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A cold-induced pectin methyl-esterase inhibitor gene contributes negatively to freezing tolerance but positively to salt tolerance in Arabidopsis

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ABSTRACT

Plant pectin methyl-esterase (PME) and PME inhibitor (PMEI) belong to large gene families whose members are proposed to be widely involved in growth, development, and stress responses; however, the biological functions of most PMEs and PMEIs have not been characterized. In this study, we studied the roles of *CbPMEI1*, a cold-induced pectin methyl-esterase inhibitor (PMEI) gene from *Chorispora bungeana*, under freezing and salt stress. The putative CbPMEI1 peptide shares highest similarity (83%) with AT5G62360 (PMEI13) of Arabidopsis. Overexpression of either CbPMEI1 or PMEI13 in Arabidopsis decreased tissue PME activity and enhanced the degree of methoxylation of cell wall pectins, indicating that both genes encode functional PMEIs. *CbPMEI1* and *PMEI13* were induced by cold but repressed by salt stress and abscisic acid, suggesting distinct roles of the genes in freezing and salt stress tolerance. Interestingly, transgenic Arabidopsis plants overexpressing *CbPMEI1* or *PMEI13* showed decreased freezing tolerance, as indicated by survival and electrolyte leakage assays. On the other hand, the salt tolerance of transgenic plants was increased, showing higher rates of germination, root growth, and survival under salinity conditions as compared with non-transgenic wild-type plants. Although the transgenic plants were freezing-sensitive, they showed longer roots than wild-type plants under cold conditions, suggesting a role of PMEs in balancing the trade-off between freezing tolerance and growth. Thus, our study indicates that *CbPMEI1* and *PMEI13* are involved in root growth regulation under cold and salt stresses, and suggests that PMEIs may be potential targets for genetic engineering aimed to improve fitness of plants under stress conditions.

1. Introduction

Low temperature is an important environmental factor that affects plant growth, development, and production. Most plants cope with cold stress by altering expressions of a variety of genes, whose products may be involved in reprogramming cellular processes (Thomashow, 1999; Zhu et al., 2007). Some of the cold-responsive genes, such as C-repeat binding factor/DRE binding factor 1 (CBF/DREB1) family transcription factors (Liu et al., 1998; Stockinger et al., 1997), play important roles in freezing tolerance. However, although transgenic plants with CBF overexpression are highly freezing-tolerant, their growth is repressed dramatically even under non-stressed warm conditions (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Tillett et al., 2012; Zhou et al., 2014), which may reduce competitiveness and in turn compromise tolerance. Therefore, the ability to maintain growth under cold stress should be an important trait for a practical freezing-tolerant plant.

Growth retardation seems common for most plants in cold conditions. Theoretically, this may be primarily due to low cellular activities as a whole at low temperature. Moreover, energy and carbon supplies from photosynthesis are reduced at low temperature since the photosynthetic apparatus is vulnerable to cold stress (Oquist and Huner, 2003). On the other hand, most plants will enhance their freezing tolerance to survive in cold conditions, and this must be achieved at the expense of other processes including growth. Such a trade-off between freezing tolerance and growth in plants has been evidenced not only in transgenic plants (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Tillett et al., 2012; Zhou et al., 2014) but also in natural species (Koehler et al., 2012). However, plants may be able to maintain relatively high growth rates while ensuring survival, although growth repression seems inevitable in cold and under other stresses. In an attempt to improve the growth of stress-tolerant plants, both drought tolerance and plant growth were enhanced successfully by double expression of DREB1A and OsPIL1 in Arabidopsis (Kudo et al., 2017). Therefore, it is expected that some natural plants may have

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evolved functional mechanisms for balancing the trade-off between stress tolerance and growth regulation.

Among the responses of plants to cold stress, cell wall adjustments are of great importance in both freezing tolerance and growth regulation (Sobkowiak et al., 2016; Yamada et al., 2002). Transcriptome studies have revealed that genes involved in cell wall biosynthesis or modification are highly responsive to cold stress (Le et al., 2015; Maul et al., 2008; Sobkowiak et al., 2016; Sobkowiak et al., 2014; Zhao et al., 2012). Several studies have indicated that cell wall properties can be associated directly with freezing tolerance. For example, the *eskimo1* (*esk1*) mutant, which causes a reduction in xylan acetylation (Yuan et al., 2013), is constitutively freezing-tolerant (Xin and Browse, 1998; Xin et al., 2007). The relation between reduced lignin and enhanced freezing tolerance was shown in mutants of TCF1 transcription factor (Ji et al., 2015).

Pectins, accounting for over 30% of polysaccharides of primary cell walls in higher plants, play important roles in plant development and abiotic resistance (Micheli, 2001; Pelloux et al., 2007). The structures and functions of cell wall pectins are closely related to the degree of methoxylation (DM), which is co-regulated by pectin methyl-esterases (PMEs) and PME inhibitors (PMEIs). It has been shown that the content and methyl-esterification degree of pectins are dynamically regulated by temperature (Baldwin et al., 2014; Solecka et al., 2008). During cold acclimation, pectin contents, PME activity, and low-DM pectins tend to increase and may play a role in freezing tolerance (Qu et al., 2011; Solecka et al., 2008). However, genetic evidence supporting a direct role of pectins in freezing tolerance is still lacking, although a number of studies have documented the involvement of pectins in other stresses (Geng et al., 2017; Lionetti et al., 2012; Lionetti et al., 2007; Sun et al., 2016; Xu et al., 2014). In plant cells, high-DM pectins are synthesized in Golgi bodies and incorporated into cell walls, where they undergo de-esterification by PME whose activity is inhibited by PMEI (Micheli, 2001). Interestingly, studies indicate that both PME and PMEI may be cold-inducible (Jeong et al., 2015; Zhao et al., 2012), suggesting the existence of a balancing mechanism in pectin regulation under cold stress.

In plants, both PME and PMEI belong to large gene families. For example, there are 67 putative PMEs and 69 putative PMEIs in the Arabidopsis genome. Functional analysis of some PME/PMEI genes reveal that they are involved in a wide range of processes such as cell elongation and organ growth (Bosch et al., 2005; Derbyshire et al., 2007), hormone signaling (Wolf et al., 2012; Wolf et al., 2014), and biotic and abiotic stresses (Geng et al., 2017; Lionetti et al., 2007). However, only 12 PMEIs and a few PMEs have been functionally characterized in Arabidopsis. Besides a large number of isoforms, the functions of PMEs and PMEIs are further complicated by their highly specific expression patterns in different tissues and in response to different stresses (Pelloux et al., 2007), suggesting that PMEs and PMEIs are tightly controlled by and are involved in plant development and environmental stresses.

C. bungeana is a subnival alpine plant highly tolerant to chilling and freezing stress (Fu et al., 2006; Wu et al., 2007). Moreover, low temperature has little effect on the growth of *C. bungeana* (Wu et al., 2008). Transcriptome analysis found that a PMEI homolog is among the top 10 cold-induced genes in *C. bungeana* (Zhao et al., 2012). In this paper, we cloned the full-length cDNA of this gene (named *CbPMEI1*) and showed that *CbPMEI1* and *AT5G62360* (*PMEI13*), a homolog of *CbPMEI1* in Arabidopsis, contributed to growth maintenance rather than freezing tolerance. We propose that *CbPMEI1/PMEI13* may play an important role in improving the fitness of natural freezing-tolerant plants in cold conditions, and plant PMEIs may be potential targets for cultivation of practical freezing-tolerant crops.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia plants were grown in soil or on Murashige-Skoog agar medium (1/2 MS) at 22 °C in a greenhouse under a 14-h/10-h light/dark cycle at a photon flux density of 100 μmol/m^2/s. Seedlings of *C. bungeana* were regenerated and cultured in the greenhouse as described previously (Zhao et al., 2012).

2.2. Construction of plasmids and generation of transgenic plants

Total RNA was extracted from matured leaves of *C. bungeana* and Arabidopsis using Trizol reagent. Full-length cDNA of *CbPMEI1* (Fig. S1) was cloned by 5′-RACE and 3′-RACE. The protein coding sequences of *CbPMEI1* and *PMEI13* were obtained by RT-PCR using total RNA from *C. bungeana* and Arabidopsis, respectively. The amplified fragments were sub-cloned into pENTR-D-TOPO vector (Invitrogen, Carlsbad, CA) to generate entry vectors and verify their sequences. To construct overexpression vectors, *PMEI* fragments in entry vectors were transferred to pEarleyGate100 (Earley et al., 2006) using the Gateway cloning system (Invitrogen, Carlsbad, CA). To construct (C-terminal) YFP-tagged vectors, *PMEI* fragments in entry vectors were transferred to pEarleyGate101 (Earley et al., 2006). Promoter sequences were PCR-amplified from genomic DNA, sub-cloned into pENTR-D-TOPO vector, and transferred to pMDC163 vector (Curtis and Grossniklaus, 2003) to generate proCbPMEI1:GUS and proPMEI13:GUS vectors. Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998). All transgenic plants were homozygotes of the T3 or T4 generation. The primer sequences are listed in Table S1.

2.3. Measurement of PME activity and pectin DM

Four-week-old soil-grown Arabidopsis seedlings were used for PME and pectin assays. Measurement of PME activity was performed as described previously (Qu et al., 2011). For pectin DM evaluation, pectins (~1 mg/ml) were extracted from leaves of two-week-old plants and saponified with 0.5 M KOH for 30 min at room temperature. After adjusting the solution to pH 7.5 with 0.1 M H_3PO_4, methanol in the solution was oxidized with alcohol oxidase, and the colored product was measured at 412 nm (Klavons and Bennett, 1986).

2.4. Immunofluorescence detection of low-DM pectins

Localization of low-DM pectins by indirect immunofluorescence was performed according to Geng et al. (2017) using LM19 monoclonal antibody (Verhertbruggen et al., 2009). Briefly, roots of four-day-old seedlings were collected in fixation solution (4% paraformaldehyde, 50 mM PIPES, 5 mM $MgSO_4$, 5 mM EGTA, pH 6.9) for two hours, and then the samples were washed three times (5 min each) with phosphate-buffered saline (PBS) and incubated with 0.2% BSA in PBS buffer for 30 min. Then the samples were incubated in a 1:10 diluted solution of LM19 antibody (Sigma-Aldrich, USA) overnight at 4 °C. After washing with PBS three times, the samples were incubated with anti-rabbit IgG coupled with fluorescein isothiocyanate for two hours at 37 °C and then washed three times with PBS. The samples were mounted on glass slides and examined under a laser-scanning confocal microscope (Olympus FV1000MPE, Japan).

2.5. Staining and microscopy

Four- or seven-day-old whole seedlings, flowers, or siliques of transgenic plants harboring proCbPMEI1:GUS or proPMEI13:GUS were used for GUS staining as described (Medina et al., 2001). Photographs were taken with a microscope (Leica DM600B, Germany). For YFP