et al., 2012). Together, these data suggest either considerable heterogeneity in the subcellular localization of this protein family or that differences in experimental approaches may have contributed to this anomalous data.

In the present study, the expression pattern of *SlGR*, *SlGRL1*, and *SlGRL2* and the subcellular localization of several GR/RTE1 proteins was assessed to determine if differential expression of these genes could provide insight into the natural role of these genes during tomato development and also to determine whether the GR/RTE1 proteins are differentially localized within the cell, which could provide insight into their altered functional characteristics (Chapter 2). *SlGR*, *SlGRL1* and *SlGRL2* are differentially expressed during tomato development with *SlGR* and *SlGRL1* displaying expression patterns that overlap with the corresponding *AtRTE1* expression in *Arabidopsis*. Reporter-gene fusions indicate that *Sl*GR, *Sl*GRL1, *Sl*GRL2, *Ph*GR, *Sm*GR proteins

are predominantly localized in the Golgi, which is slightly conflicting with the reported sub-cellular localization of *At*RTE1, *At*RTH, and their rice homologs. Together, these data provide insight into the role of this gene family in ethylene signaling, providing data to help guide additional functional characterization.

Results

SlGR, *SlGRL1* and *SlGRL2* are differentially expressed during tomato development

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 with 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 (Barry and Giovannoni, 2006). However, a robust and comprehensive view of the expression of *SlGR, SlGRL1* and *SlGRL2* was not performed. In this study, qRT-PCR was performed using RNA extracted from several tomato tissues. *SlGR* shows maximal transcript levels accumulating in the seeds (Figure 4.1A). Dissection of the seeds revealed that the majority of this expression was associated with the testa (Figure 4.1D). *SlGRL1* expression is also predominantly associated with the testa of developing seeds but is also expressed throughout fruit development with an increase in transcript abundance detected at the breaker stage of fruit ripening and in senescing flowers (Figure 4.1B, C). *SlGRL2* is also widely expressed in tomato tissues with high expression levels detected in seeds and anthers together with increased

expression detected during fruit ripening (Figure 4.1A, B and C). In contrast to *SlGR* and *SlGRL1* 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 (Figure 4.1D). Although the expression level of *SlGRL2* is dramatically higher in the fruit peel of red ripening stage than mature green stage, the expression of *SlGRL2* is almost uniformly distributed across the pericarp, peel, and flesh of dissected tomato fruits at the breaker stage of ripening (Figure 4.1E, F). Considering the increasing expression of *SlGRL2* during tomato fruit ripening, the dramatically high expression of *SlGRL2* in the fruit peel of red fruit is probably a result of the ripening process. Similar expression patterns were observed in dissected tomato stem tissues (Figure 4.1G). 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.

***SlGR*, *SlGRL1* and *SlGRL2* are differentially expressed in response to ethylene treatment and following wounding**

Ethylene is related to the biotic and abiotic stress responses of plants (Bleecker and Kende, 2000; Guo and Ecker, 2004). The expression of some components in ethylene signaling, such as *LeETR2*, *LeCTR1*, *OsETR2*, *PhETR2*, is influenced by wounding, ozone, flooding, or salt treatment etc (Leclercq et al., 2002; Moeder et al., 2002; Adams-Phillips et al., 2004; Watanabe et al., 2004; Yau et al., 2004; Wang and Kumar, 2007). The expression pattern of *SlGR*, *SlGRL1* and *SlGRL2* in response to the ethylene and wounding was also assessed by q-RT-PCR. Following 12 h of ethylene treatment, the expression of *SlGR* and *SlGRL2* was slightly

suppressed, however the expression of *SlGRL1* increased following ethylene treatment with a peak of transcript accumulation observed after 2 h of treatment (Figure 4.2A). The expression of *SlGRL1* was also influenced by wounding with transcript accumulation occuring at 8 hours after wounding, which is similar to the expression patterns of wounding-related genes *proteinase inhibitor 1(PIN1)* (Lee et al., 1986) and *PIN2* (Thornburg et al., 1987) that were utilized as positive controls (Figure 4.2 B, C, and D). Together, *SlGR*, *SlGRL1* and *SlGRL2* are differentially expressed in response to ethylene and wounding.

Transient expression of YFP-gene fusions in tobacco suggests that GR/RTE1 related proteins are localized to the Golgi

Several components of the ethylene signaling pathway are localized within the endomembrane system and separate studies have reported either dual localization of RTE1 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). Similarly, two GRL1 orthologs from rice, *Os*GRL1a/*Os*RTH1 and *Os*GRL1b/*Os*RTH2 are localized to the Golgi and ER membranes (Zhang et al., 2012). Furthermore, the *Arabidopsis* GRL2 ortholog *At*RTH and the rice GRL2 ortholog *Os*GRL2/*Os*RTH3 have recently been reported to be dual localized to the ER and the nucleus (Zhang et al., 2012). To determine the subcellular localization of *Sl*GR, *Sl*GRL1, and *Sl*GRL2, N-terminal yellow-fluorescent protein (YFP) fusions of each protein were constructed and transiently expressed with the ER and Golgi marker ERD2-GFP (Boevink et al., 1998) in tobacco (*Nicotiana tabacum*) leaves. The localization of each fusion protein was examined in the transformed leaves after 48 hours expression by confocal laser scanning microscopy (CLSM).

These analyses revealed that *Sl*GR *Sl*GRL1 and *Sl*GRL2 colocalize with the ER and Golgi marker ERD2-GFP (Figure 4.3). However, the colocalization signal was mainly associated with the "dot" like structures associated with the Golgi, and not the "net" like structures of the ER (Held et al., 2008) (Figure 4.3). Together, these data suggest that *Sl*GR *Sl*GRL1 and *Sl*GRL2 appear to be localized predominantly in the Golgi at steady state. In order to explore the relationship between protein localization and functional differences of GR/RTE1 family proteins, *Ph*GR, *Ph*GRL1, and *Sm*GR were also fused to YFP and transiently expressed with ERD2-GFP in tobacco epidermal leaves. CLSM images suggested that *Ph*GR, *Ph*GRL1, and *Sm*GR are also predominantly localized within the Golgi (Figure 4.4). Together, these data suggest that the distinct functional properties of these proteins cannot be attributed to differential subcellular localization.

Predominant Golgi localization of *Sl*GR, *Sl*GRL1, and *Ph*GR proteins was confirmed by stable expression in *Arabidopsis*

Transient expression in leaf epidermal cells of tobacco suggested that *Sl*GR, *Sl*GRL1, and *Ph*GR are predominantly Golgi localized proteins (Figure 4.3, 4.4). To confirm these data, N-terminally tagged YFP fusions of *Sl*GR, *Sl*GRL1, and *Ph*GR were transformed into the *etr1-2/rte1-3* double mutant. Transgenic lines expressing each fusion protein were confirmed by CLSM (data not shown). In addition, the *CaMV35::YFP-SlGRL1* and *CaMV35::YFP-PhGR* construct were able to complement the *rte1-3* allele whereas the *CaMV35::YFP-SlGR* construct does not fully complement indicating that the YFP-tagged proteins behave similarly to the MYC-tagged and non-tagged versions of the proteins (Figure 2.6, 2.7, 3.3 and 4.5). Each YFP-fusion protein line

was crossed with stably transformed *Arabidopsis* lines expressing either GFP-HDEL, ST-GFP, or mCherry-VTI12, which target GFP/RFP to the ER, Golgi, or trans-Golgi network (TGN), respectively (Haseloff et al., 1997; Boevink et al., 1998; Geldner et al., 2009). The localization of each fusion protein was examined in the cotyledons or root of the F1 progeny derived from each cross (Figure 4.6, 4.7, and 4.8). These analyses reveal that *Sl*GR, *Sl*GRL1, and *Ph*GR appear to be localized predominantly within the Golgi and not the ER or TGN as each fusion protein co-localized with the ST-GFP marker but not the GFP-HDEL or mCherry-VTI12 markers.

Discussion

Differential expression of the *GR/RTE1* family of tomato

Subfunctionalization within multigene families is frequently manifest at the level of expression variation in which individual genes assume distinct temporal or spatial patterns of expression within a particular tissue type or at a developmental stage (Force et al., 1999; Freeling, 2009). In regard to the ethylene signaling pathway, most characterized components, with the exception of *EIN2*, are encoded by multigene families in all higher plants that have been studied and many examples exist describing the differential expression of individual genes (Wilkinson et al., 1995; Payton et al., 1996; Zhou et al., 1996; Hua and Meyerowitz, 1998; Lashbrook et al., 1998; Tieman and Klee, 1999; Bleecker and Kende, 2000; Klee, 2004; Barry and Giovannoni, 2006; Wang and Kumar, 2007). This differential expression has the potential to impact the stoichiometry of the ethylene signaling complexes, particularly in plants like tomato where there are multiple genes encoding the ethylene receptors, CTR1-like proteins and the GR/RTE1 family

(Zhou et al., 1996; Lashbrook et al., 1998; Tieman and Klee, 1999; Klee, 2002; Leclercq et al., 2002; Moeder et al., 2002; Adams-Phillips et al., 2004; Barry and Giovannoni, 2006). The impact of this phenomena on ethylene responsiveness is not understood but evidence that control of gene expression is important for maintaining an appropriate ethylene response is observed through the characterization of the *Gr* mutant which is caused by a promoter deletion that causes ectopic expression of *SlGR*, leading to reduced ethylene responsiveness in several plant tissues (Barry and Giovannoni, 2006) (Chapter 2). Therefore, regulation of *GR/RTE1* expression is likely important for maintaining an appropriate ethylene response and differential expression of members of the *GR/RTE1* family could potentially influence ethylene responsiveness. Expression analysis revealed that *SlGR* expression relatively low in most of the tissues examined except for in the seeds where transcript abundance peaks in the testa (Figure 4.1D). In contrast, *SlGRL1* expression is more widely expressed and is ripening and ethylene-related (Figure 4.1C and 4.2A). 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 the single copy gene *AtRTE1*. For example, *AtRTE1* 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 the ethylene precursor ACC. *SlGRL2* is also widely expressed in tomato tissues with high expression levels detected in seeds and anthers together with increased expression detected during fruit ripening (Figure 4.1A, B and C). Data available from the e-FP browser (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi?primaryGene=AT3G51040&modeInput=Absolute) show that *AtRTH* is highly expressed in many *Arabidopsis* tissues, and especially highly associated with seeds, which is

similar to the expression pattern of *SlGRL2*, although the functions of these two genes are still unknown.

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 RTE1, EIN2 and CTR1 and occur within the ER and Golgi membranes (Clark et al., 1998; Chen et al., 2002; Gao et al., 2003; Gao et al., 2008; Bisson et al., 2009; Chen et al., 2010; Dong et al., 2010). Our data indicate that *Sl*GR, *Sl*GRL1, *Sl*GRL2, *Ph*GR, *Ph*GRL1, and *Sm*GR are localized primarily to the Golgi membranes at steady state (Figure 4.3, 4.4, 4.6, 4.7, and 4.8). These data are in broad agreement with previous studies that have localized *At*RTE1, *At*RTH, *Os*GRL1a/*Os*RTH1, *Os*GRL1b/*Os*RTH2, and *Os*GRL2/*Os*RTH3 to the endomembrane system (Zhou et al., 2007; Dong et al., 2008; Zhang et al., 2012). However, these studies suggested that while GR/RTE1 family members were predominantly localized to the Golgi, some fluorescence attributed fusion proteins was also detected in the ER. Such slight discrepancies may be linked to different expression levels of the transgenes whereby over-expression of the transgenes may lead to saturation of the export machinery of some organelles and partial retention of the protein in that location, while under non-saturating conditions the proteins may translocate more freely through the endomembrane system. Nonetheless, additional experimentation, including immunolocalization or separation of organelles by centrifugation followed by immunoblot analysis, will be required to address these discrepancies as they may have implications to current models of ethylene receptor function as

well as the function of the GR/RTE1 proteins and conservation of signaling mechanisms across species.

The potential for interaction between GR/RTE1 family proteins and the tomato ethylene receptors

Differential expression patterns of ethylene signaling components likely leads to altered composition of these components within plant cells resulting in the formation of variable signaling complexes. Based on current models of the function of GR/RTE1 proteins in the ethylene signaling pathway (Dong et al., 2010), it is possible that *Sl*GR and *Sl*GRL1 interact with the tomato ethylene receptors. Furthermore, our previous data indicate that *Sl*GR and *Sl*GRL1 are able to influence distinct, yet overlapping ethylene responses in tomato (Barry and Giovannoni, 2006) (Chapter 2). The mechanisms through which these differences occur are not understood but may involve differential interaction with specific ethylene receptors or interactions with same receptors but at different affinities. Differential interactions between ethylene signaling components have been reported. For example, in tomato, the *Le*CTR1, *Le*CTR3, and *Le*CTR4 proteins interact with the tomato subfamily-1 receptors (*Le*ETR1, *Le*ETR2, and NR), whereas *Le*CTR2 only interacts with *Le*ETR1 and *Le*ETR2 (Lin et al., 2008; Zhong et al., 2008). In *Arabidopsis,* functional differences between the ethylene receptors has been proposed to be due to their different affinities for the CTR1 protein with the more prominent role of *At*ETR1 attributed to a stronger interaction with CTR1 (Clark et al., 1998; Hall and Bleecker, 2003; Wang et al., 2003). Moreover, EIN2 may also possess different interaction affinities with individual ethylene receptors (Bisson and Groth, 2010). Bimolecular fluorescence

complementation (BiFC) experiments suggest that *At*RTE1 may differentially interact with the subfamily 1 receptors of *Arabidopsis* with interactions observed by co-expression of N-terminal fusion cYFP-*At*RTE1 and *At*ETR1-nYFP constructs in tobacco leaf epidermal cells, *Arabidopsis* root cells, and *Arabidopsis* cotyledon epidermal cells (Dong et al., 2010). A weak BiFC signal was also observed following co-expression of cYFP-*At*RTE1 and *At*ERS1-nYFP constructs although, as yet there is no genetic evidence to suggest that *AtRTE1* function is mediated through *AtERS1* (Resnick et al., 2006; Resnick et al., 2008; Rivarola et al., 2009; Dong et al., 2010). Potential interactions between members of the tomato GR/RTE1 family and the tomato ethylene receptors are still unknown. Preliminary data based on CLSM imaging suggests that the tomato ethylene receptors co-localize with ERD2-GFP within the endomembrane system of tobacco epidermal cells, with perhaps some fluorescence associated with the Golgi (data not shown). Taken together, with the endomembrane localization of *Sl*GR, *Sl*GRL1, and *Sl*GRL2, these data suggest that there is the potential for protein-protein interactions between these proteins and the tomato ethylene receptors.

Materials and Methods

Plant Growth and Treatments

Tomato plants, cultivar Ailsa Craig (AC) (Barry et al., 2005) were grown in Jiffy-7 Peat Pellets (http://www.hummert.com/) in a growth chamber for the first four weeks of growth under 16-h light/ 8-hour dark at 28^{o}C and 65% relative humidity, and then transferred to peat-based compost supplemented with fertilizer in greenhouses, which equipped with heating and cooling systems

and supplemental lighting at Michigan State University, East Lansing, MI. Tobacco (*Nicotiana tabacum* cv. Petit Havana) of 5-6 weeks old was used in transient transformation and grown in growth chamber under 16-h light/ 8-hour dark at 22^{o}C with 50 μE light intensity and 65% relative humidity.

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 days and breaker +7 stages of fruit ripening. Columella and locular gel were removed from the fruits and the pericarp 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 previously described (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 previously described (Nonogaki et al., 1992). Dissected stem tissues were collected from 5-week old green house grown tomato plants. For experiments designed to determine potential ethylene regulation of gene expression, leaflets were harvested from four week-old growth chamber grown plants (see above for details) treated with 10 μl l^{-1} ethylene for the specified time periods. Wounding experiments were performed on 4 week-old growth chamber grown plants. Wounding of leaflets was performed as previously described (Shackel et al., 1991) with the exception that leaves were punctured by unopened small scissors tips instead of glass microcapillary tips.

The *Arabidopsis thaliana etr1-2/rte1-3* transgenic plants with Columbia (Col-0) background (Resnick et al., 2006) were used as parent strains for *CaMV35S::YFP-SlGR*, *CaMV35S::YFP-*

SlGRL1, and *CaMV35S::YFP-PhGR*. Seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) together with the ethylene-signaling mutants *etr1-2* and *etr1-2/rte1-3* (Resnick et al., 2006) and the fluorescently tagged organelle reporter lines ST-GFP and GFP-HDEL (Haseloff et al., 1997; Boevink et al., 1998) were grown in growth chambers at 22^{o}C under 16-h light/ 8-hour dark at 145 µmol m^{-2} s^{-1} and 65% relative humidity, after three days cold treatment at 4^{o}C. The *Arabidopsis* triple response screen was performed using a slightly modified version of a previously published protocol (Alonso et al., 2003). *Arabidopsis* seeds were sterilized with 70% ethanol for 10 minutes, followed by three washes with 100% ethanol. Surface sterilized seeds were dried on sterile filter paper in a laminar flow hood and dried seeds were sprinkled onto 0.8% phytagar containing 1×Murashige and Skoog salts, pH 6.0 and 1% sucrose supplemented with 1-aminocyclopropane-1-carboxylic acid (ACC) at 100 µM. Plates were placed at 4^{o}C for 3 days, exposed to light for 12 h, and then incubated at room temperature in the dark for 6 days. Hypocotyl and root lengths were measured using ImageJ (http://rsbweb.nih.gov/ij/).

qRT-PCR analysis

Total RNA was extracted using the RNeasy® Mini Kit and subjected to on column DNase treatment (Qiagen, http://www.qiagen.com). 1µg of RNA was used for reverse transcription using SuperScript™ III First-Strand Synthesis System (Invitrogen, http://www.lifetechnologies.com/). Gene-specific primers for *SlGR*, *SlGRL1*, and *SlGRL2* were designed by Primer Express 3.0 (Applied Biosystems, http://www.lifetechnologies.com) (Table 4.1). Wound-induced *PIN1* and *PIN2* genes were used as positive controls in wounding experiments using primers previously described (Lee et al., 1986; Thornburg et al., 1987). The

PCR reactions were performed with FAST SYBR® Master Mix, 2x (Applied Biosystems) in a 10 µL volume containing 25 ng of cDNA template. PCR amplification was performed using an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems) at the Research Technology Support Facility of Michigan State University, East Lansing, MI. The qRT-PCR program included a preliminary step of 2 min at 50 ℃, 10 min at 95 ℃, followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ 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 *clathrin adaptor complexes medium subunit (CAC)* genes of tomato for normalization as previously described (Exposito-Rodriguez et al., 2008).

Construct assembly

DNA fragments were amplified by PCR using *Pfu* Ultra™ DNA polymerase (Agilent Technologies). YFP-tagged *SlGR*, *SlGRL1*, *SlGRL2*, *PhGR*, *PhGRL1*, *SmGR*, and *PhGRL1Δ9* were assembled using Gateway cloning technology. The purified PCR products were cloned into the pENTR-D-TOPO vector (Invitrogen). After digested with *Mlu*I, they were inserted into the binary vector pEarleyGate 104 (*CaMV35S::YFP*-Gateway-OCS 3') by LR reaction (Invitrogen) to create *CaMV35S::YFP-SlGR*, *CaMV35S::YFP-SlGRL1*, *CaMV35S::YFP-SlGRL2*, *CaMV35S::YFP-PhGR*, *CaMV35S::YFP-PhGRL1*, *CaMV35S::YFP-SmGR*, *CaMV35S::YFP-SlGRL1Δ9*, respectively. CFP-tagged *ETR1*, *ETR2*, *NR*, *ETR4*, *ETR5*, and *ETR6* were assembled in the similar way, except that the corresponding fragments were inserted into the binary vector pEarleyGate 102 (35S-Gateway-CFP-HA tag-OCS 3'). All constructs were transferred into

Agrobacterium tumefaciens strain GV3101. All primers used for assembling constructs are listed in Table 4.1. The fidelity of each construct was verified by DNA sequencing.

Transient expression in *Nicotiana tabacum*

Transient expression of fluorescent protein tagged versions of genes of interest in tobacco (*Nicotiana tabacum* cv. Petit Havana) was performed as previously described (Batoko et al., 2000). Briefly, 3 ml overnight cultures of recombinant *A. tumefaciens* (strain GV3101) grown at 28℃ in YEB media (per liter: 5 g of beef extract, 1 g of yeast extract, 5 g of sucrose, and 0.5 g of $MgSO_4$-$7H_2O$; supplemented with 50 ug ml^{-1} kanamycin and 10 ug ml^{-1} rifampicin) were harvested by centrifugation at 4000 *g* for 5 minutes and the pellet washed and resuspended to a density of 0.05 OD_{600} in induction media (50 mM MES, pH 5.6; 2mM NaH_2PO_4; 0.05% w/v glucose; 200 μM Acetosyringone). The YFP or CFP-tagged cultures (YFP–*Sl*GR, YFP-*Sl*GRL1, YFP–*Sl*GRL2, YFP–*Ph*GR, YFP–*Ph*GRL1, YFP–*Sm*GR, YFP-*Sl*GRL1Δ9, ETR1-CFP, ETR2-CFP, NR-CFP, ETR4-CFP, ETR5-CFP, and ETR6-CFP) were mixed with *ERD2-GFP* culture respectively, and injected into the abaxial leaf surface of 5-6 week-old plants using 1 ml needleless syringes. Following 48 h of expression fluorescence of the reporter genes was determined by confocal laser scanning microscopy.

Stable expression in *Arabidopsis thaliana*

The *Agrobacterium tumefaciens* strain GV3101 contained *CaMV35S::YFP-SlGR*, *CaMV35S::YFP-SlGRL1*, *CaMV35S::YFP-PhGR* constructs were transformed into *Arabidopsis*

etr1-2/rte1-3 double mutant 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 confocal laser scanning microscopy.

Confocal laser scanning microscopy

Zeiss 510 Meta laser scanning confocal microscope and Olympus Fluoview FV41000 laser scanning confocal microscope were used in this research. Zeiss 510 Meta laser scanning confocal microscope (Thornwood, NY) configured with fully automated Zeiss Axio observer inverted microscope. Blue-shifted GFP was excited with 458 nm Argon laser line and fluorescence emission was detected with a 475-525 band pass filter. YFP was excited with the 514 nm Argon laser line and fluorescence emission was detected with a 520-555 nm band pass filter. Fluorescence emissions were collected sequentially to avoid fluorescence crossover. Images were collected using the Plan Apocromat 63× oil objective (NA 1.4). Olympus Fluoview FV41000 laser scanning confocal microscope (Center Valley, PA) configured with fully automated IX81 olympus inverted microscope. The parameters are very similar to that of the Zeiss Meta. The images were collected sequentially, using the PlanApoN 60x oil (NA 1.42) objective. The blue-shifted GFP was excited using the 458 nm Argon laser line, and the fluorescence emission was collected from 475-520 nm. The YFP was excited using the 514 nm Argon laser line, and the fluorescence emission was collected from 530-630 nm. CFP was excited with 514 nm wavelength, and emission collected from 530-565 nm.

DNA sequence analysis

DNA sequences were assembled using Sequencher™ version 4.7 (Genecodes Corporation, Ann Arbor, MI http://genecodes.com).

Statistical analysis

Statistical analyses were performed using SAS (SAS Institute, www.sas.com). The genotypic constituents were evaluated by Student's *t-test* and LS Means.

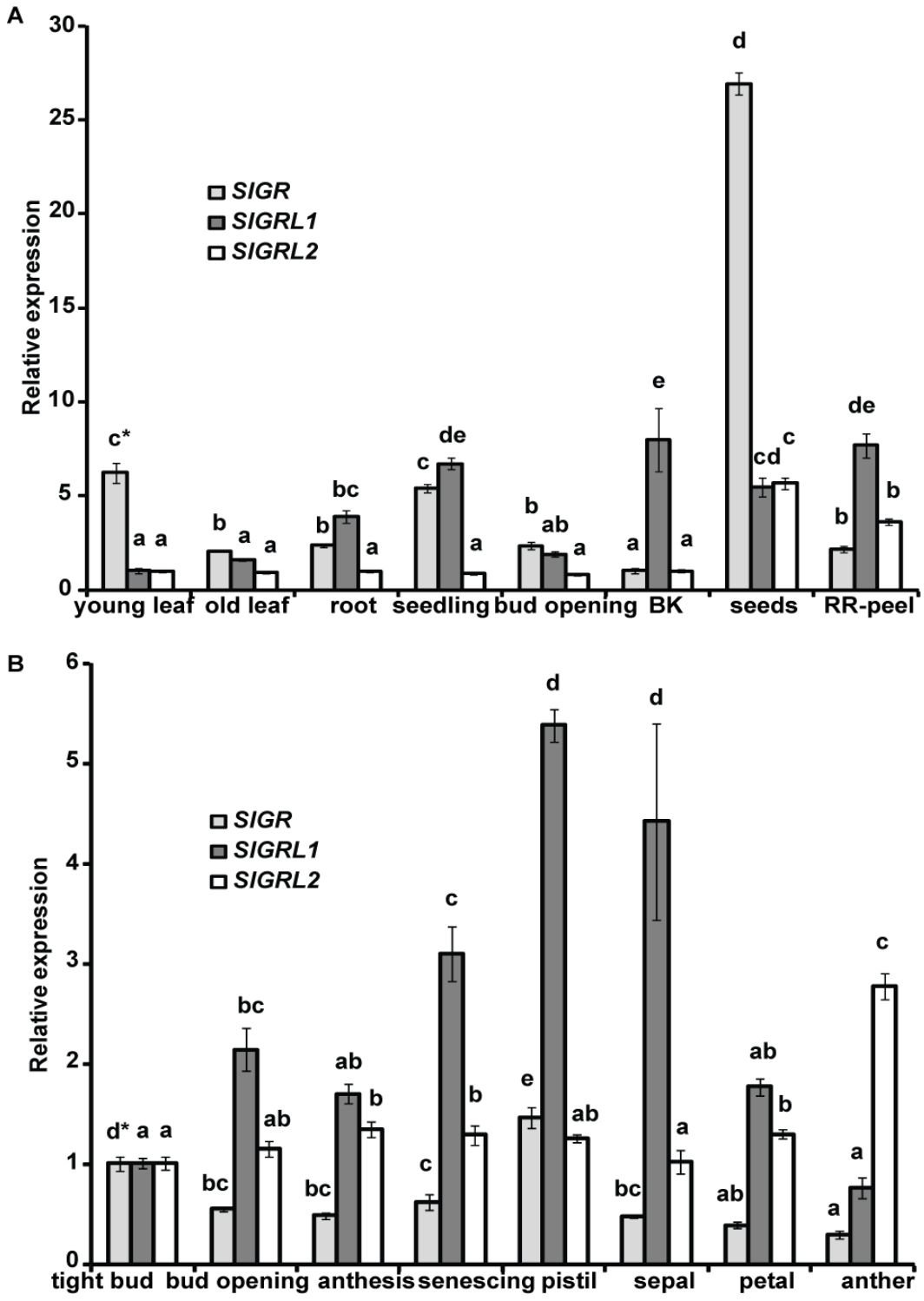

A
Relative expression
SIGR
SIGRL1
SIGRL2
young leaf
old leaf
root
seedling
bud opening
BK
seeds
RR-peel
B
Relative expression
SIGR
SIGRL1
SIGRL2
tight bud
bud opening
anthesis
senescing
pistil
sepal
petal
anther

Figure 4.1 (cont'd)

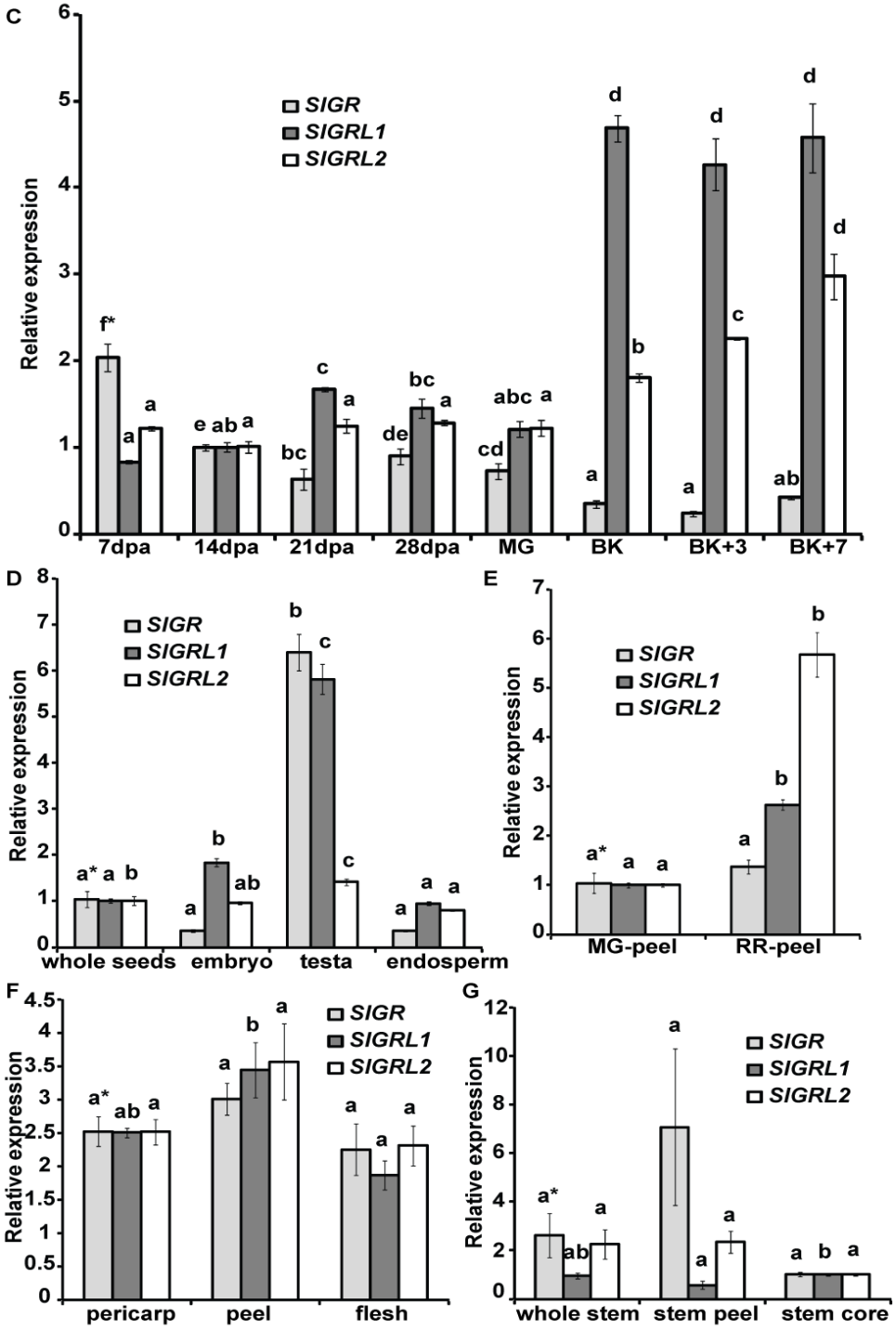


Figure 4.1 Expression of *SlGR*, *SlGRL1* and *SlGRL2* during tomato development. A, The relative expression level of *SlGR*, *SlGRL1* and *SlGRL2* in various tomato tissues as determined by qRT-

PCR. BK refers to fruit at the breaker stage of development. RR-peel refers to peel isolated from red-ripe fruits. Data are presented relative to the expression levels of *SlGR* in BK fruit, *SlGRL1* in young leaf, and *SlGRL2* in BK fruit. B, Relative expression levels of *SlGR*, *SlGRL1* and *SlGRL2* 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. Data are presented relative to the expression levels of *SlGR, SlGRL1,* and *SlGRL2* in whole flowers at the tight bud stage of development. C, Relative expression levels of *SlGR, SlGRL1* and *SlGRL2* during tomato fruit development and ripening. Fruit were harvested at various days post anthesis (dpa), at the mature green (MG), breaker (BK) and 3 and 7 days post breaker (BK+3 and BK+7). Data are presented relative to the expression levels of *SlGR, SlGRL1*, and *SlGRL2* in 14dpa fruit. D, Relative expression levels of *SlGR, SlGRL1* and *SlGRL2* in whole and dissected seeds isolated from mature green fruits. Data are presented relative to the expression levels of *SlGR, SlGRL1*, and *SlGRL2* in whole seeds. E, Relative expression levels of *SlGR, SlGRL1* and *SlGRL2* in peels isolated from different fruit development stages. MG-peel refers to peel isolated from mature green fruits. RR-peel refers to peel isolated from red-ripe fruits. Data are presented relative to the expression levels of *SlGR, SlGRL1*, and *SlGRL2* in MG-peel. F, Relative expression levels of *SlGR, SlGRL1* and *SlGRL2* in whole pericarp, peel and flesh of tomato fruits at the breaker stage of development. Data are presented relative to the expression levels of *SlGR, SlGRL1*, and *SlGRL2* in whole pericarp. G, Relative expression levels of *SlGR, SlGRL1* and *SlGRL2* in whole stem, stem peel and stem core of 5-week old tomato plants in green house. Data are presented relative to the expression levels of *SlGR, SlGRL1*, and *SlGRL2* in stem core. Experimental details are provided in the Materials and Methods section. For all experiments, data are presented as the mean ±SE of three biological and three technical replicates. *Statistical analysis is presented for

each gene across the tissue, or developmental stages. *Means followed by the same letter are not significantly different at α=0.05 level.

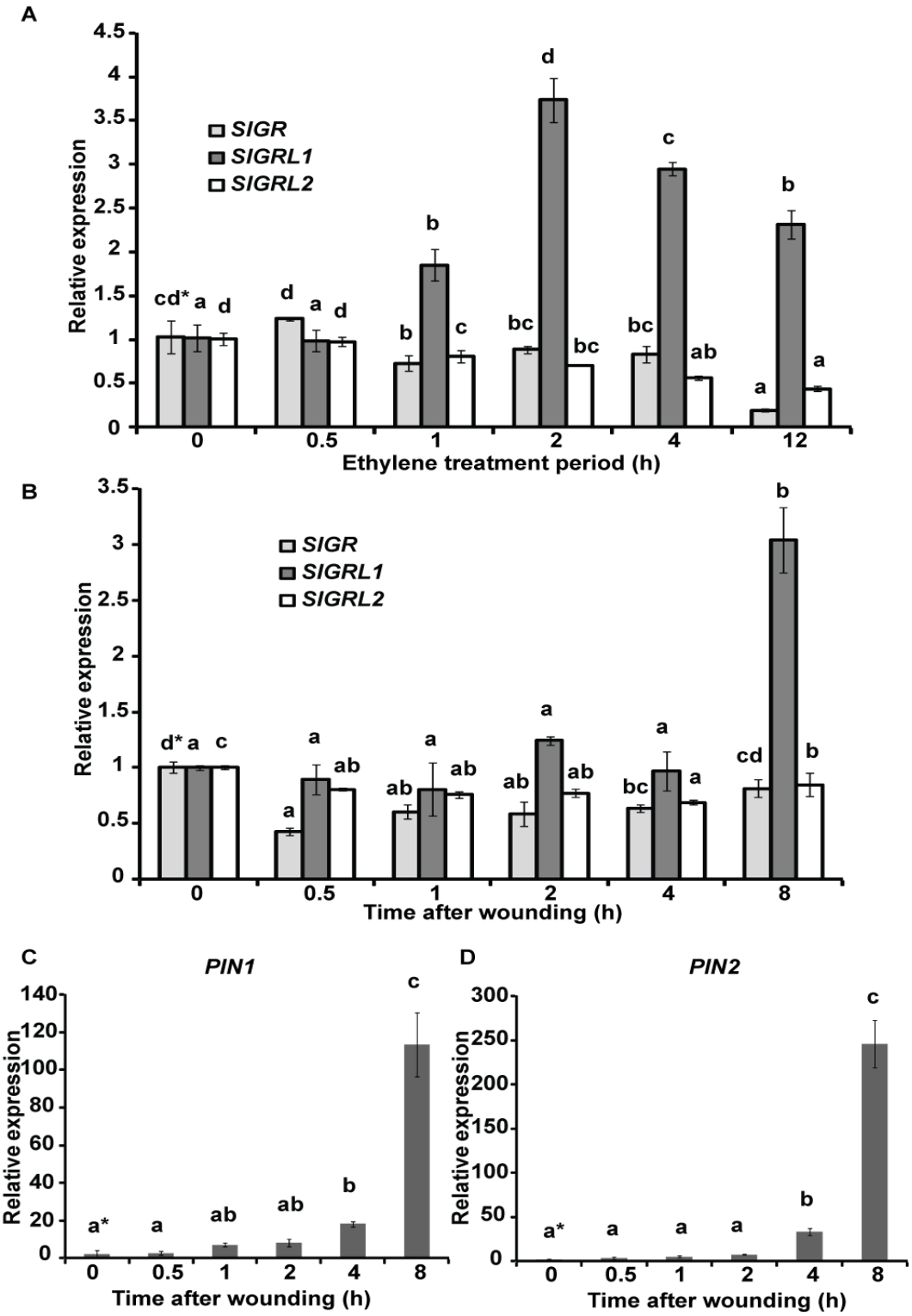


Figure 4.2 Expression of *SlGR*, *SlGRL1* and *SlGRL2* in response to ethylene and wounding. A, Relative expression levels of *SlGR*, *SlGRL1* and *SlGRL2* in leaves of 4-week old plants treated

with 10 μl l^{-1} ethylene for the specified time points. Data are presented relative to the expression levels of *SlGR*, *SlGRL1*, and *SlGRL2* at the 0 hour time point. B, Relative expression levels of *SlGR*, *SlGRL1* and *SlGRL2* in leaves of 4-week old plants with wounding treatment for the specified time points. Data are presented relative to the expression levels of *SlGR*, *SlGRL1*, and *SlGRL2* at the 0 hour time point. C and D, Relative expression levels of wounding-related genes *PIN1* (C) and *PIN2* (D) in the same samples shown in (B). Data are presented relative to the expression levels of *PIN1* (C) and *PIN2* (D) at the 0 hour time point. Experimental details are provided in the Materials and Methods section. For all experiments, data are presented as the mean ±SE of three biological and three technical replicates. *Statistical analysis is presented for each gene across the tissue, or developmental stages. *Means followed by the same letter are not significantly different at α=0.05 level.

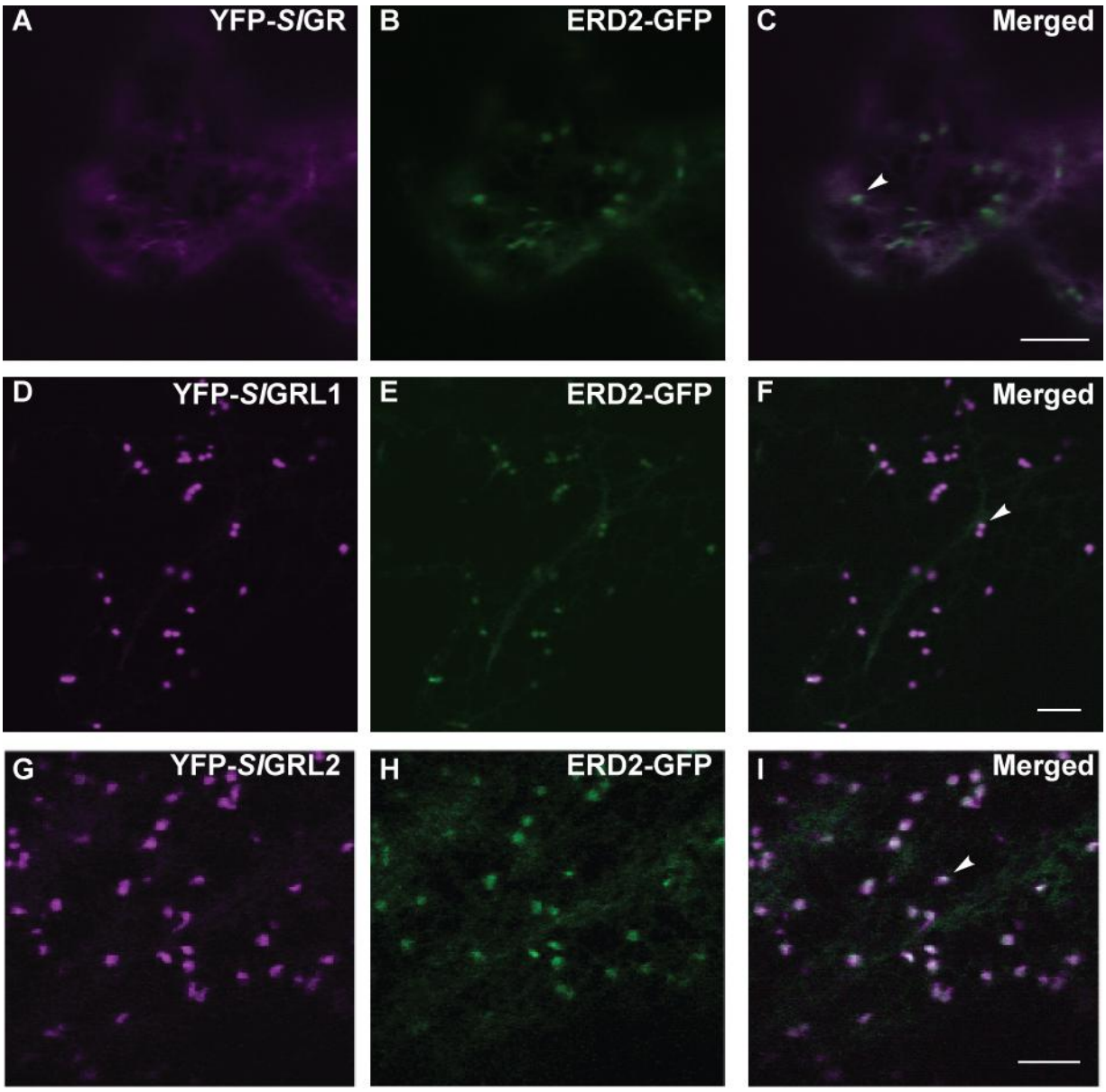


Figure 4.3 Subcellular localization of *Sl*GR, *Sl*GRL1, and *Sl*GRL2 in tobacco epidermal leaf cells. ERD2-GFP as an ER and Golgi marker was transiently expressed with YFP-*Sl*GR, YFP-*Sl*GRL1, and YFP-*Sl*GRL2, respectively. A, D, and G represent the emission channel for YFP-*Sl*GR(A), YFP-*Sl*GRL1(B), and YFP-*Sl*GRL2(G), while B, E, and H represent ERD2-GFP fluorescence. The white merged color of the GFP and YFP fluorescence demonstrated the co-

localization of ERD2 with the *Sl*GR(C), *Sl*GRL1(F), and *Sl*GRL2(I) at the Golgi membrane. Images were captured using a Zeiss 510 Meta laser scanning confocal microscope. GFP emission was excited with 458 nm wavelength, and detected with a 475-525 nm band pass filter. YFP was excited with 514 nm wavelength, and detected with a 520-555 nm band pass filter. Note the co-localization of YFP-*Sl*GR, YFP-*Sl*GRL1, and YFP-*Sl*GRL2 with ERD2-GFP in the "dot" like structures of the Golgi (indicated by arrows) in the merged panel. Scale bar=5 μm.

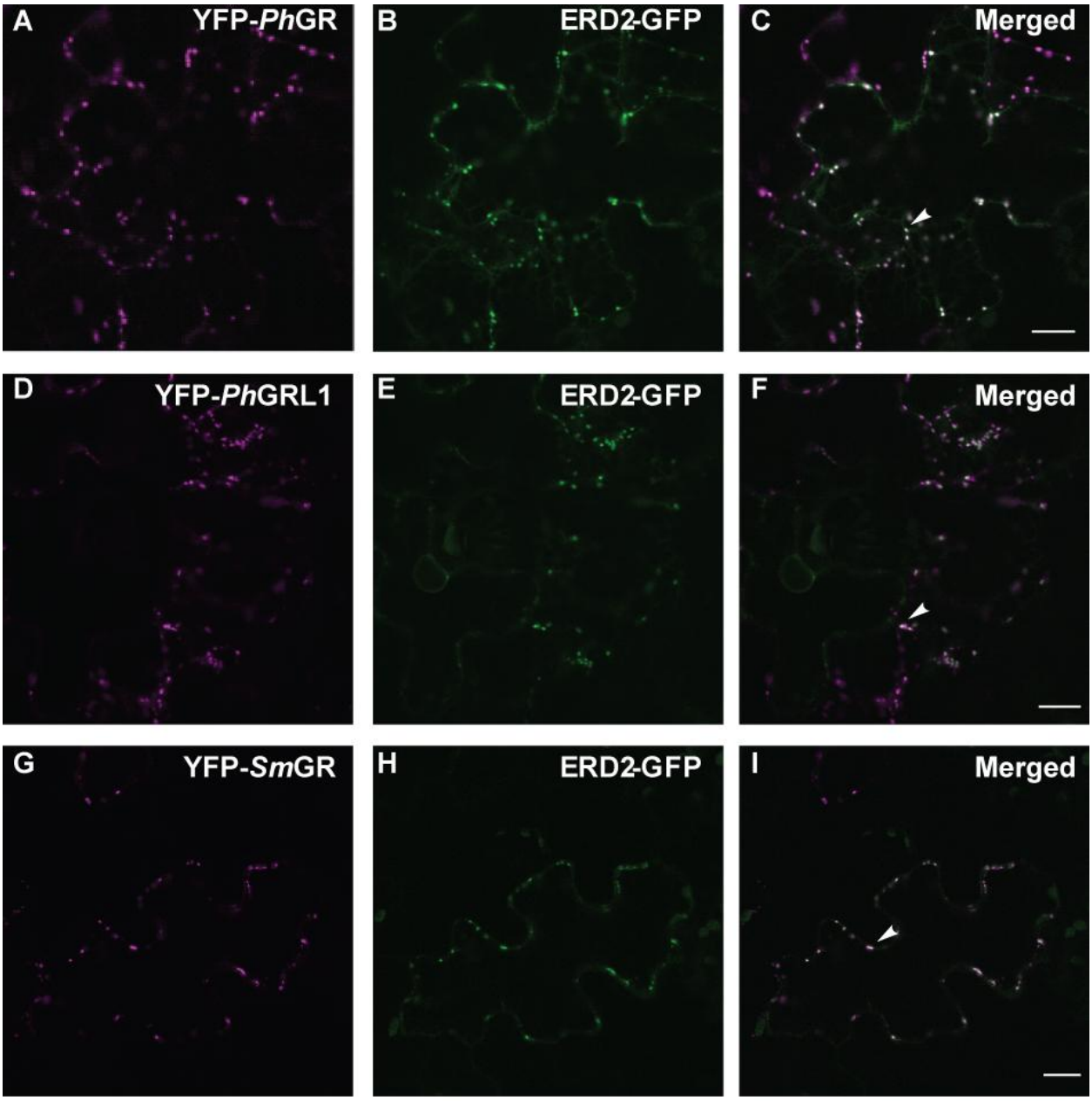
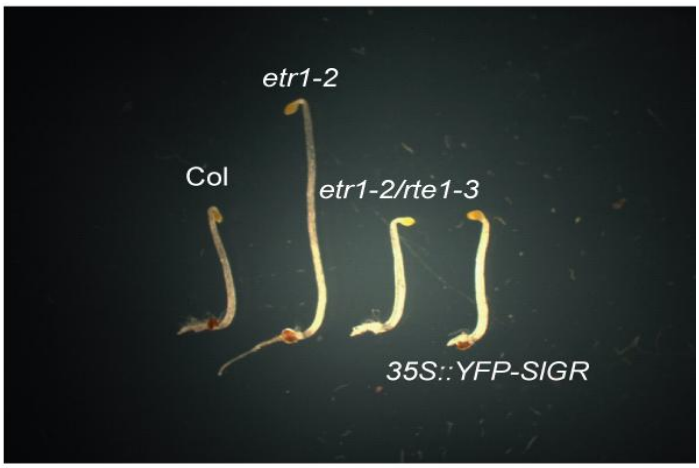


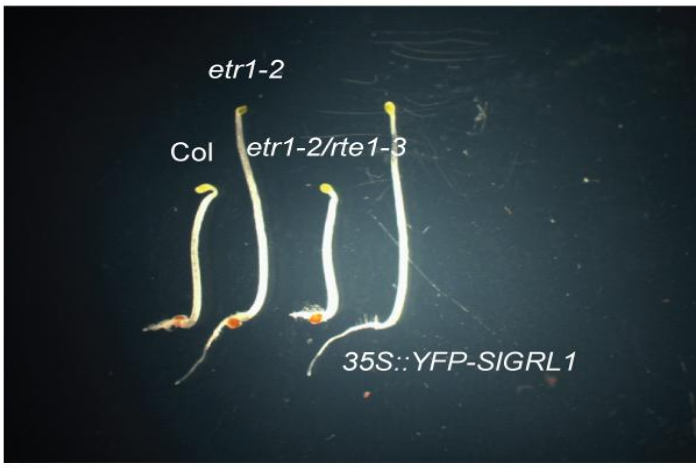
Figure 4.4 Subcellular localization of *Ph*GR, *Ph*GRL1, and *Sm*GR in tobacco leaf epidermal cells. ERD2-GFP as an ER and Golgi marker was transiently expressed with YFP-*Ph*GR, YFP-*Ph*GRL1, and YFP-*Sm*GR respectively. A, D, and G represent the emission channel for YFP-*Ph*GR(A), YFP-*Ph*GRL1(D), and YFP-*Sm*GR(G), while B, E, and H represent ERD2-GFP fluorescence. The white merged color of the GFP and YFP fluorescence demonstrated the co-

localization of ERD2 with the *Ph*GR(C), *Ph*GRL1(F), and *Sm*GR(I) at the Golgi membranes. Images were captured using an Olympus Fluoview FV41000 laser scanning confocal microscope. GFP emission was excited with 458 nm wavelength, and collected from 475-520 nm. YFP was excited with 514 nm wavelength, and collected from 530-560 nm. Note the co-localization of YFP-*Ph*GR, YFP-*Ph*GRL1, and YFP-*Sm*GR with ERD2-GFP in the "dot" like structures of the Golgi (indicated by arrows) in the merged panel. Scale bar=10 μm.

A



B



C

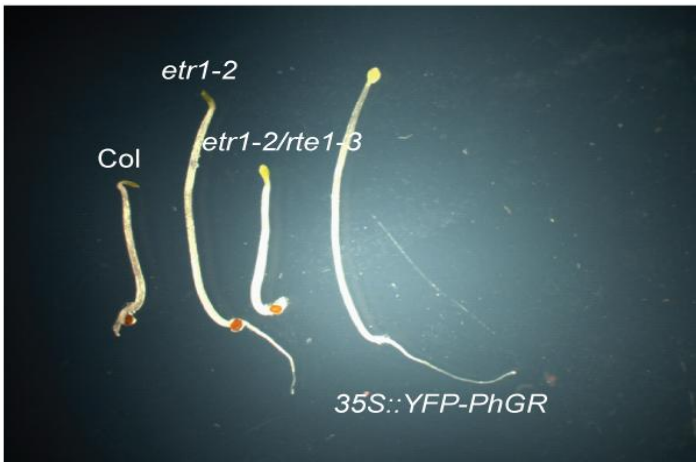


Figure 4.5 Differential complementation of *rte1-3* by YFP-tagged versions of *SlGR*, *SlGRL1*, and *PhGR*. A, The triple response phenotype of *Arabidopsis* seedlings from Col-0, *etr1-2*, and *etr1-*

2/rte1-3, together with a transgenic line expressing a *CaMV35S::YFP-SlGR* construct. B, The triple response phenotype of *Arabidopsis* seedlings from Col-0, *etr1-2*, and *etr1-2/rte1-3*, together with a transgenic line expressing a *CaMV35S::YFP-SlGRL1* construct. C, The triple response phenotype of *Arabidopsis* seedlings from Col-0, *etr1-2*, and *etr1-2/rte1-3*, together with a transgenic line expressing a *CaMV35S::YFP-PhGR* construct. Seedlings were grown in the dark at room temperature for six days on 100 μM ACC. Note, the elongated phenotypes of the *CaMV35S::YFP-SlGRL1* and *CaMV35S::YFP-PhGR* lines demonstrating complementation of the *rte1-3* mutant allele whereas the *CaMV35S::YFP-SlGR* lines do not complement the *rte1-3* mutant allele. See Figure 2.6 and Figure 3.3 for comparison with MYC-tagged versions of each construct. In each case the YFP-tagged transgenic lines shown were utilized for crosses to the organelle reporter lines utilized for subcellular localization of *YFP-SlGR*, *YFP-SlGRL1*, and *YFP-PhGR* in stably transformed *Arabidopsis* plants (see Figure 4.6, 4.7, 4.8).

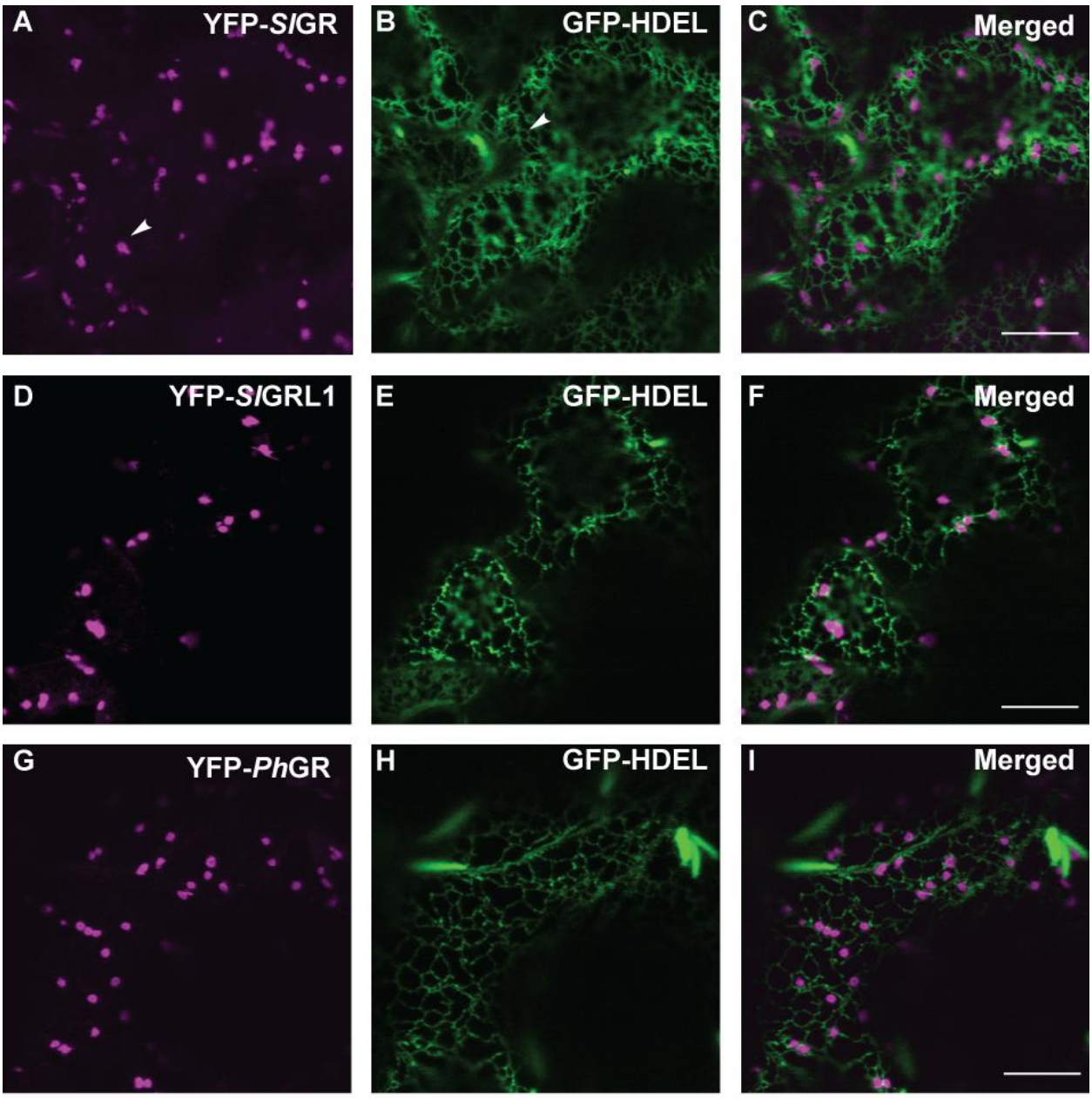


Figure 4.6 Lack of co-localization of *Sl*GR, *Sl*GRL1, and *Ph*GR with an ER membrane marker line in *Arabidopsis*. Confocal microscopy images of live cotyledon epidermal cells of F1 progeny derived from crosses between *Arabidopsis* plants expressing either *CaMV35S::YFP-SlGR*(A-C), *CaMV35S::YFP-SlGRL1*(D-F), or *CaMV35S::YFP-PhGR*(G-I) with the ER marker line 6×*CaMV35S::GFP-HDEL*. Images were captured using a Zeiss 510 Meta laser scanning

confocal microscope. GFP emission was excited with 458 nm wavelength, and detected with a 475-525 nm band pass filter. YFP was excited with 514 nm wavelength, and detected with a 520-555 nm band pass filter. Note the non-overlapping fluorescence signals in crosses incorporating the ER marker line. Arrows highlight the “net” like structure of the ER, and “dot” like structure of the Golgi, respectively. Scale bar=10 μm.

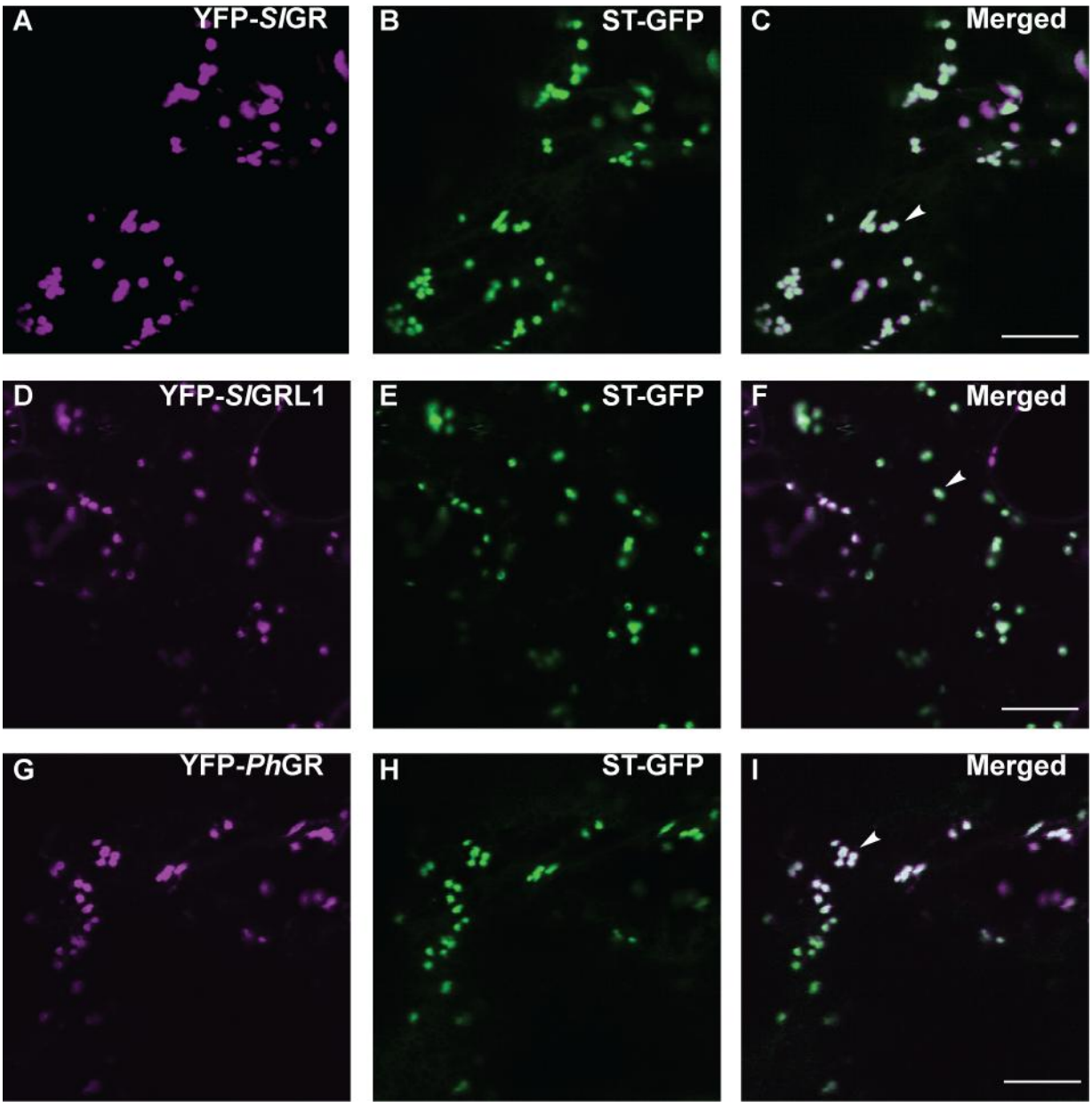


Figure 4.7 Subcellular localization of *Sl*GR, *Sl*GRL1, and *Ph*GR at the Golgi membrane in *Arabidopsis*. Confocal microscopy images of live cotyledon epidermal cells of F1 progeny derived from crosses between *Arabidopsis* plants expressing either *CaMV35S::YFP-SlGR*(A-C), *CaMV35S::YFP-SlGRL1*(D-F), or *CaMV35S::YFP-PhGR*(G-I) with the Golgi marker line 6×*CaMV35S::ST-GFP*. Images were captured using a Zeiss 510 Meta laser scanning confocal microscope. GFP emission was excited with 458 nm wavelength, and detected with a 475-525

nm band pass filter. YFP was excited with 514 nm wavelength, and detected with a 520-555 nm band pass filter. Note the overlapping fluorescence signals in progeny of either *CaMV35S::YFP-SlGR*, *CaMV35S::YFP-SlGRL1*, or *CaMV35S::YFP-PhGR* lines expressing *ST-GFP*, demonstrating Golgi localization. Arrows highlight the "dot" like structure of the Golgi. Scale bar=10 μm.

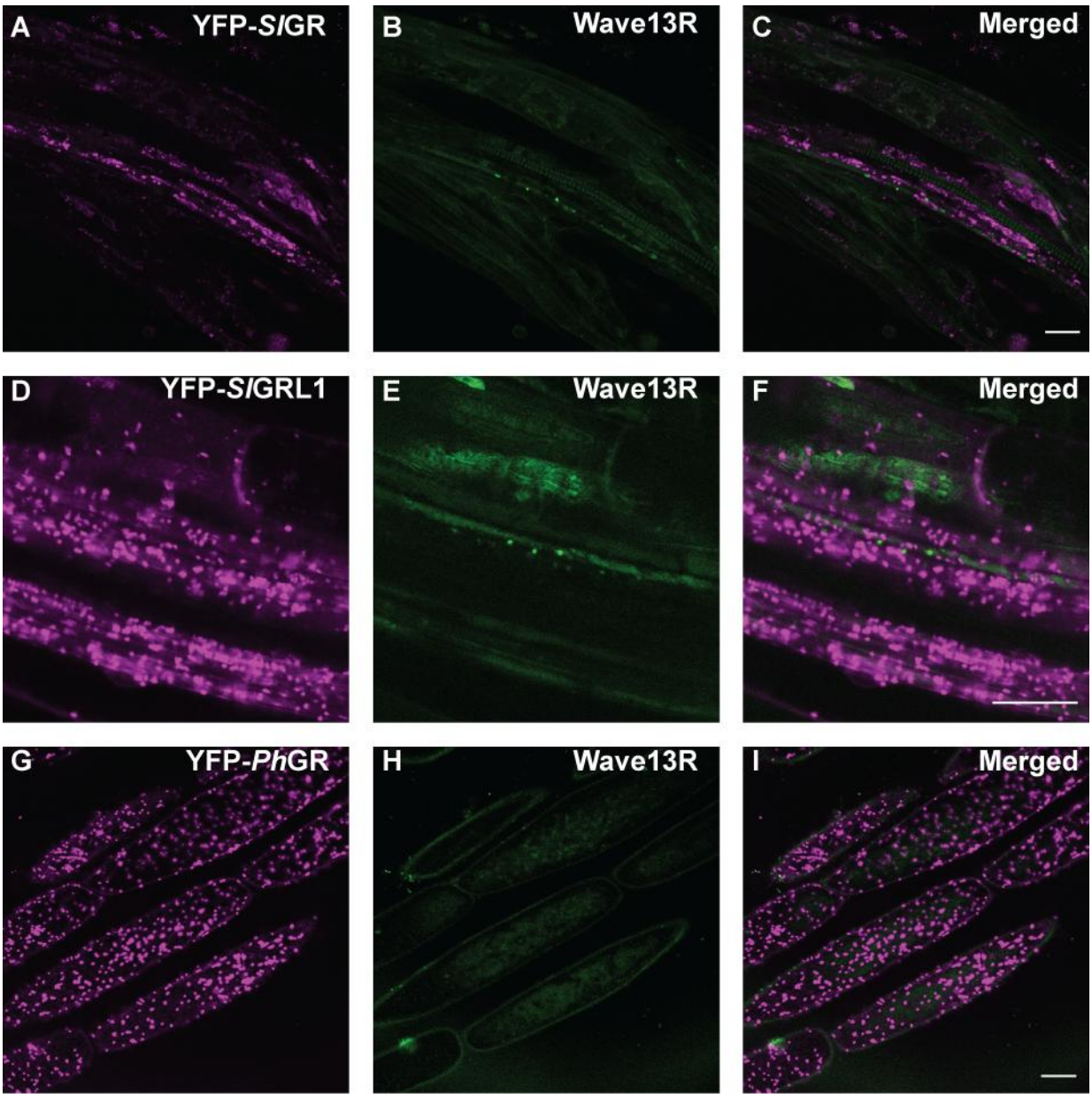


Figure 4.8 *Sl*GR, *Sl*GRL1, and *Ph*GR are not associated with the *trans*-Golgi network (TGN). Confocal microscopy images of live root cells of F1 progeny derived from crosses between *Arabidopsis* plants expressing either *CaMV35S::YFP-SlGR*(A-C), *CaMV35S::YFP-SlGRL1*(D-F), or *CaMV35S::YFP-PhGR*(G-I) together with the TGN marker line Wave13R (mCherry-VTI12), which fused the mCherry protein with the SNARE protein VTI12. Images were captured using an Olympus Fluoview FV41000 laser scanning confocal microscope. YFP emission was excited

with 514 nm wavelength, and collected from 530-560 nm. RFP emission originated from Wave13R line was excited with 559 nm wavelength, and collected from 570-630 nm. Note the non-overlapping fluorescence signals in crosses incorporating the TGN marker line. Scale bar=20 μm.

Table 4.1 Oligonucleotide primers used in this study

Primer	**Sequence**	**Use**
PETGRL1ENT-F	5´-CACCATGGATTTAGAACCTGCCTACA-3´	Construct assembly
PETGRL1ENTSTOP-R	5´-CTAGGAATCCAACAGACTTTTTACAC-3´	Construct assembly
GRQ-F	5´- AGAGCTTGAGGAATCATGAATGC-3´	qRT-PCR
GRQ-R	5´- GGGTGAATACGCCATTGACAT-3´	qRT-PCR
GRL1Q-F	5´- TGCAAAATTTCATGTCGCCATA-3´	qRT-PCR
GRL1Q-R	5´- AGGCAGGTTCCAAATCCATTAA-3´	qRT-PCR
GRL2Q-F	5´- GTGCTGCTGCCTTTCTCCTT-3´	qRT-PCR
GRL2Q-R	5´- AGATTCATCATGGTTCTCGACATATT-3´	qRT-PCR
PIN1Q-F	5´- CTTCTTCCAACTTCCTTTG-3´	qRT-PCR
PIN1Q-R	5´- TGTTTTCCTTCGCACATC-3´	qRT-PCR
PIN2Q-F	5´- AATTATCCATCATGGCTGTTCAC-3´	qRT-PCR
PIN2Q-R	5´- CCTTTTTGGATCAGATTCTCCTT-3´	qRT-PCR

Table 4.1 (cont'd)

CACQ-F	5 ´- CCTCCGTTGTGATGTAACTGG-3 ´	qRT-PCR
CACQ-R	5 ´- ATTGGTGGAAAGTAACATCATCG-3 ´	qRT-PCR

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Chapter 5 Conclusions and future directions

Introduction

The GR/RTE1 family of proteins is conserved in plants animals and protozoa although with the exception of GR and RTE1, which are known to influence ethylene responsiveness, the role of these proteins is unknown. In this study, the role of the GR/RTE1 family proteins in ethylene signaling was further investigated. Unlike most eudicot families that contain two GR/RTE1 homologs, with high homology to RTE1/GRL1 and RTH/GRL2, members of the Solanaceae family also contain an additional phylogenetically distinct family member defined by *Sl*GR that is able to influence ethylene responses in tomato. *SlGR*, *SlGRL1* and *SlGRL2* are differentially expressed during tomato development and in response to ethylene treatment and each encodes a protein that is predominantly localized to the Golgi. A combination of over-expression in tomato and complementation of the *rte1-3* mutant allele indicates that *SlGR* and *SlGRL1* influence distinct ethylene responses suggesting the existence of separate ethylene-signaling modules in tomato, that are influenced either individually by *Sl*GR or *Sl*GRL1 or together by both proteins. In contrast, over-expression of *SlGRL2* in tomato did not reveal any altered ethylene-related phenotypes suggesting that this gene may not be involved in ethylene signaling. Phylogenetic analysis and sequence alignments indicated that putative *GR* orthologs are relatively more divergent than the putative *GRL1* orthologs of the Solanaceae family. This sequence divergence led to different functional characteristics of the putative Solanaceae GR orthologs both in terms of their ability to influence ethylene responsiveness in tomato and to complement the *rte1* mutant of *Arabidopsis*. Utilizing a comparative sequence approach and mutagenesis, a set of 10 amino acids were identified within the putative GR orthologs that are important for these proteins to complement the *rte1* mutant.

Together, these data provide considerable new insight into the role of the *GR/RTE1* family in controlling ethylene responses in plants, particularly, the role of these proteins in the Solanaceae family. However, several intriguing questions and new research directions have arisen as a result of this study.

Discrepancies in the subcellular localization of GR/RTE1 proteins

Components in the upstream section of the ethylene signaling pathway are localized to the endomembrane system. The *Arabidopsis* ethylene receptors localize to the endoplasmic reticulum (ER) and Golgi membranes (Chen et al., 2002; Ma et al., 2006; Dong et al., 2008). The CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) is connected to the ER through binding to the ethylene receptors (Clark et al., 1998; Cancel and Larsen, 2002; Gao et al., 2003). ETHYLENE INSENSITIVE 2 (EIN2) is also located at the ER membrane where it interacts with all five ethylene receptors in *Arabidopsis* (Bisson et al., 2009). The presence of ethylene leads to proteolytic cleavage at the carboxyl-terminal of EIN2, and as a result, its C-terminal is released and rapidly translocates to the nucleus (Qiao et al., 2012).

Separate studies have investigated the subcellular localization of the *At*RTE1 protein. Transient expression in onion epidermal cells suggested that *At*RTE1 is localized to the Golgi whereas transient expression in *Arabidopsis* protoplasts and stable transformation in *Arabidopsis* suggested dual localization to both the ER and the Golgi (Zhou et al., 2007; Dong et al., 2008). The distant homolog of AtRTE1, *At*RTH, was recently reported to be localized to the ER and

nucleus by transient expression in onion epidermal cells as was its putative rice ortholog, *Os*GRL2/*Os*RTH3 (Zhang et al., 2012). In contrast, transient expression in onion epidermal cells suggested dual localization of *Os*GRL1a/*Os*RTH1 and *Os*GRL1b/*Os*RTH2 to the Golgi and ER membranes (Zhang et al., 2012). Together, these data suggest either considerable heterogeneity in the subcellular localization of this protein family or that differences in experimental approaches may have contributed to this anomalous data.

In the present study, the subcellular localization of several GR/RTE1 proteins was assessed. Reporter-gene fusions indicate that *Sl*GR, *Sl*GRL1, *Sl*GRL2, *Ph*GR, *Sm*GR proteins are predominantly localized in the Golgi, which is slightly conflicting with the reported sub-cellular localization of *At*RTE1, *At*RTH, and their rice homologs. For example, we did not detect localization of any of the Solanaceae GR/RTE1 homologs to the ER or the nucleus. These discrepancies in the data between different studies have implications for current models of ethylene signaling and need to be resolved. For example, *At*RTE1 is thought to interact with the ETR1 receptor in the ER (Zhou et al., 2007; Dong et al., 2008; Dong et al., 2010). However, we have shown that *SlGRL1* and *PhGR* are able to complement the *rte1-3* mutant allele, suggesting that they function in a similar way to *At*RTE1, yet YFP fusions of *Sl*GRL1 and *Ph*GR are localized predominantly in the Golgi and we did not observe any fluorescence in the ER. It is possible, that a proportion of the GR/RTE1 protein pool can localize to the ER or that the receptors may also be localized to the Golgi. A problem with current understanding of the subcellular localization of the GR/RTE1 family of proteins and the ethylene receptors is that some of the predictions of subcellular localization are based on interpretation of transiently expressed fusion proteins in onion epidermal cells and protoplasts and high level of transgene

expression in these systems may lead to a failure of protein export from the ER to the intended subcellular location. Similarly, in our study over-expression of the transgenes may have led to saturation of the Golgi export machinery causing the fusion proteins to be trapped within the Golgi. Further studies are needed using combinations of fluorescently tagged proteins and subcellular fractionation studies, together with immunolocalization approaches to resolve the subcellular localization of these proteins.

Potential protein-protein interactions between the GR/RTE1 family and the ethylene receptors

Phenotypic analysis of transgenic lines over-expressing *SlGR* and *SlGRL1* in tomato indicates that these genes influence distinct subsets of ethylene responses. This hypothesis is supported by the finding that *SlGR* and *SlGRL1* possess a differential ability to complement the *rte1-3* mutant allele of *Arabidopsis*. Furthermore, over-expression of *SlGRL1* in a *Gr* mutant background defines the existence of distinct ethylene signaling modules in tomato that we hypothesize are comprised of distinct ethylene signaling components that differentially influence ethylene responsiveness. For example, in some tissues or responses a module susceptible to the action of either *Sl*GR or *Sl*GRL1 operates while in other situations both modules operate to cooperatively influence ethylene responses.

The identity of these modules remains unclear but based on the functional properties of AtRTE1, which binds to the ETR1 N-terminus to promote ethylene signaling (Dong *et al.*, 2010), we propose that *Sl*GR and *Sl*GRL1 will also likely interact with the tomato ethylene receptors. It is

possible that *Sl*GR and *Sl*GRL1 will interact with different members of the ethylene receptor family or they could potentially interact with the same receptor but with different affinities. In support of the hypothesis that *Sl*GR and *Sl*GRL1 interact with separate ethylene receptors, tomato contains two ethylene receptors that are homologous to the ETR1 receptor of *Arabidopsis*, *Le*ETR1 and *Le*ETR2. Based on the *Arabidopsis* model where *At*RTE1 is required for ETR1 function, it is predicted that both *Le*ETR1 and *Le*ETR2 will require the activity of a GR/RTE1 protein for optimal function. The potential of *Sl*GR and *Sl*GRL1 to interact with the tomato ethylene receptors will need to be determined in future research, potentially using a combination of the mating-based split-ubiquitin system (Grefen et al., 2008), Co-immunoprecipitation, or bimolecular fluorescence complementation. In addition, we cannot completely rule out the possibility that the protein levels derived from transgene over-expression are not equal between or within transgenic lines and there remains a slight possibility that some of the phenotypic variation observed between our different transgenic lines may be attributed to different levels of protein derived from the expression of the transgenes.

Identifying amino acid residues and domains of GR/RTE1 proteins that are important for their functional characteristics

Phylogenetic analysis and sequence alignments revealed that putative *SlGR* orthologs are more divergent than the putative *SlGRL1* orthologs in the Solanaceae family. The sequence divergence of the putative GR orthologs led to the hypothesis that these proteins possess altered functional properties and influence distinct ethylene responses. This hypothesis was confirmed through a combination of over-expression in tomato and complementation of the *rte1-3* mutant allele. *SlGR*

is unable to fully complement the *rte1-3* allele whereas *SlGRL1* and the putative *GR* orthologs, *PhGR* from *Petunia hybrida* and *SmGR* from eggplant (*Solanum melongena*), are able to almost fully complement loss of *rte1* function. Sequence alignments coupled with site-directed mutagenesis identified a set of 10 amino acids that are required for the ability of *Sm*GR and *Ph*GR to complement the *rte1* mutant.

This analysis provides a starting point for more in depth analysis of the specific role of domains and amino acids of *Sl*GR and related proteins that are involved in regulating ethylene responsiveness. Additional mutagenesis will be needed to further narrow down the identity of the important amino acid residues that influence ethylene responsiveness. Considering the importance of these amino acids in ethylene signaling, it is possible that they may influence the ability of the GR/RTE1 proteins to bind the ethylene receptors either qualitatively or quantitatively by influencing binding efficiency.

GR/RTE1 proteins have an undefined biochemical function

Although several members of the GR/RTE1 family of proteins are known to influence ethylene responsiveness in plants, possibly through direct interaction with the ethylene receptors, the biochemical function of these proteins remains unknown. Furthermore, we have shown that *SlGRL2* appears not to influence ethylene responses when over-expressed in tomato and no role in ethylene responsiveness has been defined for the putative orthologous proteins from *Arabidopsis* and rice, *At*RTH and *Os*RTH3 (Dong et al., 2010; Zhang et al., 2012). Furthermore, a single GR/RTE1 family homolog is present in metazoan and protozoan genomes although the

function of these proteins is also unknown. However, as metazoans and protozoans are not known to signal using ethylene, it is highly likely that the GR/RTE1 family of proteins have a broader role in cellular physiology that may be conserved across kingdoms. Defining this role will require additional functional studies in animal and plant systems.

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