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Expression patterns and regulation of *SlCRF3* and *SlCRF5* in response to cytokinin and abiotic stresses in tomato (*Solanum lycopersicum*)

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ABSTRACT

Cytokinin is an influential hormone involved in numerous aspects of plant growth and development. A group of transcription factors–cytokinin response factors (*CRFs*) has been included as a side branch to cytokinin signaling pathway which also constitute a subset of the AP2/ERF family of transcription factor proteins. This study examined the expression patterns of two transcription factor genes *SlCRF3* and *SlCRF5* in tomato (*Solanum lycopersicum*) to determine their regulation in response to cytokinin and a variety of abiotic stress conditions. Analyses conducted during different developmental stages by RT-PCR or GUS reporter gene expression revealed that these genes are differentially expressed in vegetative and reproductive organs. qRT-PCR experiments were also performed to study regulation by the hormone cytokinin and abiotic stress conditions such as flooding, drought, osmotic, oxidative, and temperature. These showed that *SlCRF3* and *SlCRF5* have different patterns of regulation in leaf, stem, and roots with *SlCRF5* showing greater induction in leaf or root tissue compared to *SlCRF3* in most cases. Additionally, knockdown analysis for *SlCRF5* revealed defects across development including leaf morphology, primary root growth, and lateral root formation. Together, these findings indicate that *SlCRF3* and *SlCRF5* are potential regulators of tomato developmental processes associated with cytokinin or abiotic stresses.

Introduction

The cytokinin response factors (CRFs) are a subset of the APETALA2/ethylene response factor (AP2/ERF) family of transcription factors found in all land plants. These genes were originally identified in arabidopsis microarray experiments of cytokinin response as transcription factor family members that were highly induced at multiple time points after exogenous application of cytokinin (Rashotte et al., 2003). Recent works have revealed the presence of 12 *CRFs* in *Arabidopsis thaliana* (*AtCRFs*) and also similar numbers in other plant genomes including 11 *Solanum lycopersicum* (*SlCRFs*) (Rashotte and Goertzen, 2010; Cutcliffe et al., 2011; Shi et al., 2012). Much of the study of CRFs has focused on the arabidopsis system (Rashotte et al., 2006; Cutcliffe et al., 2011), although initial examinations of CRFs in tomato has shown that some *SlCRFs* are induced by cytokinin in leaves and that one *SlCRF* gene is expressed in the vasculature of various organs similar to *AtCRFs* (Shi et al., 2012; Zwack et al., 2012). One goal of this study was to conduct a more thorough examination of expression patterns and cytokinin induction for the two *SlCRF* genes, *SlCRF3* and *SlCRF5* in different organs over different stages of tomato development.

While it is clear that a subset of CRFs is cytokinin regulated, little information is known of the roles of *CRFs* in processes other than cytokinin regulation in any plant species. The genes that have been studied in this way in some manner include *PTI6*/*SlCRF1*, other *SlCRFs* 2–11, and tobacco stress-induced 1 (*TSI1*) gene (a CRF member), which are linked to disease resistance and some stress responses (Zhou et al., 1997; Park et al., 2001; Gu et al., 2002; Shi et al., 2012). Arabidopsis CRF expression data from abiotic stress microarray experiments (Winter et al., 2007) or qPCR/GUS analysis also revealed that several *AtCRFs* appear to be abiotic stress responsive (Compton, 2012; Zwack et al., 2013). Abiotic stresses can be highly detrimental to plant growth and can greatly reduce crop yields in plants, including tomato (reviewed in Pandey et al., 2011; Qin et al., 2011; Duque et al., 2013). These stresses cause damage at tissue and cellular levels that can rupture membranes, breakdown photosynthetic machinery components, as well as result in cell death. As such, the regulation of these stresses to generate stress resistant plants is of great importance to plant growers. Cytokinin has been strongly linked to abiotic stress responses in a number of studies examining cytokinin signaling, metabolism and biosynthesis where changes in cytokinin levels, responsive genes,

Abbreviations: ABA, abscissic acid; AP2/ERF, APETALA2/ERF; BA, benzyladenine; CRF, cytokinin response factor; *SlCRF3*, *Solanum lycopersicum* Cytokinin Response Factor 3; *SlCRF5*, *Solanum lycopersicum* Cytokinin Response Factor 5.

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or receptors dramatically altered plant growth under stress conditions (reviewed in Nishiyama et al., 2011; Peleg and Blumwald, 2011; Wilkinson et al., 2012). Since some *SlCRF*s are previously known to be highly induced in response to cytokinin treatment (Shi et al., 2012), we conducted here additional study focusing on abiotic stress responses of these genes to provide insight into the link between cytokinin regulated transcription factors and abiotic stress conditions.

Analysis of loss-of-function CRF mutations in arabidopsis revealed that the CRFs function redundantly to regulate the development of embryos, cotyledons, and leaves (Rashotte et al., 2006). In order to investigate potentially similar development effects of reduced CRF levels during tomato development, we generated antisense transgenic plants with decreased expression levels of *SlCRF5*. Phenotypic analysis revealed a potentially critical role for *SlCRF5* in overall tomato development. Together, this study presents baseline information on the regulation of *SlCRF3* and *SlCRF5* genes relevant for future research focused on the study of tomato development.

Materials and methods

Plant materials and growth conditions

The tomato (*Solanum lycopersicum* L.) dwarf cultivar Micro-Tom plants were grown in Sunshine Mix #8 soil under a 16:8 h light: dark photoperiod at 150 μE, with a 26 °C day (light), 22 °C night (dark) temperature in controlled-environment chambers. Sterilized seeds were germinated in magenta boxes containing 0.8% agar gel with MS salts (4.8 g/L), added Gamborg B5 Vitamins, and 2% sucrose. The pH of the medium was adjusted to a final value of 5.7.

Arabidopsis thaliana (L.) Heynh. (Col-0 ecotype) plants were germinated on Petri dishes containing 0.8% agar gel with MS salts (4.8 g/L) and 1% sucrose medium of pH 5.7. Plants were grown under a light: dark photoperiod at 100 μE, with 22 °C day (light), 18 °C night (dark) temperature in controlled-environment chambers.

Generation of transgenic plants

Plasmids for expression analysis and antisense were generated using the Invitrogen GATEWAY^{TM} cloning system according to the manufacturer's instructions. For expression analysis, the promoter regions (∼2 kb upstream of ATG) of *SlCRF3* and *SlCRF5* were amplified from the genomic DNA and cloned into the destination vector pKGWFS7 as described in Zwack et al. (2012). To generate the antisense construct, coding sequence of *SlCRF5* was cloned in the destination vector pK2WG7. All vectors were sent to the Plant Transformation Research Center (PTRC) at the University of California Riverside for transformation of tomato plants. For expression analysis in arabidopsis, *Agrobacterium tumefaciens* C58 cells were transformed with destination vector pKGWFS7 harboring *SlCRF5* promoter via electroporation, and plants were transformed using the floral dip method (Clough and Bent, 1998). For all transgenics, three independently selected homozygous lines were used for analyses.

Histochemical analysis

GUS activity was analyzed in tomato and arabidopsis organs of transgenic lines. Tissues and organs from these lines of different developmental stages were vacuum infiltrated for 20–30 min with X-gluc buffer (Weigel and Glazebrook, 2002) before incubation at 37 °C for 2–4 h for arabidopsis and overnight for tomato followed by clearing of tissues in 70% ethanol at room temperature. Whole tissues or free hand sections were then examined using a Nikon Eclipse 80i microscope and photos were taken with a Qimaging Fast 1394 digital camera.

Cytokinin treatment

Tomato plants were grown in soil as described above, from which leaves and stems of 15, 25, and 35 d old plants were excised, placed in water, and gently shaken for 2 h prior to treatment. Cytokinin (N^6-benzyladenine; BA) of different concentrations (see text) or the vehicle control DMSO were then added to shaking tissues for various times (see text). For treatment of roots, plants were grown hydroponically in CYG germination pouches from Mega International. Seedlings at 14 DAS (days after sowing) were treated for 24 h by directly adding cytokinin or control DMSO to the growth pouches. After treatment roots (Root tips (RT) – encompassing the meristem and the elongation zone; lateral roots (LR), and whole roots (WR, including RT and LR) were removed from solutions or germination pouches, patted dry with paper towel and immediately flash-frozen in liquid nitrogen (N_2), and stored at −80 °C until RNA extraction. Pro_{SlCRF5}:*GUS* homozygous plants (both tomato and arabidopsis (grown on agar plates)) were also treated with cytokinin in the same manner as described above.

Stress treatments

Water stress

For flooding stress treatment, 25 d old plants grown under standard conditions in soil were placed in trays to maintain water logged conditions for 1, 4, and 7 d. For drought experiments, 25 d old well watered plants were left unwatered for 7 d and rewatered to examine recovery from drought conditions. Leaf, stem and root samples were collected after 7 d of wilting, and 1, 3, 6, 12 h after rewatering and compared to control plants grown under standard conditions. Root treatments were performed in CYG germination pouches.

Mannitol, H_2O_2, and ABA treatment

Soil grown 25 d old plants were treated with 200 mM mannitol, 10 and 20 mM H_2O_2, and 50 and 100 μM ABA for 3 h and leaf, stem, and root tissues were collected and immediately flash-frozen in liquid N_2.

Temperature

Magenta box grown plants and treatments were performed at 25 d of age. For cold treatment, magenta boxes were kept in 4 °C for 24 h, and heat treatment was performed by moving the plants in magenta boxes from 26 °C to a 45 °C water bath for 1 h, after which leaves, stems, and roots were collected and immediately flash-frozen in liquid N_2.

RNA isolation, cDNA synthesis, and expression analysis

For expression analysis, tissues were harvested from 15, 25, and 35 d old plants and immediately flash-frozen in liquid N_2. Total RNA was extracted from these samples and other treated plants as described in Shi et al. (2012). For RT-PCR or qRT-PCR analysis, 500 ng of total RNA was converted into cDNA with Quanta qScript cDNA supermix and diluted 20X before use in PCR reactions. *SlCRF3* and *SlCRF5* expression analysis in various organs by RT-PCR was started with a one-step cycle of 2 min at 95 °C, followed by 29 cycles of 30 s at 94 °C, 45 s at 56 °C, and 50 s at 72 °C, and a 5 min final extension at 72 °C, using gene specific primer as mentioned in Shi et al. (2012).qPCR was performed using cytokinin or stress treated cDNA samples with SYBR-Green chemistry in an Eppendorf Mastercycler ep realplex using primers as in the RT-PCR. Each reaction contains 9 μL of SYBR-Green supermix, 2 μL of cDNA template, 1 μL