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Research article

AmDREB2C, from *Ammopiptanthus mongolicus*, enhances abiotic stress tolerance and regulates fatty acid composition in transgenic *Arabidopsis*

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ABSTRACT

Dehydration-responsive element-binding (DREB) transcription factors (TFs) play a vital role in plant response to abiotic stresses. However, little is known about DREB TFs in plants adapted to harsh environments and in the formation of polyunsaturated fatty acids (PUFAs), a major membrane component closely associated with plant stress tolerance. Here, we characterized *AmDREB2C* in *Ammopiptanthus mongolicus* (Maxim. ex kom.) Cheng F., a desert evergreen broadleaf shrub with a high tolerance to harsh environments. *AmDREB2C* encodes a canonical DREB2-type TF, and the protein was localized in the nucleus. *AmDREB2C* had the highest expression levels in leaves of naturally growing shrubs in the wild during the winter season of a year of sampling. The expression was also induced by cold, heat and drought stresses in laboratory-cultured seedlings. Moreover, *AmDREB2C* was most abundantly expressed in young leaves and immature seeds rather than other tissues of the shrubs. Constitutive expression of *AmDREB2C* in *Arabidopsis* enhanced freezing, heat and drought tolerances of the transgenic plants, likely through inducing the expression of important stress-responsive genes. The transgene also increased the level of linolenic acid (C18:3), a major PUFA in most plant species, in leaves and seeds of the transgenic plants. Correspondingly, the transcription of *FAD3*, *FAD7* and *FAD8*, three genes encoding fatty acid desaturases (FADs) responsible for the production of C18:3, showed a differential up-regulation in these two organs. This study provides new insight into the underlying molecular mechanisms of *A. mongolicus*' ability to endure harsh environments and DREB TF regulation of fatty acid desaturation.

1. Introduction

Abiotic stresses such as drought and extreme temperatures adversely affect growth and productivity of plants, particularly crops. Plants have developed a diverse set of mechanisms to cope with these environmental stresses. Identifying key stress-related genes is an essential prerequisite for understanding the adaptive mechanisms at the molecular level and for engineering stress-tolerant crops (Wang et al., 2016). Extensive studies have demonstrated that the genes encoding several families of transcription factors (TFs) play a central role in the regulation of stress-responsive gene expression and stress tolerance improvement in plants (Wang et al., 2016). Among these, the genes coding for dehydration-responsive element-binding proteins (DREBs) or C-repeat binding factors (CBFs) are most attractive due to their central role in the regulatory networks for plant response to drought, cold, salt and heat stresses, as well as their promising application in stress tolerance improvement of crop plants by genetic manipulation (Lata and Prasad, 2011).

The DREB TFs belong to the plant-specific APETALA2/ethylene responsive factor (AP2/ERF) family. Each member in the DREB subfamily is characterized by the presence of a highly conserved AP2/ERF DNA-binding domain consisting of approximately 60 residues near the N terminus and a diversified transcription activation domain (TAD) at the C-terminal region. In addition, one or two nuclear localization signals (NLSs) at the N-terminal region and a conserved Ser/Thr-rich negative regulatory domain (NRD) adjacent to the AP2/ERF domain are also

Abbreviations: ABA, abscisic acid; AP2/ERF, APETALA2/ethylene responsive factor; CBF, C-repeat binding factor; CRT, C-repeat; DRE, dehydration-responsive element; DREB, dehydration-responsive element-binding protein; FAD, fatty acid desaturase; FAME, fatty acid methyl ester; GFP, green fluorescent protein; Hsf, heat stress transcription factor; HSP, heat shock protein; NLS, nuclear localization signal; NRD, negative regulatory domain; P5CS, Δ-1-pyrroline-5-carboxylate synthetase; PPT, phosphinothricin; PUFA, polyunsaturated fatty acid; SD, standard deviation; TAD, transcription activation domain; TF, transcription factor

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mostly present (Sakuma et al., 2002, 2006a; Lata and Prasad, 2011). Based on the similarity in the DNA-binding domain, the DREB TFs can be further divided into six subgroups from A-1 to A-6 (Sakuma et al., 2002) or four groups from I to IV (Nakano et al., 2006), implying functional differentiation among distinct subgroups or groups of the DREB-type TFs.

Since the first isolation of *AtDREB1A, AtDREB1B*, *AtDREB1C* (also named *AtCBF3*, *AtCBF1* and *AtCBF2*, respectively), and *AtDREB2A* from *Arabidopsis thaliana*, numerous other *DREB*- or *CBF*-orthologous genes have been identified in a wide variety of plant species, especially in herbaceous plants such as rice (*Oryza sativa*), maize (*Zea mays*), and soybean (*Glycine max*) (Nakano et al., 2006; Lata and Prasad, 2011; Liu et al., 2013; Mizoi et al., 2013). Most of the characterized *DREBs* belong to the A-1 and A-2 subgroups, commonly known as *DREB1s/CBFs* and *DREB2s*, respectively. The two types of *DREBs* differ in their responses to different abiotic stresses. In general, the *DREB1/CBF* genes, such as *AtDREB1A/AtCBF3*, *AtDREB1B/AtCBF1*, and *OsDREB1B*, are primarily involved in the response to low temperatures. Overexpression or constitutive expression of most *DREB1s/CBFs* from different plant species displays significantly improved tolerance to cold, drought, and/or salt stresses in transgenic plants (Lata and Prasad, 2011; Wang et al., 2016). In contrast, the *DREB2* genes commonly exhibit a more complex stress-response pattern than *DREB1s/CBFs*. For instance, *AtDREB2A*, *AtDREB2C*, and *OsDREB2A* mainly participate in plant response to drought, heat and salt stresses (Sakuma et al., 2002, 2006b; Schramm et al., 2008; Chen et al., 2010; Matsukura et al., 2010), while *GmDREB2A;2*, *MsDREB2C* (from *Malus sieversii* Roem.) and *EsDREB2B* (from *Eremosparton songoricum*) were induced not only by drought, heat and high salinity but also by cold stress (Mizoi et al., 2013; Zhao et al., 2013; Li et al., 2014). Over-expressing or constitutively expressing *DREB2s*, such as *AtDREB2A CA* (constitutive active form), *OsDREB2B* and *MsDREB2C*, in transgenic plants not only conferred drought, salt and/or cold tolerances but often enhanced heat tolerance (Sakuma et al., 2006a, 2006b; Matsukura et al., 2010; Lata and Prasad, 2011; Zhao et al., 2013). Thus, the *DREB2* genes might provide a more suitable strategy for improving plant tolerance to a complex suite of abiotic stresses. However, previous investigations have mainly focused on *DREB1s/CBFs* from different species (Lata and Prasad, 2011; Wang et al., 2016). Identifying more *DREB2s*, especially those in plants adapted to extreme environments will help to elucidate the adaptive strategies of these plants to persist in harsh environments.

The membrane systems of plant cells are primary sites of freeze-induced injury (Thomashow, 2001), and can also be injured by heat, drought and salt stresses (Upchurch, 2008). Polyunsaturated fatty acids (PUFAs), principally linolenic acid (C18:3) and linoleic acid (C18:2), are important structural components of plant cell membranes and closely associated with membrane fluidity during abiotic stresses (Iba, 2002; Upchurch, 2008). Stress acclimating or stress-tolerant plants have the capacity to adjust membrane fluidity by changing the levels of PUFAs, especially that of C18:3, to maintain an environment suitable for the function of important integral membrane proteins and membrane integrity while under stress (Upchurch, 2008). The production of PUFAs in membrane lipids is mainly controlled by a class of membrane-bound fatty acid desaturases (FADs), such as FAD2 and FAD6 (both are responsible for the formation of C18:2 from oleic acid [C18:1]) as well as FAD3, FAD7 and FAD8 (which are responsible for the desaturation of C18:2 to from C18:3) (Iba, 2002; Zhang et al., 2012; Román et al., 2015). The genes encoding these FADs have been extensively studied in a variety of plant species, and most of them have been shown to play crucial roles in the production of the corresponding 18-C PUFAs and/or tolerance of plants to cold, heat, drought and/or salt stresses (Iba, 2002; Upchurch, 2008; Domínguez et al., 2010; Zhang et al., 2012; Wang et al., 2014; Román et al., 2015). However, the upstream regulators controlling *FAD* expression and PUFA production are largely unknown.

The DREB TFs induce the transcription of multiple stress-responsive genes that mostly contain the dehydration-responsive element (DRE) or C-repeat (CRT) element in their promoters (Lata and Prasad, 2011; Wang et al., 2016). These target genes encode various functional proteins such as dehydrins, protective enzymes and heat shock proteins (HSPs), and some regulatory proteins such as heat stress TFs (Hsfs) and zinc finger TFs. All these proteins have a direct function by protecting plant cells from damage by abiotic stresses or an indirect function by promoting new stress signaling cascades (Sakuma et al., 2006a, 2006b; Schramm et al., 2008; Chen et al., 2010; Matsukura et al., 2010; Mizoi et al., 2013). Despite extensive studies on DREBs in the regulatory networks of plant response to abiotic stresses, DREB regulation of the production of PUFAs has remained unknown. Therefore, investigations on the connection of DREBs with *FAD* expression and fatty acid desaturation in plants are needed.

Ammopiptanthus mongolicus (Maxim. ex kom.) Cheng F. is the only evergreen broadleaf relic shrub that is endemic to the central Asian desert. It has a very high tolerance to abiotic stresses, such as freezing, drought, heat and high salinity, and thus, is increasingly used as a model species for studying stress tolerance (Wu et al., 2014; Gao et al., 2016). Recently, a dozen of its stress-tolerant genes have been isolated and characterized (Song et al., 2013). In addition, thousands of cold- and drought-regulated genes as well as a batch of drought-responsive microRNAs have been identified in this species (Wu et al., 2014; Gao et al., 2016). We have identified several drought- and cold-induced *DREBs* in the *A. mongolicus* transcriptome (Wu et al., 2014); however, no DREB- or CBF-like genes have been functionally characterized in this species to date. For this study, we cloned *AmDREB2C* and investigated its primary function in stress tolerance by observing expression levels in field-grown plants in their natural conditions and lab-grown seedlings in controlled settings. We also introduced *AmDREB2C* in *Arabidopsis* to test the gene's function in another species. The transgene played key roles in plant response to freezing, heat and drought stresses by inducing the expression of multiple stress-inducible genes in the transgenic plants. Interestingly, we observed a substantial increase in the C18:3 level in both leaves and seeds of the transgenic plants, which may have potentially been caused by the up-regulation of specific omega-3 FAD gene expression. Our study not only provides new evidence of the potential underlying mechanisms of *A. mongolicus*' ability to tolerate harsh environments, but also revealed a novel role of the DREB-type TFs in the regulation of fatty acid composition.

2. Materials and methods

2.1. Field sampling and stress treatments of A. mongolicus

Various tissue samples were collected for expression analysis from naturally-grown adult *A.mongolicus* plants in the southern suburb of Hohhot, Inner Mongolia, China. Young leaves of the plants were sampled at 9:30 to 11:00 during the first five days in each month from July 2014 to May 2015. Flowers were sampled in late April 2015, and the young leaves, young twigs, lateral roots, and immature seeds were sampled in late May 2015. All samples were immediately frozen in liquid nitrogen and then stored at −76 °C for RNA extraction.

Ammopiptanthus mongolicus seedlings were also subjected to abiotic stresses of cold, heat, dehydration, high salinity, and ABA treatment for expression analysis. Seeds were surface-sterilized and cultured in pots of sand, six seeds per pot, as described previously (Wu et al., 2014). After 1.5 months after the planting date, the seedlings were exposed to one of the five treatments. For the cold treatment, seedlings were placed into a low temperature-programmable incubator (Percival LT-36VL, Percival, USA) for 48 h. The temperature was set at 4 °C for the first 24 h, 0 °C for the next 12 h, and −6 °C for the last 12 h in dim light of ~50 μmol · m^{-2} · s^{-1} (the same light level was used for all dim lighting below). The cooling rate was 0.1 °C · min^{-1}. For the heat treatment, seedlings were placed in an electric incubator (UF110, Memmert, Germany) at 42 °C in the dark for 48 h, and the pots were watered every 8 h to avoid drought stress. The dehydration treatment