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RESEARCH ARTICLE

Heterologous expression of *Lolium perenne* antifreeze protein confers chilling tolerance in tomato

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Abstract

Antifreeze proteins (AFP) are produced by certain plants, animals, fungi and bacteria that enable them to survive upon extremely low temperature. Perennial rye grass, *Lolium perenne*, was reported to possess AFP which protects them from cold environments. In the present investigation, we isolated *AFP* gene from *L. perenne* and expressed it in tomato plants to elucidate its role upon chilling stress. The T_1 transgenic tomato lines were selected and subjected to molecular, biochemical and physiological analyses. Stable integration and transcription of *LpAFP* in transgenic tomato plants was confirmed by Southern blot hybridization and RT-PCR, respectively. Physiological analyses under chilling conditions showed that the chilling stress induced physiological damage in wild type (WT) plants, while the transgenic plants remained healthy. Total sugar content increased gradually in both WT and transgenic plants throughout the chilling treatment. Interestingly, transgenic plants exhibited remarkable alterations in terms of relative water content (RWC) and electrolyte leakage index (ELI) than those of WT. RWC increased significantly by 3-fold and the electrolyte leakage was reduced by 2.6-fold in transgenic plants comparing with WT. Overall, this report proved that *LpAFP* gene confers chilling tolerance in transgenic tomato plants and it could be a potential candidate to extrapolate the chilling tolerance on other chilling-sensitive food crops.

Keywords: *Lolium perenne* antifreeze protein, chilling tolerance, genetic transformation, transgenic tomato

1. Introduction

Being sessile in nature, plants constantly encounter several abiotic and biotic stresses that adversely affect plant growth, development and survival (Smйkalovб *et al*. 2014). Among them, abiotic stresses are very critical, as they are uncontrollable and unpredictable, which will potentially limit plant survival and thus lead to reduced yield (Wang *et al*. 2013; Velada *et al*. 2014). The temperature stress imposed on plants has huge effect on agriculture. For instance, it has been reported that decrease of 1°C in the world average temperature might result in 40% reduction in rice production (Hale and Orcutt 1987). Hence, there exists a pressing need to overcome the crop loss due to negative impact of chilling stress on plant growth and survival.

Generally, temperate plants can increase their chilling tolerance by exposing to cold nonfreezing temperature *via* an adaptive response called cold acclimation (Thomashow 1999). Whereas, the plants of tropical and subtropical

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regions are sensitive to chilling stress and the negative effects of chilling stress are known to be higher in these plants, as they lack the cold acclimation mechanism (Thomashow 1999; Chinnusamy *et al*. 2007). Moreover, many important food crops such as rice, maize, soybean, cotton and tomato are chilling-sensitive, which are unable to acclimate cold and also they cannot withstand the chilling stress when exposed to low temperature (Chinnusamy *et al*. 2007).

Overwintering plants have natural ability to resist ice crystal formation in the cell by inhibiting its growth (Pudney *et al*. 2003). Proteins isolated from many grass species have shown to possess antifreeze properties that help them to cope up with the cold conditions (Antikainen and Griffith 1997). Antifreeze proteins (AFP) are a class of proteins found in a range of over-wintering plants that protect them from the damage imposed by chilling stress (Kumar *et al*. 2014). *Lolium perenne* belongs to the Poaceae family, which are adapted to grow in the colder Northern hemisphere (Sandve *et al*. 2011) and reported to withstand freezing (Kuiper *et al*. 2001). Superior antifreeze protein responsible for this activity was identified from *L*. *perenne*, which possessed two putative opposite-facing sites with surface complimentary to the prism face of ice (Kuiper *et al*. 2001). The *LpAFP* gene consists of 354 bp that encodes a protein of about 118 amino acids (13.5 kDa) with a semi-conserved seven-amino acid repeat X-X-N-X-V-X-G on its entire length (Sidebottom *et al*. 2000; Middleton *et al*. 2009). AFP reduces the freezing point of the solute non-colligative, the process termed as thermal hysteresis (TH), by inhibiting the recrystallization of the growing ice crystals (Kuiper *et al*. 2001). Besides that the AFP was also described as protector of cells from damage during non-freezing conditions (Tomczak *et al*. 2002) mainly due to their interactions with: i) the integral membrane proteins (Rubinsky *et al*. 1991); ii) the membrane lipids (Hays *et al*. 1996); and iii) the membrane, which modifies the acyl chain's order in the bilayer core (Tomczak *et al*. 2002).

Tomato (*Solanum lycopersicum*) is one of the most consumed vegetables worldwide with the production of 1.70 million tons (http://www.fao.org/statistics/en/) in 2014 and is also one of the most preferred garden crops (Fan *et al*. 2015). In recent years, the consumption rate of tomatoes has substantially increased worldwide and during last two decades, the production and harvesting of tomato has doubled (Bergougnoux 2014). Across the globe, Asia dominates the tomato market where India ranked the second after China in tomato production. Tomato is one of the preferred vegetables in routine human diet, which achieved a great familiarity over the last century (Harish and Sathishkumar 2011; Badimon *et al*. 2017). Apart from the dietary source, tomatoes are also considered as an excellent source of antioxidants, phytochemicals such as lycopene, β-carotene, ascorbic acid, lutein, tocopherol, phenolic compounds and it is also cholesterol-free. These compounds are shown to promote positive health benefits like antitumorigenic effects in human system, reduced risk of cardiovascular and different types of cancer (Harish *et al*. 2012). Besides its role in human diet, tomato plants are being extensively used in research, mostly due to its simple diploid genetics, short life cycle and relatively simple genome (~900 Mb) (Zhou *et al*. 2013; Kumar *et al*. 2014).

Nevertheless, tomato is a thermophilic crop that makes it more sensitive to chilling conditions (Zhou *et al*. 2013). Any modifications in terms of enhancing the chilling-tolerant ability of tomato plants will represent important biotechnological breakthrough in high-altitude farming. Transgenic technologies are presenting promising results in improving plant traits, such as enhanced chilling tolerance, by introducing single or multiple genes involved in response to chilling stress. A number of reports showed that the transgenic plants expressing AFP from various sources proved enhanced chilling tolerance (Holmberg *et al*. 2001; Zhu *et al*. 2010; Lin *et al*. 2011; Denga *et al*. 2014; Sun *et al*. 2014). As an attempt to develop chilling-tolerant tomato plants that could aid in high altitude farming, here we are reporting for first time the development of transgenic tomato plants by expressing the *AFP* isolated from *L*. *perenne* and successfully demonstrated the chilling-tolerant capability of these transgenic lines.

2. Materials and methods

2.1. Gene isolation, cloning and plant expression using gateway technology

Young leaves from cold acclimated *L*. *perenne* plants were used for total RNA isolation using Qiagen RNeasy RNA Isolation Kit (Qiagen, USA). And 1 μg of total RNA was used for cDNA synthesis using the random hexamer primer and M-MuLV reverse transcriptase (Fermentas, USA) following manufacturer's instructions.

The *LpAFP* gene (GenBank accession number: AJ277399) was amplified of each gene-specific primer (LpAFP-f101 and LpAFP-r102, both flanked by the attB sequences by Phusion DNA polymerase (New England Biolabs, USA) using a My-cycler Thermal Cycler (Bio-Rad, USA). The amplicon generated was purified using PCR Purification Kit (Qiagen, USA) and cloned into the entry vector pDONR/Zeo using the BP clonase II (Invitrogen, USA) following the procedure described by manufacturer's protocol. Reaction product was then transformed into *Escherichia coli* competent cells. The sequencing-confirmed plasmid was recombined with the destination vector pEARLY