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Physiological and biochemical changes of *CBF3* transgenic oat in response to salinity stress

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ABSTRACT

Salinity is a major abiotic constraint affecting oat productivity. Several physiological and biochemical traits have been found to be related to yield maintenance under salinity. The impact of introducing the *Arabidopsis CBF3* gene controlled by the rd29A stress-inducible promoter in T_2 transgenic oat on salinity tolerance and associated physiological changes were studied. Compared with the non-transgenic control, transgenic T_2 plants exhibited greater growth and showed significant maintenance of leaf area, relative water content, chlorophyll content, photosynthetic and transpiration rates as well as increased levels of proline and soluble sugars under high salt stress. These physiological changes delayed leaf-wilting symptoms, increased tolerance and reduced yield loss. At a salinity stress level of 100 mM, the *CBF3*-overexpressing transgenic oat showed a yield loss of 4–11% compared with >56% for the non-transgenic control. These results demonstrate that stress-inducible over-expression of *CBF3* may have the potential to enhance abiotic stress tolerance in oat.

1. Introduction

Plant development, growth and productivity are adversely influenced by environmental abiotic stresses such as salinity [1]. The excess of salts in soil or irrigation water exposes plant functions and metabolism to severe stress [2]. These conditions significantly decrease the yield of current crop species and limit their expansion, and constraint the introduction of new crop species [3].

Plant acclimation to salinity stress involves cascades of transcriptional control and genetic regulations at different molecular levels throughout the plant life cycle [4]. These changes in gene expression mediate a wide range of biochemical and physiological processes necessary to re-establish cellular homeostasis [5]. Recently, significant improvements in salinity tolerance have been achieved in different crops through traditional breeding [6]. However, progress remains slow due to many factors such as: (i) the complexity of the trait and inefficiency of selection methods, (ii) the limited information available regarding the inheritance of tolerance and selection indices, (iii) the lack of genetic variability in the crop's gene pool, (iv) the focus on improving yield and quality and (v) the inadequate understanding of genotype and stress interactions [5,7].

A promising approach for augmenting genetic improvement of stress tolerance involves the activation of stress-inducible signal transduction pathways in transgenic plants [8]. The downstream genes encode structural proteins or enzymes involved in the synthesis of metabolites that trigger different physiological responses [8,9]. A number of regulatory genes, associated with stress tolerance response, to be used for genetic transformation have been identified including the *Arabidopsis* CBF/DREB (cold-binding factor/dehydration responsive element binding) transcriptional factors family. These transcription factors contain an AP2/ERF (APETALA2/Etylen Responsive element binding Factor) DNA-binding domain that recognizes dehydration responsive/C-repeat (DRE/CRT) elements. These elements are present in many COR genes that regulate gene expression in response to abiotic stresses [10]. Overexpression of *CBF3* under the control of the CaMV 35S promoter was found to increase tolerance to drought, salinity, and freezing stresses. However, phenotypic abnormalities were apparent under normal growth conditions [11]. Therefore, the use of rd29A stress-inducible promoter was found to increase stress tolerance and minimize the negative effects of the CBF family including *CBF3* in different heterologous systems such as tobacco [12], sugarcane [13], maize [14], potato [15], peanut [16] and tall fescue [17].

Oat is an important cereal crop used in human and animal diets [18] as it has high contents of both phytochemicals and fibers

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[19]. Salinity stress is a major cause of oat crop yield loss [20]. Although oat is moderately salt tolerant compared with the other cereals [21], salinity decreases its seed germination, inhibits establishment, reduces almost all growth parameters and subsequently limits crop production in a genotype-dependent manner [22,23].

This paper describes the physiological and biochemical changes associated with the introduction of the *Arabidopsis CBF3* gene under the control of the rd29A stress-inducible promoter in transgenic oat under different salinity levels measured by various parameters.

2. Materials and methods

2.1. Construct and transformation

The plasmid pCAMBIA1201 vector containing the β-glucuronuridase (*gus*) and hygromycin (*hpt*) resistance genes was used. The *CBF3* open reading frame (ORF) was amplified from *Arabidopsis* (ecotype Columbia) genomic DNA by Polymerase Chain Reaction (PCR) using the F: 5′-GGATCCATGAACTCATTTTCTGC-TTTTTCT-3′, and R: 5′-GGTACCTTAATAACTCCATAACGATACGTC-3′ with a Bam*HI* and Kpn*I* sites added to the primers [14]. The PCR product was sequenced and cloned into the pCambia vector. Furthermore, a Hind*III* and Bam*HI* fragment of rd29A was cloned into the same pCambia vector generating the binary vector pCaM3RD (Fig. 1a). Oat transformation using the hexaploid Ogle wild-type cultivar was performed according to the protocol published by Gasparis et al. [24]. Selection using 20 mg/l hygromycin and monitoring using *gus* assay [25] were applied throughout callus proliferation, regeneration, plant growth and rooting.

2.2. Plant materials

Four PCR positive homozygous transgenic oat lines of the hexaploid Ogle cultivar (Agrogle 1, Agrogle 2, Agrogle 3 and Agrogle 4) were used in this study. The chosen independent lines were carrying a single copy of the *CBF3* gene. Seeds from independent T_1 plants derived from initial transformants showing 3:1 Mendelian segregation ratios and expressing *CBF3* gene were harvested separately. Approximately 50 T_2 seeds harvested from each T_1 plant in each line were germinated on medium containing 20 mg/l hygromycin. The full batch of the tested T_2 seeds derived from the same T_1 plant was discarded when any of these seeds failed to germinate. This screening process was continued repeatedly until all tested T_2 seeds harvested from one T_1 plant were germinated and produced seedlings. Non-transgenic control seeds were germinated on medium with and without hygromycin. The young positive hygromycin resistant T_2 as well as the control seedlings were analyzed by PCR and GUS staining, then transferred to soil and used for molecular analyses and evaluation into two greenhouses using salt treatments.

2.3. Molecular analyses

2.3.1. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from *CBF3* transgenic oat lines (T_2) and non-transgenic control (non-stressed and exposed to stress for two weeks) using Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was obtained using the SuperScript™ III One-Step RT-PCR with Platinum® Taq Polymerase (Invitrogen, Carlsbad, CA). The PCR program was as follows: 52 °C for 30 min; 94 °C for 2 min; 40 cycles of 94 °C (15 s), 56 °C (30 s), and 68 °C (1 min); and a final extension of 68 °C for 5 min. The following primers were used to amplify the *CBF3* gene F: 5′-AACTCATTTTCTGCTTTTTCTGAAATG-3′, and R: 5′-TTAATAACTCCATAACGATACGTC-3′. Oat β-actin was used as a housekeeping reference gene and was amplified using the following primers F: 5′-GAGCTACGAGCTTCCTGATGC-3′ and R: 5′-TCCACGTCGCACTTCATGA-3′. The PCR products were visualized and analyzed using the QIAxcel automated analysis of DNA fragments system. The PCR product size was approximately 648 bp for the *CBF3* gene and 150 bp for the oat β-actin.

2.3.2. Southern blot analysis

The independence of the lines and the confirmation of the *CBF3* transgene transmission into the oat T_2 transgenic plants were performed by Southern-blot hybridization using the *CBF3*-coding sequence as a probe. Genomic DNA from transgenic and non-transgenic control oat plants was isolated as described by [26]. About 15 μg of genomic DNA was digested with Kpn*I* restriction enzyme, electrophoresed in 0.8% (w/v) agarose gel, transferred onto Hybond-N+ (Amersham-Pharmacia Biotech) membranes, and fixed with a UV crosslinker (Stratalinker Crosslinker 1800, Stratagene, CA). Probe labeling and detection were obtained with the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Co., USA) following the manufacturer's protocol.

2.4. Salinity treatments

Salinity treatments were applied according to [27]. Briefly, young seedlings were individually transferred into Baccto Professional Planting Soil Mix (70–80% horticultural sphagnum peat, 20–30% perlite, pH 5.5–6.5) in small pots (8 cm × 6 cm × 4 cm). The pots were kept in water filled flat-bottom trays for 1 week. Two-week-old seedlings from each transgenic line and non-transgenic control were transferred to two-gallon pots for another week before starting the salt treatments. The environmental conditions in the greenhouses were set to 25 °C, with about ~50% relative humidity. Natural illumination was augmented for 16 h per day with fluorescent light levels of 800–900 $\mu mol/m^{-2}/s^{-1}$. Sodium chloride (NaCl) was added in five concentrations of 0, 100, 150, 200 and 250 mM. The plants were watered with the saline solution once a day for 2 weeks, followed by 1 week of plain water irrigation for plant recovery after the salt shock. Afterwards, the salt treatments were continued for 30 days.

2.5. Measurements of physiological variables

The physiological variables were measured at flowering.

2.5.1. Relative water content (RWC) and chlorophyll content

Leaf disks were weighed immediately after sampling for fresh weight (FW) determination and floated on distilled water for 16 h. Turgid leaf disks were then rapidly blotted to remove surface water and weighed to obtain turgid weight (TW). Leaf discs were dried in an oven at 60 °C for 48 h to obtain the dry weight (DW). The RWC was calculated using the formula given by [28]:

$$\mathrm{RWC}(\%) = \left[\frac{\mathrm{FW} - \mathrm{DW}}{\mathrm{TW} - \mathrm{DW}}\right] \times 100$$

For chlorophyll content measurement, the Minolta SPAD-502 chlorophyll meter was used according to [29], to acquire an estimate of the leaf chlorophyll content. Five measurements were made from the same leaf positions. The results were then averaged for each pot.

2.5.2. Water soluble carbohydrates and proline content

Water-soluble carbohydrates were measured on freeze-dried tissues. Samples of 200 mg dry weight were extracted according to [30]. The determination was based on the phenol-sulfuric acid method [31]. The amount of sugars was determined by reference to a standard curve prepared with D-glucose solutions. Proline content was measured on leaves of the same age and size. Proline was