Research article

Overexpression of codA gene confers enhanced tolerance to abiotic stresses in alfalfa

Hongbing Li a,b,1, Zhi Wang a,c,1, Qingbo Ke a,d, Chang Yoon Ji a,d, Jae Cheol Jeong a, Haeng-Soon Lee a,d, Yong Pyo Lim c, Bingcheng Xu b, Xi-Ping Deng b, Sang-Soo Kwak a,d, *

a Plant Systems Engineering Research Center, Korea Research Institute of Bioscience and Biotechnology (KRRIBB), Daejeon 305-806, Republic of Korea
b State Key Laboratory of Soil Erosion and Dryland Farming on the Loess Plateau, Institute of Soil and Water Conservation, Chinese Academy of Science and Ministry of Water Resources, Northwest A & F University, Yangling 712100, Shaanxi, PR China
c Department of Horticulture, Chunnam National University, Daejeon, Republic of Korea
d Department of Green Chemistry and Environmental Biotechnology, Korea University of Science & Technology, Daejeon, Republic of Korea

ARTICLE INFO

Article history:
Received 5 September 2014
Accepted 16 October 2014
Available online 18 October 2014

Keywords:
codA Glycinebetaine Alfalfa SWPA2 promoter Salt stress Drought stress Oxidative stress Biomass

ABSTRACT

We generated transgenic alfalfa plants (Medicago sativa L. cv. Xinjiang Daye) expressing a bacterial codA gene in chloroplasts under the control of the SWPA2 promoter (referred to as SC plants) and evaluated the plants under various abiotic stress conditions. Three transgenic plants (SC7, SC8, and SC9) were selected for further characterization based on the strong expression levels of codA in response to methyl viologen (MV)-mediated oxidative stress. SC plants showed enhanced tolerance to NaCl and drought stress on the whole plant level due to induced expression of codA. When plants were subjected to 250 mM NaCl treatment for 2 weeks, SC7 and SC8 plants maintained higher chlorophyll contents and lower malondialdehyde levels than non-transgenic (NT) plants. Under drought stress conditions, all SC plants showed enhanced tolerance to drought stress through maintaining high relative water contents and increased levels of glycinebetaine and proline compared to NT plants. Under normal conditions, SC plants exhibited increased growth due to increased expression of auxin-related IAA genes compared to NT plants. These results suggest that the SC plants generated in this study will be useful for enhanced biomass production on global marginal lands, such as high salinity and arid lands, yielding a sustainable agricultural product.

© 2014 Elsevier Masson SAS. All rights reserved.

1. Introduction

Environmental stress, including drought and high salinity, is one of the most important agricultural problems affecting plant growth and crop yield. It is estimated that arid and semi-arid regions account for approximately 30% of the total worldwide area and that nearly half of all irrigated lands are salt-affected (Sivakumar et al., 2005; Mali et al., 2012). As the world population is increasing fastly and is expected to reach 9 billion by 2050 (Varshney et al., 2011), the amount of available agricultural land is shrinking due to urbanization, industrialization, desertification, climatic change, and/or habitat use. Thus, there is a need to utilize salt- and drought-affected land to meet world food and energy requirements (Reguera et al., 2012).

Plants have evolved built-in mechanisms to cope with different types of abiotic and biotic stresses imposed by unfavorable environments (Vij and Tyagi, 2007; Kathuria et al., 2009). One of the basic strategies adopted by most plants is the accumulation of low molecular weight water-soluble compounds known as “compatible solutes” (Yancey et al., 1982; Bohnert et al., 1995; Chen and Murata, 2002). In general, these compounds accumulate to high concentrations under water or salt stress and protect plants from stress through different modes of action, including contributing to cellular osmotic adjustment, detoxifying reactive oxygen species (ROS), protecting membrane integrity, and stabilizing enzymes/proteins (Yancey et al., 1982; Bohnert and Jensen, 1996).

Among compatible solutes, glycinebetaine (GB) is a particularly effective protectant against abiotic stresses (Chen and Murata,
2008). GB is a quaternary amine that accumulates in a variety of plants, animals, and microorganisms in response to abiotic stress (Rhodes and Hanson, 1993; Chen and Murata, 2008). Levels of accumulated GB are generally correlated with the extent of stress tolerance (Rhodes and Hanson, 1993). GB effectively stabilizes the quaternary structures of enzymes and complex proteins, and it maintains the highly ordered state of membranes at non-physiological temperatures and salt concentrations (Papageorgiou and Murata, 1995). In photosynthetic systems, GB stabilizes the oxygen-evolving photosystem II protein–pigment complex under high NaCl concentrations (Murata et al., 1992; Papageorgiou and Murata, 1995). GB can accumulate rapidly in GB-synthesizing plants in response to environmental stresses, including salinity, drought, and low temperature (Rhodes and Hanson, 1993). Exogenous application of GB improves the growth and survival rate of plants under a variety of stress conditions (Park et al., 2004; Chen and Murata, 2008). Studies of GB have focused on GB-mediated tolerance to various types of stress and at various stages of the plant lifecycle (Sulpice et al., 2003; Park et al., 2004).

Increasing evidence from a series of in vivo and in vitro studies of the physiology, biochemistry, genetics, and molecular biology of plants strongly suggests that GB performs an important function in plants subjected to environmental stresses (Sakamoto and Murata, 2000, 2002). Therefore, pathways for enhanced production of GB have been introduced into transgenic plants to impart stress tolerance (Sakamoto and Murata, 2000; Chen and Murata, 2002, 2008, 2011). The most successful genetic engineering of GB biosynthesis has been achieved by expressing choline oxidase in plants, which is encoded by a soil bacterial (Arthurbacter globiformis) codA gene and can directly convert choline into GB and H2O2 (Ikuta et al., 1977; Deshnium et al., 1995). This gene has been introduced into various plants, such as Arabidopsis (Hayashi et al., 1997; Alia et al., 1998; Sulpice et al., 2003), Brassica (Prasad et al., 2000), tomato (Park et al., 2004; Goel et al., 2011), rice (Mohanty et al., 2002; Su et al., 2006; Kathuria et al., 2009), maize (Quan et al., 2004), and potato (Ahmad et al., 2008, 2010). Most transgenic plants with the ability to synthesize GB show enhanced tolerance to various types of environmental stress at all stages of their lifecycle (Chen and Murata, 2008, 2011).

Alfalfa (Medicago sativa L.) is a perennial forage legume of great agronomic importance worldwide, which provides abundant forage for animals and can improve soil fertility. The deep root system of alfalfa can help prevent soil and water loss in dry lands (Osborn et al., 1997). As a perennial forage crop, alfalfa is a fairly hardy species and has a relatively high level of drought and salt tolerance compared with many food crops. Although alfalfa can be grown under slightly saline-alkaline conditions and in semi-arid areas, its productivity is significantly reduced under high salinity and drought stress conditions. Developing alfalfa germplasm with increased salt and drought tolerance would provide a long-term solution to this problem. Genetic engineering has proven to be a revolutionary technique for generating transgenic plants with enhanced tolerance to various abiotic stresses, as one can transfer a desired gene from any genetic resource and alter the expression of existing gene(s). Since the first report of genetic transformation in alfalfa (Deak et al., 1986), many transgenic alfalfa plants have been obtained by gene transfer methods (Avraham et al., 2005; Zhang et al., 2005; Ramon et al., 2009; Tang et al., 2013). However, there are no reports on the improvement of stress tolerance in alfalfa by expressing a bacterial codA gene, despite the fact that alfalfa is a GB accumulating plant (Wood et al., 1991). A robust expression system with an appropriate promoter is an important prerequisite for efficient expression of foreign genes in plant cells. We previously isolated an oxidative stress-inducible sweet potato peroxidase (SWPA2) promoter from cell cultures of sweet potato (Kim et al., 2003). The SWPA2 promoter exhibits higher expression after various stress treatments than the 35S promoter of the Cauliflower mosaic virus (CaMV 35S promoter) in transgenic tobacco and potato plants (Kim et al., 2003; Tang et al., 2006). In previous studies, we used the SWPA2 promoter to generate various transgenic plants with enhanced tolerance to various environmental stresses, including sweet potato, potato, tall fescue, and poplar (Tang et al., 2006; Lee et al., 2007; Lim et al., 2007; Kim et al., 2011).

In this study, we successfully targeted choline oxidase (codA) cDNA derived from A. globiformis to alfalfa (cv. Xinjiang Daye) chloroplasts under the control of the stress-inducible SWPA2 promoter. The overexpression of the codA transgene resulted in enhanced tolerance to NaCl and drought stress on the whole plant level. To the best of our knowledge, this is the first report regarding the engineering of a GB biosynthesis pathway in alfalfa plants.

2. Materials and methods

2.1. Plant material and plant expression vector

The Xinjiang Daye cultivar of alfalfa (Medicago sativa L.), referred to high stress resistance (Wang et al., 2009a, 2009b), was used for the generation of transgenic alfalfa plants. Alalfa seeds (cv. Xinjiang Daye) were provided by Prof. Bo Zhang from Xinjiang Agriculture University, China. The codA gene expression vector was constructed according to a previous description (Ahmad et al., 2008). The codA gene was kindly provided by Prof. Norio Murata, National Institute for Basic Science, Japan. In brief, the plant expression vector pGAH/codA containing the transit peptide (TP) and the nos terminator (T-nos) under the control of the CaMV 35S promoter (Hayashi et al., 1997) was constructed. The TP, codA, and nos terminator fragments were excised from pGAH/codA and ligated to the oxidative stress-inducible SWPA2 promoter (Kim et al., 2003), after which the cassette was cloned into the HindIII and EcoRI sites of the pCAMBIA2300 binary vector, with the NPTII gene serving as a selectable marker. The resulting vector was transformed into Agrobacterium tumefaciens (EHA105 strain) for alfalfa transformation.

2.2. Plant transformation and regeneration

The alfalfa seeds were surface-sterilized with 0.5% sodium hypochloride solution for 5 min, thoroughly rinsed 7–8 times with distilled water and germinated on Murashige and Skoog (MS) medium (pH 5.7) (Murashige and Skoog, 1962) with half-strength of vitamins and salt under a 16/8 h light/dark cycle, with a light intensity of 350 μmol m−2 s−1 and a relative humidity of 65% at 25 ± 2 °C. After 5 days of germination, the hypocotyls were utilized for plant transformation via A. tumefaciens (EHA105 strain) containing the SWPA2 promoter–codA cassette. The transformed cells were selected on Schenk and Hildebrandt (SH) medium (Schenk and Hildebrandt, 1972) containing 2.0 mg L−1 2, 4-dichlorophenoxyacetic acid (2, 4-D), 0.2 mg L−1 kinetin, 250 mg L−1 cefotaxime and 50 mg L−1 kanamycin. Shoots were regenerated from the calli by transferring to MS medium containing 1.0 mg L−1 benzylaminopurine (BAP), 0.3 mg L−1 1-naphthylacetic acid (NAA), 250 mg L−1 cefotaxime, and 50 mg L−1 kanamycin. During the experiments, the cultures were maintained in a culture room at 25 ± 2 °C under a 16 h photoperiod. Regenerated shoots were transferred to MS medium for rooting. The rooted plantlets were then acclimated in pots in the growth chamber for 1 week before they were transferred to soil.
2.3. Methyl viologen treatment and ion leakage analysis

The oxidative stress tolerance assay was conducted according to the method of Kwon et al. (2002). Six leaf discs (8 mm in diameter) collected from alfalfa plants at the same position were floated on a solution containing 0.4% (w/v) sorbitol and 5 μM MV, incubated in the dark for 12 h to allow diffusion of the MV into the leaves and then subjected to continuous light treatment (150 μmol m⁻² s⁻¹) at 25 °C. The loss of cytoplasmic solutes following MV treatment (based on the electrical conductance of the solution) was measured with an ion conductivity meter (model 455C, Istek Co., Seoul, Korea) over a time period ranging from 0 to 24 h and compared with the total conductivity of the solution following tissue destruction. The extent of cellular damage was quantified by ion leakage, which is a measure of membrane disruption.

2.4. Salt and drought stress treatments

Plants were propagated under sterile conditions in Petri dishes containing MS medium supplemented 100 mg L⁻¹ kanamycin. For stress tolerance evaluations, plantlets rooted in MS medium were transferred to pots filled with equal quantities of soil and grown in a growth chamber under a 16-h photoperiod at a light intensity of 100 μmol m⁻² s⁻¹ and 60% (w/v) relative humidity at 25 °C. For salt and drought stress analysis on the whole plant level, 4-week-old similarly sized alfalfa plants grown at 25 °C under a light intensity of 100 μmol m⁻² s⁻¹ were irrigated with 250 mM NaCl solution once every 3 days for 2 weeks and then with tap water for 1 week. The tolerance of transgenic plants to salt stress was estimated based on the contents of glycinebetaine (GB), chlorophyll, and malondialdehyde (MDA) in the leaves after treatment.

Drought stress was imposed by withholding the water supply to the plants, which were maintained at 25 °C under a light intensity of 100 μmol photons m⁻² s⁻¹. Before withholding the water supply, the plants were irrigated with similar quantities of water from trays placed underneath the pots for 1 week. After 1 week of water withholding, the plants were again irrigated and permitted to recover from the drought conditions. The degree of drought stress was assessed by measuring the free proline content and relative water content (RWC) in leaves after 5 days of water withholding. RWC (%) was measured using the procedures described by Ahmad et al. (2008). The following formula was utilized: RWC (%) = [(FW-DW)/(TW-DW)] × 100, in which FW = immediate weight of freshly collected leaves, TW = turgid weight of leaves after incubation in water for 6 h at 20 °C in the light, and DW = dry weight of the same leaves after drying at 80 °C for 48 h. RWC% was measured in the fourth fully expanded leaf from the shoot apical meristem.

2.5. PCR and RT-PCR analysis

Genomic DNA was extracted from leaves of alfalfa plants using the protocol described by Kim and Hamada (2005). The PCR was conducted with purified genomic DNA in PCR premix (Cat. no. K-2012, Bioneer, Korea) using two convergent primers complementary to the codA gene (Table 1). The amplification reactions consisted of 94 °C for 5 min (1 cycle), followed by 30 cycles (94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min) and a final extension cycle of 7 min at 72 °C. The PCR products were separated on a 1% agarose gel, stained with ethidium bromide, and visualized under UV. All subsequent experiments were conducted on cloned plants from the T₀-generation of transgenic plants.

Leaves from 4-week-old plants grown in soil were treated with 5 μM MV, 250 mM NaCl solution, or withdrawing water for 1 week to activate the SWP/A2 promoter and induce codA expression. Total RNA was extracted using the TRizol reagent (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase I to remove any contaminated genomic DNA. For semi-quantitative and quantitative expression analysis of codA, the actin gene and various IAA genes (MsIAA3, MsIAA5, MsIAA6, MsIAA7, and MsIAA16) (Wang et al., 2014) in alfalfa plants, total RNA (2 μg) was used to generate first-strand cDNA using an RT-PCR kit (Topscript™ RT DryMix) according to the manufacturer’s instructions. For the Semi-quantitative RT-PCR analysis, the PCR conditions were identical for both codA and actin as described in the genomic PCR analysis, except for the extension time 30-s/cycle. And the PCR products were separated and visualized as above description of genomic PCR analysis. Quantitative real-time PCR (q-RT-PCR) was performed in a fluorometric thermal cycler (DNA Engine Opticon 2, MJ Research, USA) using the fluorescent dye EverGreen according to the manufacturer’s instructions. Transcript levels were calculated relative to the controls. Data represent means and standard errors of three biological replicates. The expression levels of the codA, actin, and various IAA genes were analyzed by q-RT-PCR using the gene-specific primers listed in Table 1. The PCR product of each alfalfa IAA gene was confirmed by sequencing analysis and further analyzed through alignment with other known plant IAA genes (data not shown).

2.6. Relative chlorophyll content

Chlorophyll contents were measured with a portable chlorophyll meter (SPAD-502, Konica Minolta, Japan) from intact fully expanded fifth leaves (from the shoot apical meristem) of individual plants. The relative chlorophyll content after salt stress (250 mM NaCl) was determined via comparison with the chlorophyll content under growth chamber conditions with 16-h photoperiod at a light intensity of 100 μmol m⁻² s⁻¹ and 60% (w/v) relative humidity at 25 °C.

2.7. Glycinebetaine analysis

Four-week-old plants grown in soil were irrigated with 250 mM NaCl solution or subjected to water withholding for 1 week to induce the expression of codA. The samples were collected after 3 days and 5 days of salt and drought treatment, respectively. GB extraction was conducted as described by Park et al. (2004) and GB

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>codA (Genomic PCR)</td>
<td>GCTGCCGGCGACAGTTCA</td>
<td>TGGCGCTATCCGGGAGCT</td>
<td>1000</td>
</tr>
<tr>
<td>codA (RT-PCR)</td>
<td>GCTGCCGGCGACAGTTCA</td>
<td>TGGCGCTATCCGGGAGCT</td>
<td>122</td>
</tr>
<tr>
<td>MsIAA3 (KJ996098)</td>
<td>GCTGCCGGCGACAGTTCA</td>
<td>TGGCGCTATCCGGGAGCT</td>
<td>125</td>
</tr>
<tr>
<td>MsIAA5 (KJ95634)</td>
<td>GCTGCCGGCGACAGTTCA</td>
<td>TGGCGCTATCCGGGAGCT</td>
<td>119</td>
</tr>
<tr>
<td>MsIAA6 (KJ95635)</td>
<td>GCTGCCGGCGACAGTTCA</td>
<td>TGGCGCTATCCGGGAGCT</td>
<td>106</td>
</tr>
<tr>
<td>MsIAA7 (KJ95636)</td>
<td>GCTGCCGGCGACAGTTCA</td>
<td>TGGCGCTATCCGGGAGCT</td>
<td>143</td>
</tr>
<tr>
<td>MsIAA16 (KJ95637)</td>
<td>GCTGCCGGCGACAGTTCA</td>
<td>TGGCGCTATCCGGGAGCT</td>
<td>149</td>
</tr>
<tr>
<td>Actin (JQ028730.1)</td>
<td>GCTGCCGGCGACAGTTCA</td>
<td>TGGCGCTATCCGGGAGCT</td>
<td>200</td>
</tr>
</tbody>
</table>
was measured via UV-VIS spectrophotometry according to Zhou et al. (2008).

2.8. Malondialdehyde analysis

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) contents according to a modified thiobarbituric acid (TBA) method (Sunarpi et al., 2005). Approximately 0.1 g of leaf tissue was ground in 0.5 ml 10% trichloroacetic acid (TCA) using a mortar and pestle. The homogenate was centrifuged at 10,000 rpm for 20 min. The reaction mixture (containing 0.4 ml extract and 0.6 ml TBA) was heated at 100 °C for 30 min, quickly cooled on ice, and centrifuged again at 10,000 rpm for 20 min. The absorbances at 450, 532, and 600 nm were determined using an ultraviolet spectrophotometer (Spectronic, Genesys™2, USA). Three biological replicates were performed.

2.9. Free proline analysis

The free proline content of drought-treated plants was measured using a spectrophotometer according to the method of Bates et al. (1973). Leaf tissue (0.1 g) was homogenized in 3 ml of sulfosalicylic acid (3%) and centrifuged at 10,000 g for 15 min. Then, 1 ml of the supernatant was added to a test tube, along with 1 ml glacial acetic acid and 1 ml ninhydrin reagent. The reaction mixture was boiled in a water bath at 100 °C for 30 min. After cooling, 2 ml toluene was added to the reaction mixture, which was then vortexed for 30 s. The upper phase (containing proline) was measured with a spectrophotometer (UV-2550, Shimadzu, Japan) at 520 nm using toluene as the blank. The proline content (μg/g FW) was quantified by the ninhydrin acid reagent method using proline as the standard (Bates et al., 1973).

2.10. Statistical analysis

Data were statistically analyzed with Statistical Package for the Social Sciences (SPSS 16). Means were separated using Duncan’s multiple range test at \( p = 0.05 \).

3. Results

3.1. Generation of transgenic alfalfa plants

Transgenic alfalfa plants overexpressing codA in chloroplasts under the control of an oxidative stress-inducible SWPA2 promoter (referred to as SC plants) were successfully generated via Agrobacterium-mediated transformation. The plant expression construct included a gene encoding a transit peptide (TP), which facilitates the targeting of choline oxidase to the chloroplast (Fig. 1A). The putative regenerated alfalfa plants were selected on SH medium containing kanamycin. An initial screening of the putative transgenic alfalfa plants was implemented by genomic PCR analysis using codA-specific primers (Table 1). The results revealed that the recombinant codA gene had been integrated into the genomes of transgenic plants in each of the 12 independent lines,
whereas no integration was detected in the NT line (Fig. 1B). We propagated the transgenic plant lines in a plant growth chamber and subjected them to semi-quantitative RT-PCR analysis with a codA gene-specific primer using RNA from MV-treated leaf discs. After stress induction by MV, induced expression of codA was detected in the leaf disc of all 12 transgenic lines, but not in the NT plants (Fig. 1C). The strongest induction of codA expression was detected in lines SC7, SC8, and SC9 after MV treatment. Plants from these three transgenic lines (referred to as SC7, SC8, and SC9 plants) were selected for further characterization.

3.2. Enhanced growth and increased expression of auxin-related IAA genes in SC plants

When acclimated NT and SC plants were grown in soil for 1 month in the growth chamber, SC plants exhibited 1.26–1.37-fold higher plant height than NT plants (Fig. 2A). To determine whether the increased growth of SC plants was related to auxin biosynthesis, we investigated the expression of auxin-responsive IAA genes, including MsIAA3, MsIAA5, MsIAA6, MsIAA7, and MsIAA16. Under normal conditions, the SC plants showed increased transcript levels of five auxin-responsive IAA genes (Fig. 2B), indicating that overexpression of codA may play a positive role in an auxin transport-related signaling pathway.

3.3. Improved tolerance to MV-mediated oxidative stress

To evaluate the methyl viologen (MV) mediated-oxidative stress tolerance of SC plants, we treated leaf discs of 1 month old SC and NT plants with 5 μM MV solution for 24 h. MV is a non-selective herbicide that produces massive bursts of ROS, which disrupt membrane integrity and lead to cell death (Bowler et al., 1991). Different extents of visible damage were observed in leaf discs from SC and NT plants (Fig. 3A). Severe necrosis was observed in NT leaf discs, whereas SC plants exhibited less necrosis. The ion leakage level of transgenic SC7, SC8, and SC9 plants was 25%, 21%, and 27%, respectively, whereas the NT plants exhibited 43% ion leakage after 24 h of MV treatment (Fig. 3B). SC plants displayed significantly (p = 0.05) less ion leakage than NT plants under MV-stress treatment. Since the heterologous gene was driven by the stress-inducible SWPA2 promoter, we examined the expression patterns of codA after MV-triggered oxidative stress by q-RT-PCR. The transcript levels of codA were significantly higher in transgenic lines SC7, SC8, and SC9 than in NT under MV treatment (Fig. 3C), indicating that overexpression of codA increases the tolerance to MV-mediated oxidative stress.

3.4. Enhanced tolerance of SC plants to high salinity stress

To evaluate the tolerance of SC plants to salt stress, we subjected 1-month-old SC and NT plants to 250 mM NaCl treatment for 14 days. This treatment had a significantly smaller impact on the growth of SC plants versus NT plants. Before salt treatment, the health status of SC and NT plants were indistinguishable (Fig. 4A). However, NT plants exhibited severely inhibited growth with yellowish leaf coloration after salt treatment, and they were dead 2 weeks later, in contrast to the vigorous growth of SC plants (Fig. 4A). Since the heterologous gene was driven by the stress-inducible SWPA2 promoter, we examined the expression patterns of codA after salt treatment by q-RT-PCR. The transcript levels of codA significantly increased in transgenic SC7, SC8, and SC9 plants after salt treatment (Fig. 4B). To determine whether overexpression of codA increased the glycinebetaine (GB) contents after salt treatment, we measured the GB contents in leaves of alfalfa plants (Fig. 4C). Before salt treatment, the GB content in the NT leaves was 7.89 μmol g⁻¹, indicating that alfalfa is a natural accumulator of GB, whereas that in SC7, SC8, and SC9 plants was 8.8, 9.0, and 7.9 μmol g⁻¹ fresh weight, respectively. After salt treatment, SC7, SC8 and SC9 plants exhibited 1.76-, 1.17-, and 1.21-fold higher GB levels than NT plants, respectively.

Fig. 3. Effect of MV-mediated oxidative stress on the ion leakage of non-transgenic (NT) and transgenic (SC) plants at 0 and 24 h after 5 μM MV treatment. (A) Differential visible damage of leaf discs. (B) Relative membrane permeability. Percentages of relative membrane permeability were calculated using 100% to represent values obtained after autoclaving. (C) Transcript levels of codA gene expression. Total RNA was extracted after 0 and 24 h MV treatment. The expression level of codA was normalized to that of the alfalfa actin gene as the internal control. Data are expressed as the mean ± SD of three replicates. Asterisks indicate a significant difference between NT and SC plants at "p < 0.05" or "p < 0.01" by t-test.
We then investigated the chlorophyll and malondialdehyde (MDA) contents in plants before and after salt stress treatment, as these values represent important stress-related physiological and biochemical parameters. Before salt stress, there was no significant difference in chlorophyll content in the leaves of NT versus SC lines. However, after salt treatment, SC7 and SC8 plants maintained high levels of chlorophyll (3.5%–5.2% reduction) compared with NT plants (Fig. 4D), indicating that the photosystem of SC plants is less affected than that of NT plants during salt stress. To test the membrane stability of the plant lines, which represents the degree of cell membrane damage under stress, we determined the MDA contents in these plants. Before salt stress, the MDA contents in SC transgenic lines were similar to those of NT plants. Under 250 mM NaCl treatment for 24 h, the MDA contents of NT plants were greater than those of SC plants overexpressing codA (Fig. 4E).

3.5. Enhanced tolerance of SC plants to drought stress

To determine whether codA is involved in the drought tolerance of transgenic alfalfa, we investigated the performance of the codA-overexpressing alfalfa plants under drought stress. Under normal growth conditions, there were no significant differences in plant growth between NT plants and the three SC transgenic lines (Fig. 5A). After drought treatment for 7 days, SC plants grew well and only some leaves turned yellow, whereas NT plants showed severe wilting and curling. Following 3 days of dehydration after drought treatment, the NT plants were nearly dead, with no recovery observed, whereas SC8 and SC9 plants fully recovered from the dehydration conditions. The survival rates of transgenic plants after drought treatment were greater than those of NT plants (Fig. 5A). The transcript levels of codA in SC7, SC8, and SC9 lines significantly increased after drought treatment (Fig. 5B). The GB contents in the leaves of SC plants also significantly increased after drought stress (Fig. 5C). After drought stress, the GB contents in SC plants exhibited 1.21-, 1.39-, and 1.24-fold increases compared with those under normal conditions, whereas the GB contents in NT plants were unchanged.

We analyzed the degree of water loss in plants after drought stress. SC plants exhibited higher relative water content (RWC) values than NT plants under drought stress (Fig. 5D), suggesting that SC plants exhibited less water loss than NT plants during the dehydration process. We also measured the contents of proline, a type of compatible osmolyte, in SC and NT plants. Under control conditions, the free proline contents were not significantly different between NT and SC plants. However, after withholding water for 5 days, SC plants accumulated higher free proline levels than NT plants (Fig. 5E).

4. Discussion

This study demonstrates the successful transformation of an elite alfalfa cultivar, producing plants harboring the chloroplast-targeted codA gene under the control of the oxidative stress-inducible SWPA2 promoter. The transgenic alfalfa plants (SC plants) exhibited increased tolerance to oxidative stress, as well as salt and drought stress, via induced expression of codA. This
observation is consistent with the results of previous studies of the heterologous expression of bacterial codA in various transgenic plants (Hayashi et al., 1997; Alia et al., 1998; Sakamoto and Murata, 2000; Park et al., 2004, 2007; Ahmad et al., 2008, 2010; Kathuria et al., 2009; Goel et al., 2011). These results indicate that engineering increased GB biosynthesis in alfalfa is a feasible approach to improving its tolerance to multiple abiotic stresses.

Many approaches have been used to engineer stress tolerance in plants, with limited success (Park and Chen, 2006). One major problem that this type of engineering can lead to undesirable or unfavorable phenotypic changes, such as growth retardation and decreased yields. To date, only positive effects of GB accumulation in transgenic plants have been reported, even in plants grown under non-stress conditions (Sulpice et al., 2003; Chen and Murata, 2011). In addition, exogenous treatment of GB can increase plant growth and yield (Xing and Rajashekar, 1999). Moreover, the codA transgenic plants exhibited increased production of flowers and seeds, even the size of flower buds and fruit compared with wild-type plants (Park et al., 2007b; Chen and Murata, 2008). Under stressful conditions, the performance of many types of transgenic plants is better than that of untransformed controls, suggesting that this technique has great potential for agricultural applications (Park et al., 2007a; Chen and Murata, 2008, 2011). The plant hormone auxin regulates a number of cellular and developmental processes, including cell division, cell growth, and differentiation (Friml, 2003). At the molecular level, auxin exerts its function by regulating the expression of numerous auxin-responsive genes, including AUXIAA genes. To confirm whether the increased growth of SC plants was related to the expression of auxin-responsive genes, we investigate the expression of MsIAA genes in these plants. Interestingly, the expression of auxin-responsive MsIAA genes, including MsIAA3, MsIAA5, MsIAA6, MsIAA7, and MsIAA16, was significantly up-regulated at the transcriptional level under normal growth conditions (Fig. 2B). However, the relationship of codA to auxin biosynthesis remains to be clarified to elucidate the possible mode of action of choline oxidase.
Transgenic lines harboring codA exhibited enhanced tolerance to a variety of stresses. As the codA gene was driven by a stress-inducible promoter, the induction patterns of codA under different stress conditions, including MV, salt and drought, verify the stable integration and transcription of foreign genes in SC plants (Figs. 3C, 4B and 5B).

Abiotic stresses, such as salinity and drought, which disturb redox homeostasis in plant cells, can induce oxidative stress by inducing a burst of ROS. ROS have been implicated in all types of stresses, as they can damage lipids, proteins, DNA, and carbohydrates. If ROS are not scavenged sufficiently in the proper period of time, senescence and programmed cell death might occur (Apel and Hirt, 2004; Miller et al., 2010). On the other hand, scavenging too many ROS may negatively affect signal transduction. Therefore, balancing ROS generation and scavenging is essential for the stress response in plants. Despite the occurrence of GB-mediated enhancement of stress tolerance in many plants, its mechanistic influence on stress signaling and perception remains unclear due to a lack of direct evidence supporting the participation of GB in antioxidant defense systems. Studies have demonstrated that GB alone does not have antioxidative activity in vitro (Smirnoff and Cumbes, 1989). Thus, its ROS-scavenging function must be indirect. The present results, which demonstrate that the transgenic plants had enhanced stress tolerance, as evidenced by the lower extent of visible damage in leaf discs and lower levels of ion leakage under challenging conditions imposed by 5 μM MV-mediated oxidative stress (Fig. 3), are consistent with previous findings.

Salt stress causes ROS production, which results in the accumulation of MDA in plants due to membrane lipid peroxidation (Gill and Tuteja, 2010). Oxidative stress-induced membrane damage and cell membrane stability have been used as efficient criteria to assess the degree of salt tolerance in plants (Meloni et al., 2003; Sairam et al., 2005). Our results show that the MDA contents were lower in transgenic alfalfa than in NT plants under salt stress conditions (Fig. 4E). These results imply that the degree of membrane injury in transgenic plants was less than that of NT plants, which is consistent with the enhanced salt tolerance phenotype of transgenic alfalfa.

Under stress conditions, alfalfa plants instinctively synthesize and accumulate more GB, since alfalfa is a natural GB accumulator. Both the SC and NT plants accumulated GB to similar levels before stress treatment (Figs. 4C and 5C). The GB content in SC plants significantly increased under salt and drought stress due to the anticipated induction of codA transcriptional expression under the control of stress-inducible SWPA2 promoter. The increased GB contents in the SC plants contributed to their enhanced tolerance to oxidative, salt, and drought stresses. A series of studies using transgenic plants have demonstrated that GB, even at low levels, can confer transgenic plants with increased tolerance to cold, freezing, drought, and salt stress (Chen and Murata, 2008, 2011). GB levels as low as 0.09 μmol g⁻¹ FW can impart chilling stress tolerance in tomato plants (Park et al., 2004), and 0.035 μmol g⁻¹ FW GB is sufficient to impart salinity tolerance in transgenic tobacco plants (Holmstrom et al., 2000). Many studies have demonstrated that GB synthesized in chloroplasts is more effective at imparting stress tolerance in transgenic plants that GB synthesized elsewhere (Park et al., 2007a). We assume that in the current study, codA, which functions in GB synthesis, was successfully targeted to the alfalfa chloroplasts, because the same transit peptide was utilized as that reported by Hayashi et al. (1997) and Ahmad et al. (2008). Mechanistically, GB stabilizes macromolecular activity and membrane integrity (Sakamoto and Murata, 2002). Indeed, we detected reduced ion leakage and MDA content in the leaf cells of codA transgenic alfalfa (Figs. 3B and 4E) in this study, suggesting improved cell membrane homeostasis in SC plants. Therefore, it can be assumed that GB protects the cell membrane from stress-induced injuries, likely through alleviating oxidative stress.

Transgenic tomato plants that synthesize GB in the chloroplast exhibit higher levels of photosynthetic activity than wild-type plants under salt stress conditions (Park et al., 2007a). Chloroplasts are the major sites of photo oxidative reactions, which abundantly produce ROS. The protective effect conferred by GB guards the machinery required for the degradation and synthesis of D1 protein under stress exerted by high salt conditions (Ohnishi and Murata, 2006). In the current study, SC transgenic plants maintained higher levels of chlorophyll and salinity tolerance during the entire period of salt stress than NT plants (Fig. 4). Therefore, we can conclude that the higher photosynthetic activity maintained by SC plants versus NT plants was the result of GB synthesis in the chloroplasts, indicating that GB synthesized in chloroplasts protects not only the photosynthetic machinery but also the antioxidant defense mechanisms of plants.

There is evidence that high levels of salt cause an imbalance of cellular ions, leading to both ion toxicity and osmotic stress (Greenway and Munns, 1980). High salt concentrations make it more difficult for roots to take up water. Additionally, high salt concentrations within the plant can be toxic due to the disturbance of essential cellular metabolic pathways, such as protein synthesis and enzyme activity. Osmotic adjustments by accumulating osmoprotectants inside the cell are essential for reducing the cellular osmotic potential against an osmotic gradient between root cells and the outside saline solution, which eventually restores water uptake into roots during salinity stress (Greenway and Munns, 1980). When suffering from abiotic stresses, many plants accumulate compatible osmolytes, such as free proline and soluble sugar (Liu and Zhu, 1997; Armengaud et al., 2004). These osmolytes function as osmoprotectants, allowing the plants to tolerate stress. In this study, we examined the contents of the osmoprotectant proline in plants. The results showed that the contents of free proline were greater under drought stress conditions, and the free proline levels in codA-overexpressing transgenic plants were greater than those in NT plants (Fig. 5E). Thus, the increased accumulation of proline contributes to the increased drought tolerance of transgenic alfalfa.

GB is a compatible solute, also known as an osmolyte, which performs an important function in plants, as evidenced by their enhanced tolerance to osmotic stress. However, the increased level of GB in transgenic plants indicates that GB plays a role other than osmoprotection. A variety of studies have demonstrated that GB exerts protective effects and stabilizes macromolecules, enzyme activity, and membranes under stressful conditions (Papageorgiou and Murata, 1995; Chen and Murata, 2002; Sakamoto and Murata, 2002). Quan et al. (2004) reported that GB-synthesizing transgenic maize withstood drought conditions better than wild-type (WT) plants, even though the GB contents in the transgenic plants were significantly lower than those of natural accumulators. Moreover, transgenic tobacco plants evidencing GB-synthesizing ability also exhibit enhanced tolerance to polyethylene glycol (PEG)-mediated osmotic stress conditions (Shen et al., 2002). According to our data, GB-synthesizing alfalfa plants evidenced enhanced tolerance to drought stress via the maintenance of higher relative water contents and higher free proline contents than the NT plants, and the transgenic plants recovered normal growth after irrigation (Fig. 5).

In conclusion, GB accumulation in transgenic alfalfa plants by the heterologous overexpression of codA significantly improved plant tolerance to oxidative, salt, and drought stress, which was mediated by the induction or activation of GB synthesis, increased proline accumulation, and improved protection of membrane integrity. Thus, this study has increased our understanding of the
molecular mechanisms of GB function in plants, although some aspects are still unclear, such as how ROS affect the biosynthesis of GB and proline at the molecular level. The relationship between GB biosynthesis and the expression of auxin-related IAA genes remains to be studied. Nevertheless, the enhanced performance of these transgenic alfalfa lines without phenotypic defects demonstrates that engineering the GB biosynthetic pathway is not only a feasible approach to improving the economically important crop alfalfa, but it also holds great promise for breeding programs of other crops. In this study, transgenic alfalfa overexpressing codA was significantly more drought and salt tolerant than NT plants. We hope that the transgenic alfalfa plants generated in this study can be used not only as a forage crop but also as a crop for covering marginal lands such as salt-affected or dry lands.

Author contributions

S.S. Kwak, H. Li and Z. Wang: conceived and designed the experiments. Z. Wang, H. Li, Q. Ke, and J.C. Jeong: performed the experiments. J.C. Jeong, H.S. Lee, B. Xu, and Y.P. Lim: analyzed the data. X. Deng and H.S. Lee: contributed reagents/materials/analysis tools. H. Li, Z. Wang and S.S. Kwak: wrote the paper.

Acknowledgments

This work was supported by grants from the Korea-China International Collaboration Project, the National Research Foundation (NRF) of Korea, the National Natural Science Foundation of China (no.51479198), the 111 project of the Ministry of Education, China (No. B12007) and the National Center for GM Crops (PJ008097), BioGreen21 Project for Next Generation, Rural Development Administration, Korea.

References

Park, E.J., 2004. Tran-..


