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Transformation of *Cichorium intybus* with the *HvBADH1* gene enhanced the salinity tolerance of the transformants

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ABSTRACT

Drought, salinity, and freezing are three major abiotic stresses that adversely affect plant growth and crop yield. Previous reports have demonstrated that the overexpression of the betaine aldehyde dehydrogenase (BADH) gene can improve the tolerance of plants to osmotic and salinity stresses. In the present study, a novel and atypical BADH gene, *HvBADH1*, was transferred into *Cichorium intybus* by an *Agrobacterium tumefaciens*-mediated method. The integration and expression of *HvBADH1* in the transformants were confirmed by PCR, Southern blot, and RT-PCR analysis. The stress tolerance traits of the transgenic plants were investigated by evaluating the phenotype of the transformed and wild-type (WT) plants, by measuring seven physiological indicators associated with stress resistance in plants, such as the K^+ and Na^+ contents, K^+/Na^+ ratio, malondialdehyde (MDA) content, chlorophyll content, the content of glycine betaine (GB), and relative conductivity in the leaves of the plants. The results revealed that the transgenic tissues had a better K^+ retention ability, a reduced sensitivity to stress-induced hydroxyl radical production, an enhanced protection of the photosynthetic system, 4.92–6.95-fold greater amount of GB, and a higher growth rate compared with the WT plant. Accordingly, we concluded that the overexpression of *HvBADH1* not only enhanced the salt tolerance of transgenic *Cichorium intybus*, but also reduced the cellular membrane damage caused by the salinity stress.

1. Introduction

Cichorium intybus L. (also known as chicory) is a short-term (2–5 years) perennial herb native to Europe, temperate and tropical Asia, and North Africa (Atta ur et al., 2008). Besides being a vegetable eaten in Asia, America, Africa, and parts of Europe (Luzina, 2013), this nutritious and high-quality forage is also considered to be a potential feed resource in livestock feeding (Liu et al., 2013). The contents of aesculetin and lactucopicrin in *Cichorium intybus* L. play an important physiological role in the plant defense against pathogens (Wulfkuehler et al., 2013), and they are also responsible for some remarkable beneficial effects on the consumers, such as antimalarial (Bischoff et al., 2004), promoting the digestive function of organs (Liu et al., 2012), and clearing the liver. These and other benefits attributed to chicory have prompted the use of the whole plant in European, Indian, and Chinese folk medicine, and its roots are used as a coffee substitute and additive. Researchers studying *Cichorium intybus* L. have focused their attention on pharmacological (Zhang et al., 2014) or agronomy research to promote biomass by developing cultivation techniques (Mathieu et al., 2014). Less attention has been given to improving the productivity of *Cichorium intybus* L. by genetic engineering approaches (Matveeva et al., 2011a, 2011b).

When some species of higher plants, *E. coli*, and yeast cells encounter abiotic stresses, such as salinity, osmotic, or low-temperature stresses, they often respond by accumulating in their cells glycine betaine (GB), a quaternary ammonium compound which acts as an effective compatible osmolytes. Indeed, since GB has a pivotal role in stabilizing the structure of proteins, maintaining osmotic balance of protoplasts, protecting some important enzymes in the abovementioned cells, it has been considered as one of the most important osmotic protective agents in many studies (Liu et al., 2011; Metris et al., 2014; Wani et al., 2013). Thus, an effective way to promote stress tolerance in plants is by over-expressing the GB synthetase genes in target plants. In higher plants, the biosynthesis of GB is carried out in a two-step reaction. The first step is catalyzed by choline monooxygenase (CMO), while the second step is catalyzed by betaine aldehyde dehydrogenase (BADH) (Ashraf & Foolad, 2007). In the plant species which can synthesize GB naturally, such as Barley (Ishitani et al., 1995) and Sorghum (Wood et al., 1996), the endogenous BADH level may be rapidly up-regulated by several-fold as a physiological response to the environmental salt and drought stresses. Meanwhile, after transformation by the exogenous BADH genes, the betaine-deficient plants exhibited an enhanced ability to accumulate GB in cells at different levels and showed an increased tolerance to harsh environmental stresses (Singh et al., 2010). This indicates that new plant germplasm resources with enhanced resistance to stresses may be created by transferring only one or a limited

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set of genes into the target commercial crops. Even though the extreme environment of the Tibetan Plateau in China, due to its very high average altitude (Wang et al., 2009), makes it one of the harshest living conditions for plants on the earth. This plateau is the original habitat of cultivated barley and many wild barleys. Due to a unique morphological trait, its caryopsis is bared, Tibetan hulless barley (*Hordeu vulgare* L. var. *nudum*. hook. f.) differs from the regular cultivated barley with a hull-covered caryopsis. In fact, as Tibetan hulless barley features prominently in the diet of the residents of this region, it is the most important cultivated crop, even an irreplaceable cereal, in some regions (Thomason et al., 2009). Moreover, compared to the normal cultivated barley and all other plants within the genus Hordeum, the hulless barley has developed a stronger resistance system to deal with general environmental stresses. However, almost all the detail information about how this stress defense system is organized and operated remains unknown. Noteworthy, *HvBADH1* is the first reported salt stress resistant gene and the only BADH cloned from Tibetan hulless barley, though the existence of two BADH genes, namely, a peroxisomal BADH (BBD_1) and a cytosolic BADH (BBD_2), were reported in the model barley species *Hordeum vulgare* L (Fujiwara et al., 2008).

In this study, a cDNA copy of *HvBADH1* (EF492983,1512 bp), which encodes the BADH protein from Tibetan hulless barley, was transferred into the genome of *Cichorium intybus* L. via an *Agrobacterium tumefaciens*-mediated method. The integration and expression of exogenous *HvBADH1* gene in the transformants were assessed by PCR, Southern blot, and RT-PCR analysis. To evaluate the effect of *HvBADH1* overexpression on the salt stress resistance traits of transgenic seedlings, several physiological indicators were examined, including the Na^+ and K^+ contents, K^+/Na^+ ratio, malondialdehyde (MDA), chlorophyll and glycine betaine (GB) contents, as well as the relative electrical conductivity (REC). In addition, the growth test of calli from transgenic and WT lines were performed to understand the effect of overexpression of *HvBADH1* on cell growth.

2. Material and methods

2.1. Plant material

The seeds of chicory were surface sterilized with 0.1% $HgCl_2$, placed on Murashige and Skoog (MS) agar medium, and subsequently germinated by incubation at 25 ± 2 °C under a 16 h · d^{-1}, 36 μmol · $(s \cdot m^2)^{-1}$ photoperiod provided by a cool white florescence lamps, and 50% humidity. After 2 weeks, the fully expanded cotyledons from the seedlings were used as explants for subsequent tissue culture and transformation. The seeds of hulless barley were surface sterilized with 0.1% $HgCl_2$, and then germinated on water soaked filter paper for 7 d. The seedlings were treated with 200 mmol · L^{-1} NaCl at room temperature for 72 h, and then the leaves of hulless barley were frozen in liquid nitrogen and preserved at −70 °C until used for gene cloning.

2.2. Construction of the HvBADH1 overexpression vector

The total RNA of the salt stressed hulless barley seedlings was isolated using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instruction. Then, the genomic DNA was removed by DNase I (Takara, Dalian, LN, China). A Revert Aid H Minus First Strand cDNA Synthesis kit 1631 (Thermo Fisher, Waltham, MA, USA) was used to synthesize the first strand of the cDNA, which was then used as template for *HvBADH1* amplification by PCR. A pair of primers, to perform the PCR amplification, was designed according to the reported sequence of *HvBADH1* (GenBank No. EF492983), the nucleotide sequences of these primers are: 5′-Cg**ggATCC(cleavage site of *Bam*H I)**ATggTCgCgCCggCCAAgATCC-3, and 5′-gC**TCTAgA(cleavage site of *Xba* I)**CTAgCCggAgCCTTgTAC-3. The PCR product corresponding to the coding region of *HvBADH1* was purified from the agarose gel, and its nucleotide sequence was determined. Then under the control of a CaMV35S promoter and a NOS poly A terminator, the cloned *HvBADH1* expression cassette was introduced into the T-DNA region of the pCAMBIA1301 binary vector (Cambia, Canberra, Australia), containing the selectable marker gene *hpt* II, which confers hygromycin B (Hyg) resistance to the transgenic plants. This vector construct, designated as pCAM-ba, was transferred into the cell of the *Agrobacterium tumefaciens* strain LBA4404 (Takara) by the freeze-thaw method to generate the *Agrobacterium* LBA4404 (pCAM-ba) for subsequent plant transformation.

2.3. Development of transgenic chicory plants

Agrobacterium LBA4404 (pCAM-ba) was incubated in liquid YEB medium containing 50 mg · L^{-1} kanamycin, 25 mg · L^{-1} rifampicin, and 100 mg · L^{-1} streptomycin, at 28 °C overnight with gentle shaking. One mL aliquots of bacterial cultures were transferred into100 mL fresh liquid LB medium and cultured for 6–8 h until the OD_{600} reached 0.5. Next, the bacterial cells were harvested and resuspended in MS liquid medium, with an OD_{600} of approximately 0.35. The cotyledon discs of the chicory seedlings with a diameter of about 5 mm were immersed in the *Agrobacterium* LBA4404 (pCAM-ba) suspension for 30 min at 25 °C in the dark, and then the excess bacteria on the explants surface were removed with sterilized filter paper. The explants were co-cultured at 25 °C in the dark for 3–4 d on MS_1 agar medium, which contained MS basal medium supplemented with 30 g · L^{-1} sucrose, 1.5 mg · L^{-1} 6-BA, 0.2 mg · L^{-1} NAA, and 100 mg · L^{-1} ascorbic acid. After co-culture, the infected cotyledon discs were rinsed 3 times in sterilized water, and subcultured on MS_1 agar medium supplemented with 25 mg · L^{-1} Hyg and 500 mg · L^{-1} cefotaxime at 25 ± 2 °C under a 16 h · d^{-1}, 36 μmol · $(s \cdot m^2)^{-1}$ photoperiod, provided by a cool white florescence lamps. About 4 weeks later, the surviving shoots were transferred to root-inducing medium MS_2, which consisted of half-strength MS basal medium supplemented with 0.2 mg · L^{-1} IBA and 30 g · L^{-1} sucrose, 50 mg · L^{-1} Hyg and 250 mg · L^{-1} cefotaxime. All the Hyg-resistant plantlets were planted in soil and kept in a standard greenhouse.

After germination seedlings from the WT line and the transgenic lines H_1, H_2, and H_3 were planted in pots with normal garden soil for 60 d, at 25 °C, under 150 μE · m^{-2} · s^{-1} and 50% humidity. Then the seedlings of each strains were divided into 2 groups, one of which was irrigated with 150 mM NaCl, while the other was irrigated with tap water. Five days after the NaCl treatment, the morphological features of the plant aerial portions were recorded by a camera.

2.4. Molecular identification of the transgenic plants

Genomic DNA was extracted from the leaf tissue of the putative transgenic and WT chicory using the CTAB protocol (Murray & Thompson, 1980). To screen the transgenic plants, a pair of gene-specific primers, Qbsen1: 5′-GTGGCAAAAGTCCTATTGTAGTATT-3′ and Qbantisen1: 5′-ATGCATCCCGCTTCGATC-3′, was used to perform PCR analysis to identify the transgenic plants, using the WT plants and the plasmid pCAM-ba as negative and positive controls, respectively. The PCR amplification conditions were 1 min pre-denaturation at 94 °C, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, elongation at 72 °C for 45 s, and a final extension step of 10 min at 72 °C.

The PCR-positive plants were further confirmed by Southern blot analysis. A 530-bp fragment amplified from *HvBADH1* was purified and used as probe. Incorporation of DIG-dUTP into the DNA probes and the detection of bound probes were performed using the DIG High Primer DNA Labeling and Detection Starter Kit II (Roche, Basel, Switzerland) following the supplier's protocol. About 30–40 μg of genomic DNA from transformant or WT plants was digested with BamH I, and then separated by electrophoresis on a 0.6% agarose gel. After transfer to a nylon membrane, the DNA fragments hybridization was performed by using the same kit as described in the probe labeling procedure.