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Overexpression of the *betaine aldehyde dehydrogenase* gene from *Atriplex hortensis* enhances salt tolerance in the transgenic trifoliate orange (*Poncirus trifoliata* L. Raf.)

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ABSTRACT

Trifoliate orange (*Poncirus trifoliata* L. Raf.), a rootstock widely used for citrus species, is salt-sensitive. Worldwide, salinity is a major abiotic stress affecting citrus growth and yield. Glycinebetaine (GB) is an important osmoprotectant involved in responses to salt stress. However, current evidence regarding the effect of salt stress on GB accumulation in trifoliate orange is limited, and the GB synthesis gene has not yet been shown to confer enhanced salt stress tolerance to this species in a transgenic context. In the current study, we first examined the change in GB level of trifoliate orange seedlings exposed to salt stress, and found that salt increased endogenous GB level in a concentration-dependent manner. A *betaine aldehyde dehydrogenase* gene (*AhBADH*) cloned from *Atriplex hortensis* was introduced into the trifoliate orange by means of *Agrobacterium*-mediated transformation. RT-PCR analysis on three selected transgenic lines showed that the *AhBADH* gene was overexpressed in each of them. GB levels in these lines were also higher than those in untransformed wild-type (WT) plants. In the transgenic lines, exposure to 200 mM NaCl resulted in significantly less serious leaf burning and defoliation, lower MDA accumulation, and higher chlorophyll contents than those in the WT plants. Moreover, when exposed to salt, shoots of transgenic plants contained lower levels of Na^+ and Cl^-, higher levels of K^+, and a higher K/Na ratio, while the same was true for the roots in most cases. Taken together, the data suggest that overexpression of the *AhBADH* gene in transgenic trifoliate orange enhanced salt stress tolerance. This may be correlated with the low levels of lipid peroxidation, protection of the photosynthetic machinery, and increase in K^+ uptake.

1. Introduction

As plants are sessile organisms, their growth, development, and productivity are strongly influenced by an array of environmental stress conditions, including drought, salinity, and temperatures. These abiotic stresses can reduce crop yield by up to 70% (Agarwal et al., 2006), posing serious challenges to the food supply for our ever expanding population. It is estimated that salinity affects nearly 400 million ha of land worldwide, accounting for approximately 3% of the world's cultivated area. In addition, many agricultural lands are exposed to secondary salinity as a result of land clearing and irrigation (Munns, 2005). Both natural and secondary salinity are among the most devastating conditions for agricultural production. Therefore, it is necessary to enhance salt tolerance of the crops in order to use new lands for cultivation and improve crop productivity.

The presence of high concentrations of NaCl in plant cells disturbs the kinetic steady state of Na^+ and Cl^- transport. This results in accumulation of these two ions at high concentrations in the apoplast, giving rise to hyperosmotic stress, ionic imbalance, and toxicity (Niu et al., 1995; Zhu, 2002). In this context, high salinity restricts uptake of water, leading to intracellular water loss, which may eventually alter the osmotic potential of the salt-exposed cells (Türkan and Demiral, 2009).

In the course of evolution, plants have developed an arsenal of elaborate defense mechanisms so as to adapt to unfavorable situations including salt stress. Plants respond to abiotic stresses by initiating cellular, physiological, and biochemical modifications. One of these involves the production and accumulation of small organic compounds called compatible solutes. The production of these compounds, which are also known as osmolytes or osmoprotectants, is a typical plant response to salt stress (Sahi et al., 2006). The osmolytes function to adjust the osmotic potential. They

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are thus essential for the coordinated regulation of vacuolar and cytoplasmic volume, which is important for maintaining cell turgor upon exposure to salt stress (Munns, 2005). Moreover, these compounds play key roles in the protection of various cellular structures and proteins required for sustaining normal physiological activity (Serraj and Sinclair, 2002; Ashraf and Foolad, 2007). On the basis of these understandings, a rationale for enhancing stress tolerance is to increase the cellular content of osmolytes by manipulating the rate-limiting enzymes involved in the synthesis of these compounds.

So far, various compounds that are produced at high levels under stress conditions have been suggested to act as osmolytes, including sugars, sugar alcohols, complex sugars, quaternary amino acid derivatives, and tertiary amines. Of these, glycinebetaine (*N*,*N*,*N*-trimethylglycine, GB), a fully *N*-methyl-substituted derivative of glycine, was demonstrated to be an important protectant against a variety of abiotic stresses in plants. This conclusion is based on several lines of evidence. First, under stress conditions, the biosynthesis of GB is upregulated in many plants, and the compound accumulates to high levels (Rhodes and Hanson, 1993; Ashraf and Foolad, 2007; Chen and Murata, 2011). Second, exogenous application of GB, or introduction of GB biosynthesis-related genes enhanced stress tolerance in a number of plant species (Jia et al., 2002; Kumar et al., 2004; Su et al., 2006; Yang et al., 2008; Chen and Murata, 2008; Abbas et al., 2010). Third, specific inhibition of *BADH* gene is associated with reduction of stress tolerance. Niu et al. (2008) have shown that transgenic non-fragrant rice with inhibited expression of *BADH2* by RNAi was more susceptible to salt stress than WT rice with normal *BADH2* expression. Recently, Fitzgerald et al. (2010) reported that under salt stress the yield of fragrant rice, which contains a loss-of-function deletion of *BADH2* gene, was significantly lower than that of non-fragrant rice possessing functional *BADH2* gene. In plants, GB is synthesized by a two-step process of oxidation of choline, catalyzed by choline monooxygenase and betaine aldehyde dehydrogenase (BADH) (Hanson et al., 1985; Ashraf and Foolad, 2007). BADH is the enzyme responsible for the direct synthesis of GB, and has long been a subject of genetic engineering with the intention to increase GB production in response to stress. Several species have been used to produce transgenic plants overexpressing *BADH* genes. In these studies, the transgenic plants accumulated GB to different levels and exhibited enhanced tolerance to heat (Yang et al., 2005), salinity (Zhou et al., 2007; Hasthanasombut et al., 2010; Liu et al., 2011), or to multiple stress conditions (Kishitani et al., 2000). These data suggest that increasing GB levels via genetic engineering of *BADH* genes might be effective in enhancing stress tolerance.

Trifoliate orange (*Poncirus trifoliate* L. Raf.) is an important rootstock widely used for citrus because of certain favorable traits, including cold tolerance and resistance to citrus tristeza virus. Nevertheless, sensitivity to salt stress impedes its use in areas with saline soil (Storey and Walker, 1999). Previously, attempts to generate salt-tolerant trifoliate orange plants included cross hybridization (Ream and Furr, 1976), *in vitro* mutagenesis (Matsumoto and Yamaguchi, 1984; Deng et al., 1990; García-Agustin and Primo-Millo, 1995), and somaclonal selection (Ben-Hayyim et al., 1987; García-Agustin and Primo-Millo, 1995). Unfortunately, these attempts have not been translated into increased resistance of whole plants (Storey and Walker, 1999). Recently, exogenous application of abscisic acid (Gómez-Cadenas et al., 2002), nitrate (Iglesias et al., 2004), and nitrogen fertilizers (Gimeno et al., 2009) and the use of arbuscular mycorrhizal fungus (Wu et al., 2010) were reported to enhance salt stress tolerance in citrus/trifoliate orange. The genetic transformation of trifoliate orange for this purpose was reported previously only by Cervera et al. (2000) who transformed the *HAL2* gene into Carrizo citrange, a hybrid of trifoliate orange. Unfortunately, the transgenic plants did not display increased tolerance to salt stress. Although GB has proven effective in other plants, to our knowledge, information regarding trifoliate orange is scarce; whether trifoliate orange accumulates GB under salt stress, and whether genetic transformation of the *BADH* gene promotes GB synthesis and is able to increase salt tolerance in this tree plant, remains unclear. However, based on previous data (Chen and Murata, 2011), we speculate that overproduction of GB may lead to enhanced salt stress tolerance in trifoliate orange. To provide clarification, we measured endogenous GB in trifoliate orange plants exposed to salt and generated and characterized transgenic plants via *Agrobacterium tumefaciens*-mediated transformation of the *BADH* gene cloned from *Atriplex hortensis*.

2. Materials and methods

2.1. Plant materials

In order to obtain *in vitro* materials for genetic transformation, seeds from mature fruits of trifoliate orange were first immersed in 1 M NaOH for 15 min to remove pectins, and then sterilized with 2.5% (v/v) sodium hypochlorite solution for 20 min, followed by three rinses in sterilized water. After removing the seed coats, the seeds were placed in 150 mm × 25 mm test tubes containing 25 mL of MT basal medium (Murashige and Tucker, 1969), supplemented with 35 g/L sucrose, and solidified with 7.5 g/L agar. Unless otherwise stated, the pH value of the media used in this study was adjusted to 5.8. The cultures were maintained at 26 ± 1 °C in the dark for 20 days before they were transferred to a 16-h photoperiod (45 μmol m^{-2} s^{-1}). Ten days later, the stem segments were used as explants for transformation. In a separate experiment, uniform and healthy 10-month-old trifoliate orange seedlings, grown in a greenhouse, were collected for salt stress experiments as described below.

2.2. Agrobacterium-mediated transformation, selection, and regeneration of trifoliate orange plants

The *A. tumefaciens* strain LBA4404, a kind gift from Prof. Shou-Yi Chen (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences), carried the *pBin438* vector with the *AhBADH* gene isolated from *A. hortensis* and the neomycin phosphotransferase II (*NPTII*) gene under the control of a CaMV35S promoter. Prior to transformation, the strain was cultured overnight at 28 °C on 20 mL of solid LB medium (10 g/L yeast extract, 5 g/L tryptone, 10 g/L NaCl, and 15 g/L agar) supplemented with 50 mg/L kanamycin (Km). A single colony was inoculated in fresh liquid LB medium containing the same antibiotic and the culture was incubated at 28 °C for 2 days. The bacterial culture was centrifuged and the pellet suspended in sterile medium composed of liquid MT supplemented with 50 g/L sucrose, 0.5 g/L malt extract, 1.5 g/L L-glutamine, and 20 mg/L acetosyringone. The suspension was incubated on a shaker at 200 rpm and at 28 °C, until the OD_{600} of the *Agrobacterium* concentration reached 0.6–0.8.

Transformation and regeneration were carried out as described by Tong et al. (2009) with minor modifications. The stems from the 30-day-old seedlings were cut obliquely into 1-cm segments, which were immersed in the suspension medium for 20 min. The segments were blotted dry on sterile soft absorbent paper, and placed horizontally on a co-cultivation medium (CM) containing MT medium, 1.0 mg/L benzylaminopurine (BA), 0.5 mg/L indoleacetic acid (IAA), and 20 mg/L acetosyringone for 3 days in the dark at 22 °C. After co-cultivation, the explants were washed three times in sterile distilled water and blotted dry on soft absorbent paper, and subsequently transferred to shoot regeneration medium consisting of CM (without acetosyringone), 400 mg/L cefotaxime,