Overexpression of *Arabidopsis* Dehydration-Responsive Element-Binding Protein 2A Confers Tolerance to Salinity Stress to Transgenic Canola

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**Abstract:** Stress responsive transcriptional regulation is an adaptive strategy of plants that alleviates the adverse effects of environmental stresses. The ectopic overexpression of Dehydration-Responsive Element Binding transcription factors (DREBs) either in homologous or in heterologous plants are the classical transcriptional regulators involved in plant responses to drought, salt and cold stresses. To elucidate the transcriptional mechanism associated with the *DREB2A* gene after removing PEST sequence, which acts as a signal peptide for protein degradation, 34 transgenic T₂ canola plants overexpressing *DREB2A* were developed. The quantitative Real time PCR of transgenic plants showed higher expression of downstream stress-responsive genes including *COR14, HSF3, HSP70, PEROX* and *RD20.* The transgenic plants exhibited enhanced tolerance to salt stress. At the high concentration of NaCl the growth of non-transformed plants had been clearly diminished, whereas transgenic line was survived. These results indicated that transformed *DREB2A* gene might improve the plant response to salinity in transgenic canola plants.

**Key words:** *DREB2A*, transcription factor, transgenics, overexpression, salinity stress, canola

**INTRODUCTION**

Various environmental stresses, such as salinity, drought, flooding and freezing cause metabolic toxicity, membrane disorganization, closure of stomata, decreased photosynthetic activity, generation of Reactive Oxygen Species (ROS) and altered nutrient acquisition (Mittler, 2006; Salavati *et al.*, 2012a, b). These stresses, subsequently, have adverse effects on plant growth and crop yields. Plants have developed a complex and sophisticated signalling network that ensures their adaptation to continuously changing surroundings (Moller and Sweetlove, 2010). To meet the increasing demands for agricultural commodities it would be urgent to redesigning of crops to cope with environmental stresses. Accordingly, a great deal of effort has been invested in perception the molecular background of stress adaptation by which plants recognize and transfer adverse environmental stimuli to trigger adaptive responses (Xiong *et al.*, 2002). In addition, great deal of effort has been invested in creating improved plants that show increased tolerance (Wang *et al.*, 2003). Adaptation mechanisms such as re-establishment of cellular homeostasis and mitigating the effects of stress-induced toxic metabolites (Pastore *et al.*, 2007), have polygenic nature. Breeding for stress tolerance using traditional methods has been limitedly successful (Cattivelli *et al.*, 2008), which has prompted interest in transgenic strategies.

The activation of stress signalling pathways in plants begins with the perception of extra-cellular signals at molecular level, followed by the generation of secondary signalling molecules, and the induction or activation of transcription factors (TFs) (Bartels and Sunkar, 2005). TFs activation allows the plant to grow and propagate under these deteriorate conditions, especially, high-salt concentration, which cause to disturbance of cellular protein homeostasis (Rae *et al.*, 2011). Regulation in transcription level is one of the most important steps for plant adaptation to stress conditions such as salinity. Transcription factors respond to stress signals, regulate the expression of many stress-related downstream genes, by specifically binding to cis-acting elements in the promotors of target genes. TFs which are related to stresses are categorized into several families, such as AP2/EREBP, bZIP, NAC, MYB, MYC, Cys2His2

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zinc-finger and WRKY (Umezawa et al., 2010). Likewise, many genes conferring stress tolerance are induced under stress conditions.

Plant stress responses have been classified to abscisic acid (ABA)-dependent and -independent regulatory pathways (Shinozaki and Yamaguchi-Shinozaki, 1997, 2000). The ABA-dependent TFs particularly bind to ABA-responsive (ABRE) core cis-acting sequences in the promoters and trigger the ABA-dependent plant stress response (Shinozaki and Yamaguchi-Shinozaki, 2000). In contrast, the AP2/EREBP super-family of TFs bind to Dehydration-responsive element/C-repeat (DRE/CRT) cis-element and trigger the expression of stress-related genes in an ABA-independent procedure (Riechmann et al., 2000). AP2/EREBP family is divided into several subfamilies such as ethylene responsive factor (ERF), APETALA2 (AP2), RAV and Dehydration responsive element binding factor (DREBs) confer stress endurance in plants and are the largest and most diverse family of proteins involved in the regulation of plant responses. These proteins, based on their involvement in signal transduction pathways under low temperature and dehydration, have been divided into two classes including DREB1 and DREB2, respectively (Agarwal et al., 2007). As DREBs induce several abiotic stress responsive genes and maintain water balance in plant system, they are appropriate candidates for generating transgenic plant with increased tolerance to drought, high salt, high temperate and cold stresses (Sakuma et al., 2006).

Dehydration-Responsive Element-Binding Protein 2a (DREB2A) initially was identified in a yeast one-hybrid screen using the CaMV35s promoter as bait (Liu et al., 1998). DREB2 genes, then, were recognized as drought, heat and salinity response genes (Liu et al., 1998; Lim et al., 2007; Sakuma et al., 2006). Recent studies suggested that DREB2s may function as multi stimulus-response factor that interact with genes and transcription factors, including members of the ABA-responsive element binding protein (AREB)/ABRE binding factor (ABF) family, in downstream gene regulation, as well as proteins during different stress conditions (Lee et al., 2009). However, overexpression of a constitutively active form of DREB2A that lacks the negative regulatory domain led to increased stress tolerance in transgenic plants (Sakuma et al., 2006; Lee et al., 2010; Ellwing et al., 2011). Domain analysis of DREB2A protein showed that its C-terminal region functioned as a transcriptional activation domain and that its middle region functioned as a negative regulatory domain in the regulation of DREB2A activity. Deletion of the negative regulatory domain resulted in the produce of a constitutive active domain of DREB2A (Sakuma et al., 2006).

In this study, transgenic canola plants overexpressing DREB2A under a CaMV35s promoter from Arabidopsis were developed to reveal its role in stress adaptation. Genes downstream of DREB2A using transgenic plants overexpressing the constitutive active form of DREB2A were analyzed and many salt-inducible genes lying downstream of DREB2A were identified. In addition, the stress tolerance of the transgenic plants and found improvement of salt stress tolerance were analyzed. These results indicate that transformed DREB2A gene into canola might improve the plant response to salt stress.

MATERIALS AND METHODS

Deletion of PEST sequence and Vector construction: pBluescript plasmid carrying DREB2A gene with accession number AB007790 (Fig. 1) received from RIKEN bioresource centre (Japan) was introduced into E.coli JM109. The region related to complete coding DNA sequence (eds) between nucleotides 405 and 495 was deleted using SOEing PCR (Sakuma et al., 2006). The SOEing PCR method uses 2 pairs oligonucleotide primers (Table 1) with two rounds of PCR. In the first PCR, to delete negative regulatory domain, first and second primer pairs were used to delete the brackets of the upstream and downstream of region, respectively. Subsequently in the second PCR, forward primer from first pair and reverse primer from second pair joint these two segments to produce a longer segment without the Ser/Thr-rich region. The PCR rounds were performed for 30 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 1.5 min. The generated products were purified and ligated into pGEM-T easy vector (Promega, Madison, WI, USA) for sequencing to verify the deletion. The new segment of DREB2A without the Ser/Thr-rich coding region was named DREB2A-CA. This modified DREB2A was cloned into the pBI121 binary vector, therefore chimeric construct comprised of DREB2A open reading sequence fused in sense orientation downstream of CaMV35s promoter and upstream to nos terminator (Fig. 2). Finally, the place and direction of DREB2A gene in plasmid was confirmed by restriction cutting with EcoRI and Hind III, Xho I.

Transformation procedure and plantlet formation: The recombinant vector pBI121(CaMV35s -DREB2A- nos terminator) was mobilized into Agrobacterium tumefaciens (LBA4404) by freeze-thawing. Transformation of canola was carried using 7-10 day-old cut surfaces hypocotyl of B. napus cv. PF7045/91.
Fig. 1: Location of DREB2A gene in the pBluescript SK plasmid

Fig. 2: Location of DREB2A-CA gene in the pBI21 plasmid

Hypocotyl explants were plated on pre-culture medium (Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 30 g L\(^{-1}\) sucrose supplemented with 100 mg L\(^{-1}\) myoinositol, 7-8 g L\(^{-1}\) 2,4-D) for 72 h at 25°C under a 16-h photoperiod. Hypocotyl explants were inoculated with the A. tumefaciens suspension supplemented with 0.2 mM acetosyringone, in 50 mL tubes for 30 sec with gentle shaking. The explants were then blotted dry on sterile filter paper for 3 min to remove excess solution before they were transferred to fortified co-cultivation medium (MS containing 20 g L\(^{-1}\) sucrose supplemented with
### Table 1: Designed primers to this experiment

<table>
<thead>
<tr>
<th>Sequences</th>
<th>Primers Description</th>
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<tr>
<td><strong>a. SOEing designed primers</strong></td>
<td></td>
</tr>
<tr>
<td>5’ AGA ATT GTT GTT TTI CTG GGA AGG GAG A 3’</td>
<td>AFF1 First Pair</td>
</tr>
<tr>
<td>5’ AGA GCT CAG TCG TTG TGG GAT TAA GCC GGC 3’</td>
<td>ARRI</td>
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<tr>
<td><strong>b. PCR designed primers</strong></td>
<td></td>
</tr>
<tr>
<td>5’ GGC ACA ATC CCA CTA TCC TT T3’</td>
<td>CaM-DRE Forwards</td>
</tr>
<tr>
<td>5’ TCT CAG CAA CCC ATT TAC CCC T3’</td>
<td>CaM-DRE Reverses</td>
</tr>
<tr>
<td><strong>c. RT-PCR designed primers</strong></td>
<td></td>
</tr>
<tr>
<td>5’ GCG TCT CGA CTA GGA CCA AG 3’</td>
<td>Forwards BnACT20</td>
</tr>
<tr>
<td>5’ AGA TAC TCC CCG CCT CCA AGG 3’</td>
<td>Reverses</td>
</tr>
<tr>
<td>5’ AAA CCA TCT TGT TGA CTC TCG GCC 3’</td>
<td>Forwards DREB2A</td>
</tr>
<tr>
<td>5’ TAT GAG AGC ACG ATT G 3’</td>
<td>Reverses HSP70</td>
</tr>
<tr>
<td>5’ TAC AACATG AGG AAC ACC ATC C3’</td>
<td>Forwards PROX</td>
</tr>
<tr>
<td>5’ ACT GAA TCG CCT GCT CAA TAG 3’</td>
<td>Reverses</td>
</tr>
<tr>
<td>5’ ACA GAC ACA ATC CCC TTG GTG 3’</td>
<td>Forwards RD20</td>
</tr>
<tr>
<td>5’ AGG TGG AAT GCT GCC CAT CC 3’</td>
<td>Reverses</td>
</tr>
<tr>
<td>5’ GAT TCG AGC ACC TAT GAC ACC GA 3’</td>
<td>Forwards Cor14</td>
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<td>5’ ATT GCC ATT CGA TTT CCC TCG GT 3’</td>
<td>Reverses</td>
</tr>
<tr>
<td>5’ GCC AGA AAA GGT GAT GGA AAC 3’</td>
<td>Reverses</td>
</tr>
<tr>
<td>5’ AGC CCT TTG TCT TGT CGT 3’</td>
<td>Reverses</td>
</tr>
</tbody>
</table>

*cutting site to EcoR I enzyme, *cutting site to Sac I enzyme, *segment a is complement with segment d, *segment c is complement with segment b, *segment b is complement with segment c, *segment d is complement with segment a. *BnACT20 was used as a standard control gene in RT-PCR experiment (Reference gene).

100 mg L⁻¹ myoinositol, 7 g L⁻¹ agar) and incubated for 2 days at 25°C under dark condition. After co-cultivation, the explants were transferred onto callus induction medium (MS containing 30 g L⁻¹ sucrose augmented with 100 mg L⁻¹ myoinositol, 6-8 g L⁻¹ agar, 1.0 mg L⁻¹ 2,4-D, 500 mg L⁻¹ cefotaxime) for 7 days at 25°C under a 16-h photoperiod and then transferred to selective medium (MS containing 30 g L⁻¹ sucrose augmented with 100 mg L⁻¹ myoinositol, 6-8 g L⁻¹ agar, 4.5 g L⁻¹ Benzaminopurine (BAP), 0.3 mg L⁻¹ Thidiazuron (TDZ), 250 mg L⁻¹ cefotaxime, 10-20 mg L⁻¹ kanamycin and 3 mg L⁻¹ silver nitrate) for the induction of transgenic callus. As a control, uninfected explants were cultured on selective medium. After 3 weeks of culture, hypocotyl calli were transferred to shoot induction medium (MS containing 10 g L⁻¹ sucrose supplemented with 100 mg L⁻¹ myoinositol, 6-8 g L⁻¹ agar, 200 mg L⁻¹ cefotaxime and 25 mg L⁻¹ kanamycin) for 4 weeks. Elongated shoots were transferred to rooting medium (MS media supplemented with 10 g L⁻¹ sucrose, 100 mg L⁻¹ myoinositol, 6-8 g L⁻¹ agar, 150 g L⁻¹ cefotaxime and 25 mg L⁻¹ kanamycin). Rooted plantlets were washed in sterile distilled water to remove traces of medium and were transferred to plastic pots (5 cm diameter) containing a mixture of sterile soil, perite and vermiculite (3:1:1). After 3 weeks, the plants were transferred to pots containing soil and grown in a greenhouse. Putative transgenic lines that showed vigorous growth on kanamycin media and their self-pollinated heterozygous progenies (T₂) were used for the experiments described below.

### PCR analysis:
PCR was carried out in a total volume of 20 µL containing 200 ng genomic DNA, 200 µM dNTPs, 2.5 U Taq DNA polymerase, Taq buffer and 10 pmol each of forward and reverse primers, which were designed within the CaMV35s promoter and the DREB2A gene, respectively (Table 1). The PCR conditions were 94°C, 1 min; 58°C, 1 min and 72°C, 1.5 min for 30 cycles. Integration of T-DNA was analyzed by PCR amplification of the DREB2A transgenic cassette in the genome of both T₁ and T₂ canola plants.

### Quantitative real-time PCR analysis:
Total RNA was isolated and purified from canola leaves according to the protocol of the RNasy plant mini kit (Qiagen, Valencia, CA, USA). cDNA was synthesized from total RNA using QuantiTect Revers Transcriptoin synthesis kit (Qiagen) and expression of the selected genes were analyzed by Real-Time PCR (RT-PCR) using the fluorescent intercalating dye SYBRGreen with a detection system (Step One Plus, Real-Time PCR System, Applied Biosystem, USA). Real-Time PCR reaction was performed using Real-Time PCR Master Mix (Applied Biosystem)
according to the manufacturer's instructions, with gene-specific primers (Table 1). The cycle threshold (Ct) as the PCR cycle at which a statistically significant increase of reporter fluorescence is first detected, was measured for two biological and two technical repeats for each sample. Relative quantity of the target gene expression level was performed using the comparative Ct method (Pfaffl, 2001). The relative value for the expression level of each target gene was calculated by the equation 
\[ R = \left( \frac{E_{\text{target}}}{E_{\text{act}}} \right) \left( \frac{C_{\text{target}}}{C_{\text{ref}}} \right) \] 
which \( \Delta C_{\text{target}} \) is (Mean control–Mean sample) for target gene and \( \Delta C_{\text{ref}} \) is (Mean control–Mean sample) for \( \beta\text{-act} \), as a standard control.

**Evaluation of transgenic T<sub>1</sub> canola lines under salt stress:** Control and transgenic T<sub>1</sub> lines were hardened and transferred to pots at 22<sup>°</sup>C with 70% humidity in a green house under white fluorescent light (600 µmol m<sup>-2</sup> s<sup>-1</sup>, 12 h light period/day) supplemented with NaCl (150 mM) for 14 days. Biomass of control and transgenic plants was then measured under salt stress.

**Statistical analyses:** The statistical significance of the results was evaluated with the Student's t-test when only two means were compared or with two-way ANOVA followed by Duncan's multiple comparisons test otherwise. Data analyses and graphical representations were performed using Microsoft Office Excel 2007 or R, a language and environment for statistical computing and graphics (Ihaka and Gentleman, 1996).

**RESULTS**

**Developing DREB2A transgenic canola plants:** To analyze the function of DREB2A in canola plants, DREB2A-C4, DREB2A 1-135:166-335 which presented the maximum activity in the transgenic plants (Nakashima et al., 2009), were over expressed in canola plants. This constitutive active form of DREB2A was over expressed in canola under the control of the cauliflower mosaic virus 35S promoter. The CaMV35s::DREB2A expression cassette was cloned into pB1121 binary vector for Agrobacterium-mediated transformation of B. napus cv. PFT045/91 using hypocotyl derived calli. Approx. 50 calli were transferred to the selection medium after cocultivation with Agrobacterium tumefaciens the DREB2A cassette and about 30 kanamycin resistant calli were obtained after two rounds of selection on 25 mg kanamycin/l supplemented medium. Resistant calli were sequentially transferred to regeneration and rooting medium for formation of shoots and roots, respectively. Thirty four plants were regenerated and transferred to pots with soil and maintained under controlled greenhouse conditions. Out of 34 putative T<sub>1</sub> transgenic lines, which were hardened and transferred to greenhouse, 4 putative transgenic lines showing vigorous growth on kanamycin media and their self-pollinated heterozygous progenies (T<sub>2</sub>) were selected for the experiments described below.

**Molecular characterization of transgenic canola lines:** To confirm the foreign gene integration in putative transgenic canola plants, PCR amplification was performed using forward primer from CaMV35s promoter and reverse primer from DREB2A gene, i.e., CaM-DRE (Table 1). Transformation experiments generated 34 independently regenerated T<sub>2</sub> plants. Thirty four putative T<sub>2</sub> transgenic lines have been regenerated from MS medium supplemented with kanamycin. These seedlings transferred to pots and were used for PCR analysis. Amongst these 34 transformed lines, 4 lines were showed the expected amplification of 518 bp DNA fragment from the integrated CaMV35s::DREB2A gene cassette (Fig. 3a). Therefore, these lines were chosen for future analysis and T<sub>1</sub> seed production. No such amplification was observed in non-transformed control. Meanwhile, to determine the expression of the DREB2A-C4 in the transformed canola plants, six T<sub>1</sub> seedlings were randomly selected of each T<sub>0</sub> plants and PCR amplification was used on selected T<sub>1</sub> plants. Template DNA from all selected transgenic plants allowed the amplification of expected 518 bp fragment (Fig. 3b). Therefore it was concluded that the CaMV35s::DREB2A gene cassette was successfully located into the transgenic T<sub>1</sub> canola plants.

**Morphological analysis of T<sub>2</sub> plants under salt stress:** Overexpression of DREB2A under the CaMV35s promoter did not show any phenotypic abnormalities either in stress or non-stress growth conditions in these transgenic lines. The growth performances of transgenic seedlings in terms of shoot were significantly higher for transgenic seedlings over the control seedlings during salt stresses (Fig. 4). Comparatively, transgenic plants grew healthily, whereas the surviving control seedlings were dwarf and developed pale green leaves during salt stress. The DREB2A transgenic T<sub>1</sub> canola plants exhibited superior growth performance in comparison to control and transgenic overexpression of DREB2A in canola conferred stress tolerance. Among transgenic lines studied, T1 plants originated from T0 KCA2 plant showed the highest performance and tolerance to salt stress. Therefore this line was used for further analysis. Furthermore, to assess the impact on biomass production the fresh weight of plants was determined two weeks after salt stress. As
shown in Fig. 5 there were significant differences in biomass between non-transgenic and transgenic plants.

Functional analysis of DREB2A transgenic canola plants: To determine qualitatively whether or not the integrated and intact DREB2A transgenes were transcribed, and the expression of the transgene in the transformed plants, RT-PCR was used. Template cDNA from all of the transgenic plants examined allowed the expression of DREB2A, whereas RT-PCR of the untransformed control did not. Therefore, it was concluded that the CaMV35s::DREB2A gene cassette was successfully located into the transgenic canola plants. The constant expression of transgene in transformed canola plant, even in non stress condition, is due to locating the DREB2A gene in downstream of the constant promoter so RT-PCR was uses for additional confirmation and overexpression of DREB2A in T1 generations, was recorded, more than 10000 times (Table 2, Fig. 6). To understand the entire transcriptional network of DREB2A in B. napus transgenic plants, some DREB2A-CA (35S::DREB2A-CA) downstream genes, which their remarkable expression ratios under abiotic stress had been previously described, were studied. These downstream genes were included heat shock factor (Zou et al., 1998), heat shock protein
Table 2: RT-PCR results

<table>
<thead>
<tr>
<th>Genes</th>
<th>Control</th>
<th>Sample</th>
<th>Expression ratio</th>
</tr>
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<tbody>
<tr>
<td>RD20‡</td>
<td>23.785</td>
<td>22.951</td>
<td>1.03**</td>
</tr>
<tr>
<td>HSF3‡</td>
<td>30.612</td>
<td>26.553</td>
<td>4.62**</td>
</tr>
<tr>
<td>HSP70‡</td>
<td>27.896</td>
<td>25.434</td>
<td>4.66**</td>
</tr>
<tr>
<td>COR14‡</td>
<td>27.258</td>
<td>35.920</td>
<td>2.254**</td>
</tr>
<tr>
<td>PEROX¹</td>
<td>29.871</td>
<td>26.868</td>
<td>7.333**</td>
</tr>
<tr>
<td>DREB2A</td>
<td>37.295</td>
<td>23.622</td>
<td>11943.454**</td>
</tr>
</tbody>
</table>

*R20 Ca²⁺-binding protein, ‡Heat shock factor, §Heat shock protein, ¶Cyclic nucleotide-gated channel, ¶ Peroxidase, ‰Ratio of miRNA in transgenic plant, to control plants; ** indicates significant at p = 0.01

Fig. 4(a-e): Transgenic lines and controls in early stage under salt stress. (a) KCA3 transgenic T₃ line, (b) KCA1 transgenic T₃ line, (c) Control line under stress treatment, (d) KCA2 transgenic T₃ line and (e) Control line in non stress situation

Fig. 5: Biomass of four-weeks-old transgenic and non-transgenic canola under stress and non stress conditions. Each value represents the mean±SE. The means were compared using the Duncan multiple range test. Letters above columns indicate a statistically significant difference (p<0.05). Standard errors are denoted by error bars.
Fig. 6: Melt curve of amplified fragments in Real-Time PCR, A: Transgenic line and B: Non-transgenic line

(Wang et al., 2004), RD20 Ca²⁺-binding protein (Takahashi et al., 2000), cold regulated protein 14, COR14, (Crosatti et al., 1995) and peroxidase (Zhu et al., 2001). The canola homologue of these genes from NCBI were selected and primers were designed (Table 1). Up-regulation of these genes was confirmed by quantitative RT-PCR (Table 2).

DISCUSSION

Regulatory effects of the cis-acting promoter element DRE, containing the core sequence A/GCCGAC, has been identified in response to drought, salt and cold stresses in Arabidopsis (Sakuma et al., 2006). Although, the function of DREB2A under these stresses especially in Arabidopsis has been studied (Liu et al., 1998; Desikan et al., 2001 and Schrumm et al., 2008), the effect of DREB2A in abiotic stress in other plants such as canola has not been previously well evaluated. The overexpression of DREB1A in transgenic Arabidopsis induced expression of many downstream stress-responsive genes under unstressed conditions and led to enhancing freezing and dehydration tolerance (Liu et al., 1998). However, It is suggested that the presence of negative regulatory domain in the central region (136–165 aa) has an inhibitory effect in the normal condition and is modified under salt/drought stress. Deletion of this region transforms DREB2A to a constitutive active form (Sakuma et al., 2006), which results in more stable transcription factor than the full-length DREB2A protein in the nucleus of transgenic plants. Therefore, in current study, to investigate the role of DREB2A in transformed B. napus under salt stress, canola plants overexpressing DREB2A-CA transgene driven by a CaMV35s promoter were developed. Thirty four transgenic canola lines were regenerated under antibiotic supplemented medium, out of 34 regenerated seedlings. Four transgenic seedlings were confirmed by PCR analysis, survived and grew healthily during salt stress conditions (150 mM NaCl), in comparison to their corresponding control seedlings (Fig. 3). During salt stress, control plants showed wilting while transgenic lines grew without any visual wilting symptoms. Performance of DREB2A transgenic canola plants was relatively superior in terms of several phenotypic growth parameters in comparison to their respective non-transgenic control during stress condition (Fig. 4). Taken together, these results indicate that transgenic overexpression of DREB2A in canola positively correlates with stress tolerance.

The constant promoter in upstream of target gene caused to high-level persistent transgene expression even in non-stress condition, 10000 times higher than non-transformed plants. On the other hand, the absence of CaMV35s: DREB2A gene cassette in genetic background of wild type plants naturally resulted in no expression in
non transformed plant. Therefore, the successful transformation of DREB2A gene into canola plant was confirmed by RT-PCR results as well as PCR results.

Several genes have been identified as functional components in the plant response to salt stress such as detoxifying enzymes like glutathione peroxidase (Roxas et al., 2000) and ion accumulation (Parida and Das, 2005). In this study, to analyze whether this transgene overexpression induces tolerance to salt stress in transgenic canola plants, the relative transcript levels of several downstream salt-stress responsive target genes were monitored. The downstream genes including peroxidase, heat shock proteins, heat shock factor, cold regulated protein 14 and RD20 Ca2+-binding protein were analyzed by RT-PCR in both transgenic and corresponding non-transgenic control canola seedlings, in transgenic (Table 2).

Most tolerant genotypes to salinity have been to date reported the significantly increased abundance of peroxidase for example in tomato (Mittova et al., 2004), Arabidopsis (Jiang et al., 2007), potato (Aghaei et al., 2008) and rice (Yan et al., 2005). Most of the transgenic improvements in salt tolerance plants have been achieved through detoxification strategy (Zhu, 2001) and also regulation of the ROS by scavenging them with antioxidant enzymes (Askari et al., 2006; Bhushan et al., 2007). This is more obvious in the case of transgenic plants overexpressing enzymes involved in oxidative protection, such as glutathione peroxidase (Roxas et al., 2000), superoxide dismutase (Meloni et al., 2003), ascorbate peroxidases (Badawi et al., 2004) and glutathione reductases (Pilon-Smits et al., 2000). In this study, expression of peroxidase was highly upregulated in transgenics in comparison with non transformed plants, more than 7 times suggesting that the expression of peroxidase was due to expression of DREB2A transcription factor in transgenic canola.

Differential regulation for genes encoding carriers and channels involved in transporting ions and organic substrates were reported in tolerant crop genotypes. Cold-regulated genes had been reported to differentially express in freezing-sensitive and -tolerance plants under both laboratory and field conditions. Accumulation of encoded proteins by cold-regulated genes implies a direct interaction between the cold regulated gene expression coming from cold induced signaling pathway and redox situation of the chloroplast (Rapacz et al., 2008). The RD20 gene, which encodes a Ca2+-binding protein is induced by dehydration (Takahashi et al., 2000), salt stress (Liu et al., 2007), and ABA treatment (Lee and Chun, 1998). In current study, upregulation of Cold-regulated gene and RD20 in transformed plant at transcript level were recorded in comparison to wild type plants, more than 3 times indicating that overexpression of DREB2A induces expression of the salt response downstream genes under even nonstressed conditions.

CONCLUSION

In order to investigate the effect of DREB2A-CA stress-inducible transcription factor on plant growth, its relationship to biomass production and expression of some downstream salt stress response genes under salinity stress, T1 generation originated from T0 KCA2 plant that showed the highest performance and tolerance to salt stress were used for complementary analysis. Functional analysis showed that of DREB2A-CA gene enhances salt tolerance in canola transgenics and up-regulates downstream salt-responsive genes not only under osmotic stress but also under non stress condition, due to constant promoter in upstream of gene. Thus, DREB2A could be considered as an important transcription factor that could be use to confer salt stress tolerance in canola plants.

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