Transgenic tobacco plants expressing *BoRS1* gene from *Brassica oleracea* var. *acephala* show enhanced tolerance to water stress

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Water stress is by far the leading environmental stress limiting crop yields worldwide. Genetic engineering techniques hold great promise for developing crop cultivars with high tolerance to water stress. In this study, the *Brassica oleracea* var. *acephala BoRS1* gene was transferred into tobacco through *Agrobacterium*-mediated leaf disc transformation. The transgenic status and transgene expression of the transgenic plants was confirmed by polymerase chain reaction (PCR) analysis, Southern hybridization and semi-quantitative one step RT-PCR analysis respectively. Subsequently, the growth status under water stress, and physiological responses to water stress of transgenic tobacco were studied. The results showed that the transgenic plants exhibited better growth status under water stress condition compared to the untransformed control plants. In physiological assessment of water tolerance, transgenic plants showed more dry matter accumulation and maintained significantly higher levels of leaf chlorophyll content along with increasing levels of water stress than the untransformed control plants. This study shows that *BoRS1* is a candidate gene in the engineering of crops for enhanced water stress tolerance.

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1. Introduction

Many reports reveal that water stress is by far the leading environmental stress limiting crop yields, which appears in many habitual forms caused by drought, salinity and extreme temperatures. To develop plant cultivars inherently resistant to water stress would help to protect crop production. However, progress in genetic improvement of crops for water-limiting environments is slow and more limited (Evenson and Gollin 2003), due to the lack of knowledge about the physiological processes limiting growth under water stress conditions, poor understanding of water stress tolerance mechanism, and lack of efficient techniques for screening breeding materials for water stress tolerance (Khush 2001). Therefore, to unveil the genetic and physiological basis of water stress tolerance is fundamental and necessary to develop crops suitable for water limiting environments. The development of stress tolerant cultivars has been one of the major objectives for most plant breeding programs. With the rapid development of genetic engineering, molecular breeding has provided a very promising approach to improve stress tolerance of crops and much progress has been made recently (Holmstrom 1996; Anil *et al* 1998; Niklas and Leif 1998; Blum 1999; Smirnoff and Bryant 1999; Ramanjulu and Bartels 2002).

Many organisms develop the ability to survive in extreme environments during long history of gene evolution, which can be used as good research material for the isolation of the stress-resistant genes for genetic im-

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provement. Recently, there are some successful attempts to transfer stress-tolerant genes into plants, and the resulting transgenic plants showed enhanced tolerance to water stress and other stresses (Xu *et al* 1996; Hsieh *et al* 2002; Bhattacharya *et al* 2004; Chandra Babu *et al* 2004).

As *Brassica oleracea* var. *acephala* was a plant source with excellent resistance to stress, recently we have cloned a new water stress responsive gene *BoRS1* from it and our earlier study showed that the expression of the *BoRS1* gene was up-regulated by many stress factors including cold, drought, salinity and abscisic acid (ABA) (Tang *et al* 2004). In order to further elucidate the biological roles and potential stress tolerance conferred by the expression of *BoRS1* gene, in this study, we introduced the *BoRS1* into tobacco through *Agrobacterium tumefaciens*-mediated transformation. The growth and physiological responses of transgenic plants under water stress were also investigated.

2. Materials and methods

2.1 *Plant material and* in vitro *culture conditions*

Surface-sterilized seeds of axenic plants of tobacco (*Nicotiana tabacum* var. *Petit Havana* SR1) were germinated under aseptic conditions on solid MS medium with 3% sucrose (pH 5·8). Leaf-discs of approximately 8 mm in length from 20-day-old seedlings were used for plant transformation. The incubation condition maintained for germination was 25°C with a 12 h photoperiod (irradiance of 50 µmol m^{-2} s^{-1} provided by white fluorescent tubes).

2.2 *Construction of plant transformation vector p2300⁺ BoRS1*

The 1865 nt open reading frame (ORF) of the *BoRS1* cDNA was amplified by polymerase chain reaction (PCR) using the forward primer RSF1 carrying *Bgl*II restriction site (5′-GAAGA TCTAA ATGGA TTTGA CACGT CCT-3′) and the reverse primer RSR1 carrying *Sac*I restriction site (5′-CGAGC TCCCC AGATT CTCAA AGTTG TT-3′). The amplified product was purified and cloned into the pMD-18T vector (Takara, Dalian, China), and digested with *Bam*HI and *Sac*I. Subsequently the resulting fragment was inserted into the *Bam*HI and *Sac*I-predigested modified expression vector pCAMBIA2300. The resulting vector, *p2300⁺BoRS1* (figure 1), contained the *BoRS1* gene driven by the CaMV35S promoter and terminated by nopaline synthase terminator (Nos).

The *p2300⁺BoRS1* was introduced into *A. tumefaciens* strain EHA105 by the freeze-thaw transformation method. Single transformed colony was transferred into liquid bacterial culture medium (LB medium with 50 mg l^{-1} rifampicin, 25 mg l^{-1} streptomycin and 50 mg l^{-1} kanamycin) and shaken at 240 rpm at 28°C for 2 days. The suspension was then mixed with sterile glycerol (1 : 1 v/v) and stored at –70°C until use.

2.3 *Plant transformation*

Tobacco was transformed with EHA105 (*p2300⁺BoRS1*) using the leaf disc method (Horsch *et al* 1988). The bacterial culture was grown to an OD_{600} of 0·5–0·8 before used for plant infection. The infected leaf explants were placed on co-cultivation medium MS_1 (MS + 0·5 mg l^{-1} BA + 0·1 mg l^{-1} NAA) on 25°C in the dark for 2 days. The shoots were regenerated on selection medium MS_2 (MS_1 + 75 mg l^{-1} kanamycin + 500 mg l^{-1} cefotaxime). The well-grown shoots (2–3 cm in length) were excised carefully and then transferred onto rooting medium MS_3 (MS + 0·1 mg l^{-1} NAA + 25 mg l^{-1} kanamycin + 250 mg l^{-1} cefotaxime). All media were supplied with 3% sucrose and 2·6 g l^{-1} phytagel (Sigma, St. Louis, MO, USA), and the pH was adjusted to 5·8. The condition for shooting and rooting was the same as that for germination. The rooted shoots were multiplied by nodal bud cultures for maintaining clones under *in vitro* conditions and some of

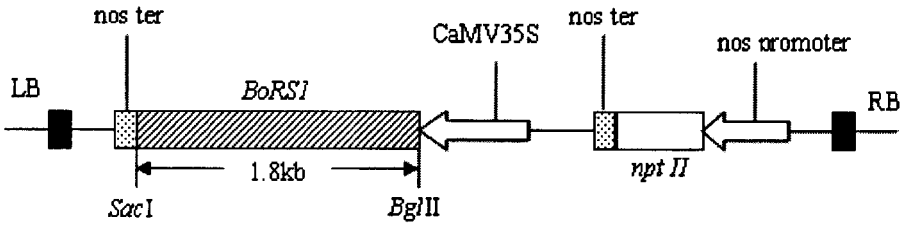

Figure 1. The schematic map of the transformation vector *p2300⁺BoRS1*. CaMV35S, cauliflower mosaic virus 35S promoter; *npt*II, neomycin phosphotransferase gene; Nos ter, 3′ termination region from *Agrobacterium nopaline* synthase gene; *BoRS1*, *Brassica oleracea* var. *acephala BoRS1*-encoding gene; RB, right border of T-DNA; LB, left border of T-DNA.

the clones were transferred to pots for hardening and then to greenhouse for further growth.

2.4 *Molecular analyses of T_0 transgenic plants*

2.4a *PCR analysis of kanamycin-resistant plants*: Total genomic DNA was isolated from leaves of the kanamycin-resistant tobacco plants as well as untransformed control plants using SDS method (Doyle and Doyle 1990). The presence of transgene in putative transgenic plants was detected by PCR method with primers RSF1 and RSR1. The expected amplified product size was about 1860 bp. The PCR was performed in a total volume of 25 µl containing 2 µl DNA, 10 pmol each of primers, 10 µmol dNTPs, 1 × PCR buffer, 1 × $MgCl_2$ and 0·5 U *Taq* polymerase (Takara, Dalian, China). The PCR reaction was carried out by denaturing the template at 94°C for 5 min followed by 35 cycles of amplification (1 min at 94°C, 1 min at 58°C and 2 min at 72°C) and by extension at 72°C for 8 min.

2.4b *Southern blot analysis*: Southern blot analysis was further used to confirm the transgenic status of plants positive detected by PCR analysis. In total, 8 independent PCR-positive tobacco plants (plant Nos RS14, RS28, RS37, RS44, RS54, RS77, RS90 and RS95) were analysed by Southern blot hybridization for the presence of the *BoRS1* transgene. Total genomic DNA was extracted from young leaves of PCR-positive plants using the cetyl trimethyl ammonium bromide (CTAB) method (Sambrook *et al* 1998). Sixty µg of plant genomic DNA and 8 µg of *p2300*$^+$*BoRS1* were digested with *Bgl*II overnight at 37°C, which did not cut within *p2300*$^+$*BoRS1*, separated by 0·8% agarose gel electrophoresis and transferred to Hybond nylon membranes (Amersham Co. Ltd, USA). The 1·4 kb fragment of the *BoRS1* was generated by PCR using the primers RSF2 (5′-ATGGA TTTGA CACGT CCTTC-3′) and RSR2 (5′-TCTGC CGCCT CGTAT CTTGTC-3′), and used as the probe in Southern blot analysis. The Gene Images random priming labelling module and Gene Images CDP-Star detection module were used for probe labelling, hybridization and detection procedures (Amersham Pharmacia, Piscataway, USA).

2.4c *Semi-quantitative one-step RT-PCR analysis*: For semi-quantitative one-step RT-PCR analysis, one µg of total RNA extracted from young leaves of transgenic plants was used as the template in RT-PCR with the forward primers RSF2 and reverse primers RSR2 by using one-step RT-PCR kit (Takara, Dalian, China). The template was reversely transcribed at 50°C for 30 min and denatured at 95°C for 2 min, followed by 25 cycles of amplification (95°C for 30 s, 59°C for 30 s, 72°C for 2 min) and by extension at 72°C for 10 min. The RT-PCR reaction for the house-keeping gene (actin gene) using specific primers actF (5′-GTGAC AATGG AACTG GAATG-3′) and actR (5′-AGACG GAGGA TAGCG TGAGG-3′) was performed as described above as the control. The quantity of PCR products was analysed with Gene analysis software package.

2.5 *Water stress tolerance analysis of T_0 transgenic plants*

Several independent transgenic tobacco plants (T_0 generations) expressing *BoRS1* gene and the untransformed control plants were micropropagated into four copies and grown under 14 h photoperiod. Each of the controls and transgenic plants was subjected to four different water stress treatments (by supplement with different concentrations of mannitol) labelled from A to D. The concentrations of mannitol supplementation were increased stepwise by 50 mM every 2 days for each group to the indicated maximum: (i) 50 mM, (ii) 100 mM, (iii) 150 mM, (iv) 200 mM.

2.6 *Genetic analysis of segregation of the* BoRS1 *gene in T_1 progenies*

Four independent T_0 primary transgenic plants expressing *BoRS1* gene, RS14, RS37, RS77 and RS95, were grown to maturity in greenhouse. T_1 seeds of these plants were harvested and then sown in soil in the greenhouse. The germinated T_1 plants were analysed for the presence of the *BoRS1* gene by PCR analysis using the method mentioned before.

2.7 *Water stress tolerance assays of T_1 transgenic plants*

Four independent transgenic tobacco T_1 lines expressing *BoRS1* gene, named RS14, RS37, RS77 and RS95, were germinated on MS media supplemented with 100 mg l^{-1} kanamycin. Meanwhile the untransformed control plant (wild-type), named WT, was germinated on MS media. The seedlings were used for water stress tolerance assays.

2.7a *Bioassay of whole plant*: Two-week-old tobacco plants were placed on MS medium under three levels of water stress treatments by supplement with different concentration of mannitol: 100 mM, 200 mM and 400 mM. A parallel experiment in MS without any mannitol was set-up as the control. Tolerance was assessed on the basis of the accumulation of dry matter and leaf relative water content (RWC) after 1 month under water stress chal-

lenge compared to the controls. Dry matter weights were determined after oven drying the tissues at 100°C for 72 h. RWC = (leaf fresh wt.– leaf dry wt.)/leaf fresh wt. Ten replicates per line per treatment were sampled for this test.

2.7b *Leaf total chlorophyll content assay*: Six-week-old tobacco plants were used in this experiment. The plants were transferred to MS medium under three levels of water stress by supplement with different concentration of mannitol: 150 mM, 300 mM and 450 mM. A parallel experiment in MS without any mannitol was set up as the control. The leaf total chlorophyll content was monitored after 15 days subjecting the plants to water stress. The chlorophyll content was determined by extraction from leaf tissue in 95% alcohol. Leaf slices weighing about 0·2 g were placed in 25 ml of alcohol in 50 ml tubes protected from light overnight. Absorbance (OD) of the extract was obtained at 665 nm and 649 nm respectively. Total chlorophyll content (mg/g fresh wt.) = $[(13{\cdot}95 \times OD_{665} - 6{\cdot}88 \times OD_{649}) + (24{\cdot}96 \times OD_{649} - 7{\cdot}32 \times OD_{665}) \times V]/(1000 \times W)$, with V being the volume of the extract and W being the fresh weight of leaf tissue in g. Three replicates per line per treatment were sampled for this test.

2.8 *Assay of seed germination under water stress*

Surface-sterilized seeds from four independent transgenic tobacco T_1 lines and untransformed control plants were plated on MS media under three levels of water stress by supplement with different concentration of mannitol: 150 mM, 300 mM and 450 mM. A parallel experiment in MS without any mannitol was set-up as the control. The tested seeds were kept at 25°C under dark condition. Germinated seeds were scored every day. After 25 days, the germination rates were counted, and three replicates per line per treatment were sampled for this test.

3. Results

3.1 *Plant transformation*

The leaf explants were transferred to fresh selection medium MS_2 after 2 days of co-cultivation on MS_1 medium. One week later, some explants had grown larger and thicker, and then shoots were appeared gradually from the edges of these explants. After one month, 138 shoots of 2–3 cm in length were cut from the base of explants and placed on rooting medium MS_3. After 2 weeks, 130 kanamycin-resistant plants developed normal roots on MS_3. These 130 plants were transplanted to perlite for further molecular analysis and functional tests. Later, some plants were transferred to soil in greenhouse for seed production.

3.2 *Molecular analyses of T_0 transgenic plants*

The presence of transgenes in putative transgenic plants was first analysed by PCR with primers specific to *BoRS1* gene. Thirty-six among 130 transformants were found to be PCR-positive for the *BoRS1* gene (data not shown).

Subsequently, to further confirm transgenic status of the PCR-positive plants and to determine the copy number of insertions in independent transgenic lines, the genomic DNA of the PCR-positive transgenic plants was digested with *Bgl*II and hybridized with labelled 1·4 kb *BoRS1* gene probe. Southern analysis confirmed the integration of the *BoRS1* gene in the genome of some transgenic lines, including RS14, RS37, RS77 and RS95. The number of the *BoRS1* gene insertions in transgenic lines varied from 1 to 3, while the control did not show any hybridization signal (figure 2). Since there is no internal *Bgl*II site in the *BoRS1* expression cassette, the number of bands in the autoradiogram represent independent event of transgene integration.

The transgenic plants confirmed by PCR and Southern hybridization were further analysed for the expression of *BoRS1* gene by semi-quantitative one-step RT-PCR. The result showed that the independent transgenic plants expressed *BoRS1* at various levels (figure 3). In the depicted gel photograph, RS77 and RS95 showed higher levels of *BoRS1* transcript, followed by RS37 and RS14.

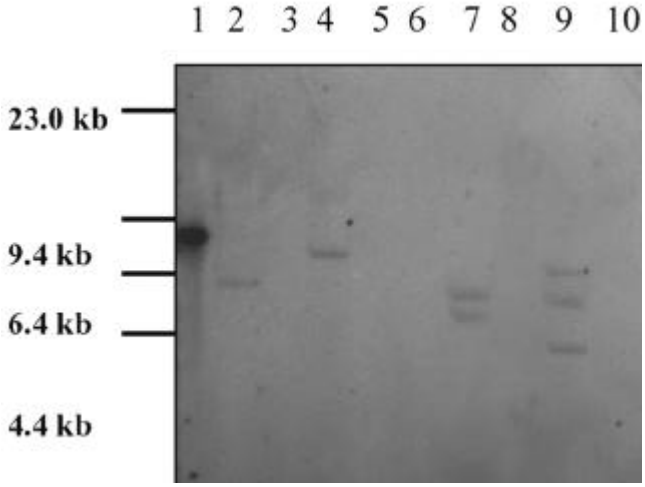

Figure 2. Southern blot analysis for the presence of the *BoRS1* gene in PCR-positive tobacco plants. Genomic DNA and *p2300*$^+$*BoRS1* were digested with *Bgl*II, separated by 0·8% agarose gel electrophoresis and hybridized with *BoRS1*-fragment probe. Lane 1, *p2300*$^+$*BoRS1* (positive control); lanes 2–9, independent plants RS14, RS28, RS37, RS44, RS54, RS77, RS90 and RS95 respectively; lane 10, untransformed plant (negative control).

3.3 *Analysis of transgenic T_1 progenies*

T_1 progenies derived from the independent primary transformants (plant Nos RS14, RS37, RS77 and RS95) expressing *BoRS1* gene were analysed for the segregation patterns of the induced *BoRS1* gene by PCR. The *BoRS1* gene in all the 4 lines was segregated at a ratio of 3 : 1 (table 1), indicating the integration of the transgenes as Mendelian manner into tobacco genomes.

3.4 *Water stress responses of T_0 transgenic tobacco plants*

Under the treatment of 50 mM mannitol, the control plants maintained the growth at the same growth rate as the transgenic plants. Under the 100 mM mannitol treatment, the leaves of the control plants became yellow much more quickly than the transgenic plants and some leaves wilted. Under 200 mM mannitol treatment, the control plants wilted quickly and died after 8 days, while the transgenic ones could grow with some leaves being wilted. After 8 days of water stress treatment, the transgenic plants were transplanted to soil in greenhouse where they blossomed and produced seeds. This result showed that the transgenic plants maintained a better growth status than the untransformed control plants under water stress.

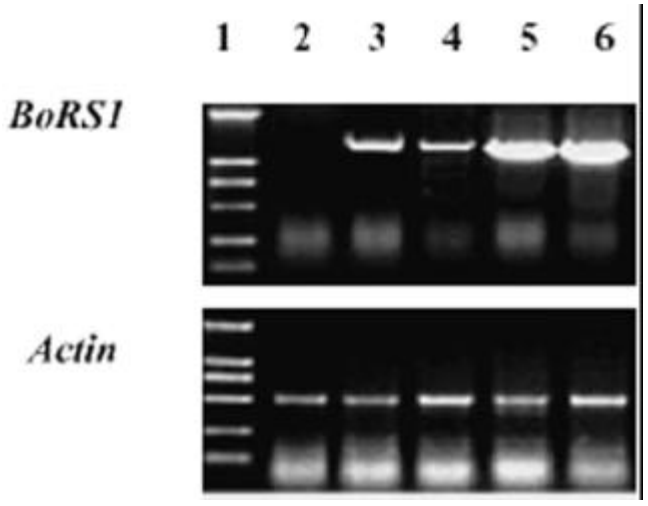

Figure 3. Semi-quantitative one-step RT-PCR analysis of transgenic tobacco lines. One μg of total RNA extracted from young leaves of transgenic plants was used as the template in the RT-PCR. Lane 1, DL2000 DNA marker; lane 2, untransformed plant; lanes 3–6, transgenic lines RS14, RS37, RS77 and RS95 respectively.

3.5 *Water stress responses of T_1 transgenic tobacco plants*

3.5a *Bioassay of whole plant*: By being cultured on the water stress medium, the leaves of the control plants became yellow much more quickly than those of the transgenic plants and some leaves wilted after 5 days under water stress. Under water stress, the transgenic plants accumulated more dry matter weight at different levels of water stress, compared to the untransformed control. However, there was variation among independent transgenic plants (figure 4a). Dry matter production under stress conditions is a good indicator of abiotic tolerance in plants (Reddy *et al* 1992). Higher dry weight accumulation from transformed plants as compared to untransformed plants clearly showed that the observed better growth of transgenic plants was due to higher biomass accumulation, not due to tissue expansion. However, there were no significant differences on the relative water content between the tested transgenic plants and the control (figure 4b).

3.5b *Leaf total chlorophyll content*: After 15 days subjecting the plants to different water stress levels, the leaf total chlorophyll content was monitored. The analysis of variance of the chlorophyll content data showed significant differences between transgenic plants and untransformed control according to the LSD test ($P < 0{\cdot}05$) (figure 5). Compared to the untransformed control, the transgenic plants showed slower decreasing along with the increase of water stress level, revealing the better tolerance to water stress. However, there also existed variation among independent transgenic plants. The transgenic plant named RS77 showed the highest level on leaf total chlorophyll content.

3.6 *Assay of seed germination under water stress*

After 25 days germinating on the water stress medium, the germination rates were counted and measured by LSD statistical analysis. Compared to the untransformed control, the transgenic lines maintained higher germination rate at different levels of water stress. There were significant differences on the germination rate between the tested transgenic lines and the control. However, there was variation among independent transgenic lines (figure 6). The transgenic plant named RS14 showed the highest level and the transgenic plant named RS37 showed the least level on the germination rate.

4. Discussion

It is important to enrich the database on new stress-responsive genes not only from the model plant, *Arabi-*

dopsis, but also from other stress-resistant plants. Furthermore, it is even more important to study the responses of plants in order to further improve the stress tolerance of crops by genetic manipulation.

Our previous study showed that the deduced amino acid residues of BoRS1 exhibited some homologies to some stress-responsive proteins, including low-temperature-induced 65 kDa protein, RD29B, RD29A and COR78, and that the percentage of hydrophilic amino acids was 79% (Tang *et al* 2004). In addition, the putative BoRS1 peptide contained 62% of random coil and the random coil and *a* helix constituted interlaced domination of the

Table 1. Segregation of *BoRS1* gene in T_1 progenies of transgenic tobacco lines.

Line	Total assayed	Expected segregation ratio	PCR for the *BoRS1* gene		
			BoRS1$^+$	*BoRS1*$^-$	c^2
RS14	60	3 : 1	45	15	0·00
RS37	60	3 : 1	43	17	0·36
RS77	60	3 : 1	39	21	3·20
RS95	60	3 : 1	47	13	0·36

Note: $a = 0{\cdot}05$, $c^2_{0{\cdot}05,1} = 3{\cdot}84$.

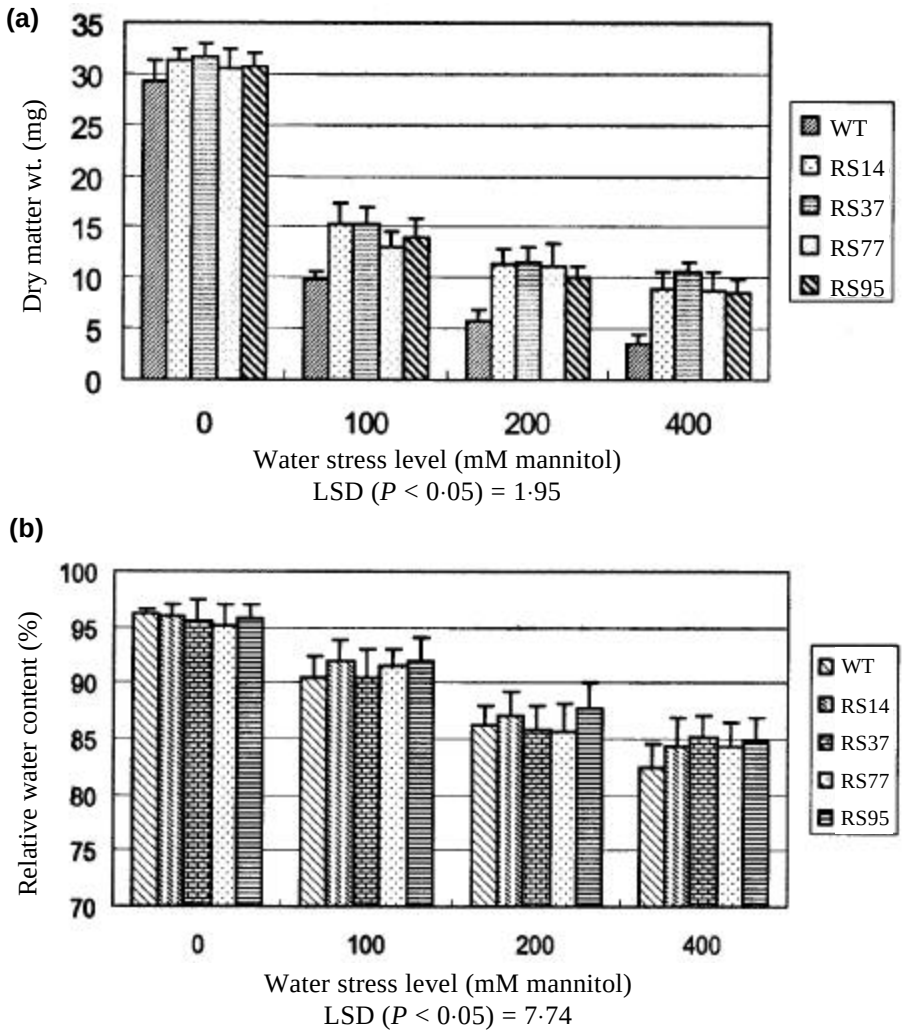

Figure 4. Bioassay of T_1 transgenic tobacco lines for water stress tolerance. WT, untransformed plant (negative control). Data represent the means of ten replicates per line per treatment ± standard error. (**a**) Dry matter accumulation. (**b**) Relative water content.

main part of the secondary structure (Tang *et al* 2004). Accumulating evidence indicates that the random coil could protect the plant cell from the damage during water stress (Close 1997). The structure of BoRS1 peptide, which is similar to LEA proteins (Baker *et al* 1998) and RD29 protein (Yamaguchi-Shinozaki and Shinozaki 1993), implies that BoRS1 protein may play a role to enhance plants' tolerant to water stress.

It is important to analyse the functions of stress-inducible genes not only to understand the molecular mechanisms of stress tolerance and responses of higher plants, but also to improve the stress tolerance of crops by gene manipulation. For evaluating the water stress tolerance, in the present study, several experiments were carried out by using transgenic tobacco plants expressing *BoRS1* and untransformed control plants. It was reported earlier that constitutive expression of proteins might hamper the normal growth of transgenic plants resulting in smaller phenotypes as compared to WT plants (Jain and Selvaraj 1997; Anil *et al* 1998). However, in the pre-

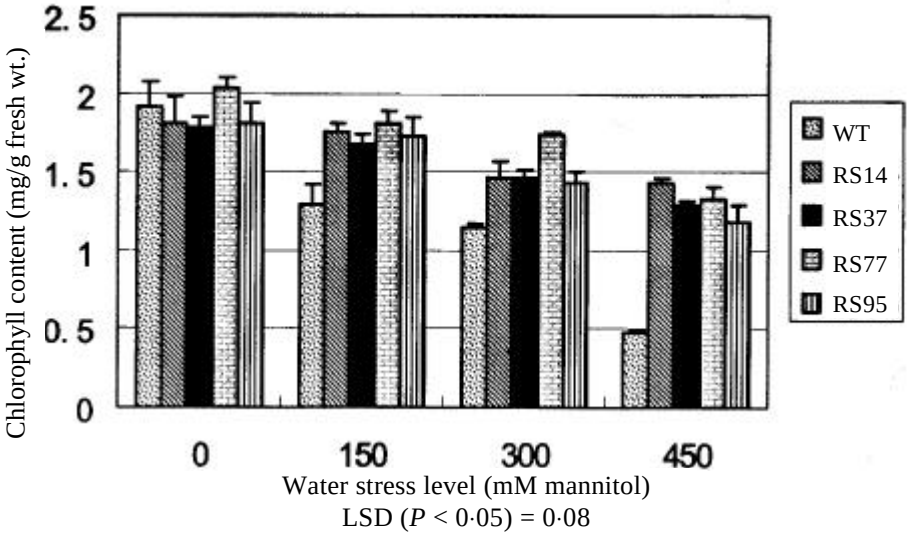

Figure 5. The leaf chlorophyll contents of T_1 transgenic tobacco lines and WT on MS medium with different levels of water stress. Data represent the means of three replicates per line per treatment ± standard error.

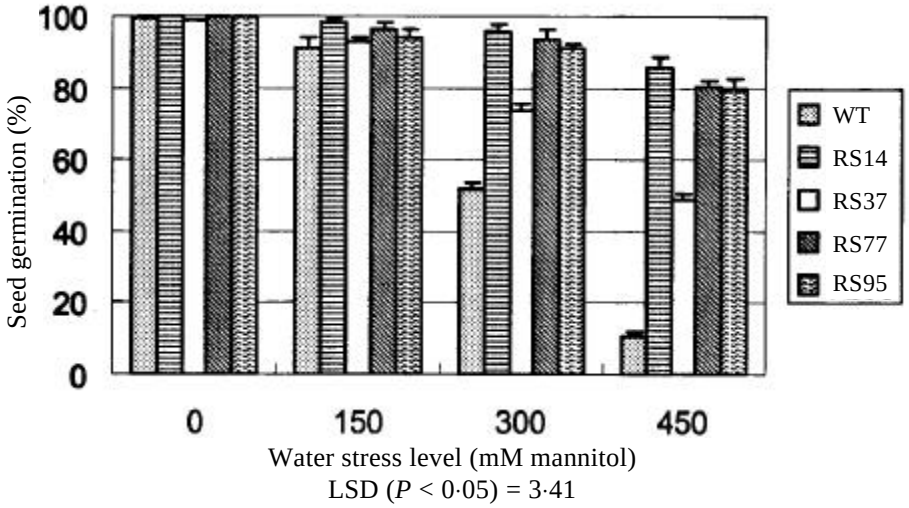

Figure 6. The germination rates of T_1 transgenic tobacco lines and WT on MS medium with different levels of water stress. Data represent the means of three replicates per line per treatment ± standard error.

sent study, though some T_0 transgenic plants showed smaller phenotypes, most of T_0 and T_1 transgenic plants had normal growth rate and showed normal phenotypes compared to the control plants. Analysis of the water stress tolerance of T_0 transgenic plants showed that the transformants exhibited better growth status under water stress compared to the untransformed control plants. The further physiological assessment of the water stress tolerance of T_1 transgenic lines revealed that transgenic lines showed better growth response, more accumulation of dry matter, greater stability in maintaining leaf chlorophyll content and the higher germination rate at increasing levels of water stress, revealing higher metabolic activity and better carbon assimilation than the untransformed control lines. Maintenance of high (favourable) plant water status, expressed as a high RWC, is an indication of water stress and other stress resistance (Bhattacharya *et al* 2004; Chandra Babu *et al* 2004). However, significant difference in RWC did not exist in the early stage of plant growth generally, variation among different lines in dry matter weight could be derived from differences in plant (shoot) size (Blum 1999), which may be a reason for no significant differences in RWC and significant differences in dry matter accumulation between the tested transgenics and the control in the present study. Our results demonstrate that over-expression of the *BoRS1* gene in tobacco can confer tolerance to water stress under the tested conditions. Meanwhile, although expressing *BoRS1* at various levels, the independent transgenic plants had similar response under water stress, suggesting that there may be no linear relationship between the expression level and the stress tolerance. For instance, line RS37, which had little expression of *BoRS1* compared with other lines, also exhibited increased tolerance as compared with the untransformed control plants, suggesting that a threshold expression of *BoRS1* is sufficient to promote stress tolerance.

Water stress signal transduction pathway is a complex network that involves multiple physiological and biochemical mechanisms and regulation of numerous genes (Bray 1997). It is difficult to imagine that the expression of a single gene in transgenic plants could promote a dramatic enhancement in water stress tolerance, leading directly to a new water stress-tolerant cultivar. However, the genetic manipulation of crop species with individual transgenes could, indeed, lead to the improved tolerance of stresses (Gisbert *et al* 2000; Chandra Babu *et al* 2004), which would be sufficient from a breeding point of view. What's more, the genetic modified plant with a single transgene is perhaps the best tool at our disposal for the genetic dissection of the complex trait.

In summary, we reported for the first time that transgenic tobacco expressing the stress responsive gene *BoRS1* from *B. oleracea* var. *acephala* showed favourable tolerance to water stress. Although further studies are necessary to demonstrate the molecular mechanism of *BoRS1* gene, the results obtained in this study would be helpful for further evaluating the biological roles of *BoRS1* gene under water stress and in genetic modification of water stress tolerance of crops.

Acknowledgements

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