18. Transcription factor AtMyb2 increased salt-stress tolerance in rice, *Oryza sativa* L.

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Abiotic stress is a major environmental stress impeding crop productivity throughout the world. Several transcription factors are shown to be transcriptional activators in ABA-dependent signal transduction and gene expression. Previous experiments have shown that overexpression of *AtMYB2* cDNA, along with *AtMYC2*, activates the *rd22* gene promoter under dehydration- and ABA-induced conditions in transgenic *Arabidopsis* plants. Several additional ABA-inducible genes were also upregulated in these plants. We report here that regulated expression of *Arabidopsis AtMYB2* cDNA in japonica rice increases plant tolerance against salt stress. Our results show that transgenic lines exhibited enhanced tolerance in terms of biomass production as well as decreased leakage of ions from rice cells.

Rice is the staple diet of about half the world's population. Considerable amounts of rice yield are lost each year due to various abiotic stresses such as salinity and drought. Various genes are induced by abiotic stresses and contribute to overcoming adverse effects (Wang et al. 2003). Abiotic-stress tolerance is a complex phenomenon that involves the expression of many genes.

Recently, a number of genes have been identified that respond to osmotic stress at the transcriptional level; they trigger a whole cascade of downstream genes involved in stress tolerance. Significant improvement in stress tolerance has been achieved by over-expressing these transcription factors in plants. For example, transgenic *Arabidopsis* plants over-expressing *AtMYC2* & *AtMYB2* showed a higher tolerance to either freezing and/or drought stress by increasing expression of downstream target genes (Abe et al. 2003).

Studies on constitutive and stress-inducible promoters have shown that transgenic *Arabidopsis* over-expressing the transcription factor *DREB1A* under the 35S promoter causes severe growth retardation under unstressed conditions. However, the use of a stress-inducible promoter, *rd29A*, for over-expression of *DREB1A* minimizes the adverse effects on plant growth in *Arabidopsis* (Kasuga et al. 1999).

A plasmid, pVS105, was constructed in pCAMBIA 1300 using the standard cloning procedure. The important components include the use of an ABA-inducible promoter complex containing 4 copies of the ABA-responsive complex ABRC1, a minimal rice *actin1* promoter, and an HVA22 intron (Su and Wu 2004). An *AtMyb2* fragment (1.2 kb), along with the 3' non-coding sequence PinII 3', was isolated from pJP33 plasmid (unpublished) as a *Pst*I fragment and inserted into the *Pst*I site of pCAMBIA 1300 to make pVS1. The ABA-inducible promoter complex was then excised as a *Hin*dIII/*Sma*I fragment, blunted at the *Hin*dIII site and ligated to pVS1 at the *Sma*I site. The selection cassette includes a hygromycin acetyltransferase gene driven by the cauliflower mosaic virus 35S promoter and has a nopaline synthase 3' non-coding region. The plasmid was mobilized into *Agrobacterium tumefaciens* strain LBA4404 harboring the pSB1 through tri-parental mating using the helper plasmid pRK2013 (Rohila et al. 2002). A japonica rice (*Oryza sativa* L.) cv. TNG-67 was used for the present study. Calli from mature seed scutellum was used as starting material for transformation and regeneration of plants as described in Rohila et al.(2002).

Production and analysis of transgenic plants. Plasmid pVS105, containing a MYB transcription factor (AtMyb2) from *Arabidopsis thaliana*, driven by an ABA-inducible promoter (*AIPC*), was introduced into calli derived from seeds of japonica rice cultivar TNG-67 through *Agrobacterium*-mediated gene transfer (Rohila et al. 2002). Over 200 putative transgenic plants were generated. The frequency of regeneration of transgenic plants was high, on the order of 71%.

Transgene integration into the transformed rice plants was analyzed by Southern blot hybridization using an α-^{32}P-labeled *AtMyb2* coding region as the probe. Out of ten plants tested, the transgene copy number was between one and four. Nearly 35% of the plants had a single copy of the transgene (data not shown).

RT-PCR with *AtMYB2*-specific primers was used to analyze the expression of the transgene as follows. Total RNA was isolated from young leaves of transgenic and control rice plants by the acid-guanidinium thiocyanate-phenol-chloroform RNA isolation method. To study the expression of the transgene, RT-PCR was performed as follows: RNA was treated with RNAse-free DNase at 37ºC for 1 hr. First-strand cDNA was synthesized from 5 microgram RNA using a Superscript II kit (Life Technologies, Inc.), according to the manufacturer's instructions. PCR assays were performed with gene-specific primers. *AtMYB2* cDNA was PCR amplified using the gene-specific forward and reverse primers: 5'-AGCAAGCC-AAACACCTAAGAT-3' and 5'-CCTGCAAGAACCAAAAACTC-3'. The PCR conditions were: 94ºC for 2 min, followed by 30 cycles of 94ºC for 30 sec, 56ºC for 30 sec and 72ºC for 1 min, with the final extension of 72ºC for 5 min. The results were normalized bycomparison

with that of the rice *actin* gene. Primers for *actin* were RAc5', 5'-CTGGGATGATATGGAGAAGATCTGG-3' and RAc3', 5'-CCGTTGTGGTGAATGAGTAACCAC G-3'. Seven-week-old R2 plants (*AtMYB2* transgenic as well as control) were subjected to 150 mM NaCl treatment for 4 days before the leaf tissue samples were collected and assayed for *AtMYB2* gene expression. As shown on the left-hand side of Fig. 1, all three transgenic lines gave a band of 417 bp, which is specific for the transgene, even though the bands in samples 1 and 2 were rather weak. As expected, a 417-bp band was also found in the plasmid DNA (lane 5), but not in the control plant sample (lane 4).

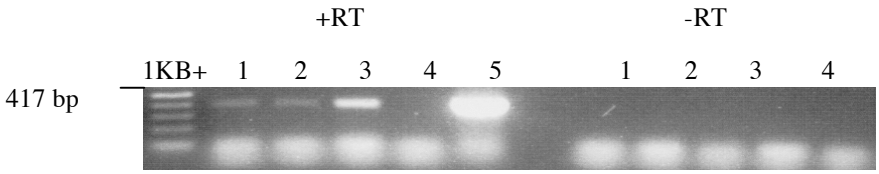

Fig. 1. RT-PCR analysis of the transgene in the leaf tissues of transgenic and non-transgenic lines of rice. Lanes 1, 2 and 3, transgenic samples; lane 4, non-transgenic control; lane 5, plasmid DNA. Left-hand panel, RT-PCR results; right-hand panel, no RT-PCR to serve as a control.

Growth performance of transgenic plants in soil under salt-stress conditions. We analyzed R2 plants in soil in the greenhouse under NaCl stress. Seven-week-old plants were used for the study. Under non-stress conditions, the transgenic plants and control plants were similar to each other (data not shown). Severe salt stress, using 150 mM NaCl for four days inhibited the growth of both the transgenic and the non-transgenic plants, but the control plants started to show wilting as well as burning of the leaf tips, one to two days earlier than the transgenic plants. The plant height, however, was similar (data not shown). Visual observation of salt-treated control and transgenic plants clearly shows the differences in salt damage. Table 1 shows the recovery of salt-stressed control (NT, non-transgenic), as well as transgenic plants. It is evident that all three transgenic lines performed better in terms of plant height, as well as fresh and dry weights. Transgenic line #6 performed the best.

Cell membrane integrity as shown by the extent of ion leakage from leaves. Salt and drought are known to damage cell membrane integrity in plants, making them more vulnerable to leaking out ions from the cells. Cell membrane stability has previously been used as a measure of plant tolerance to drought and salt stresses (Rohila et al. 2002). We measured the intactness of the membranes by electrical conductivity of the solution in which the control and transgenic leaves were soaked. As is evident from Table 2, the electrical conductivity of the samples in all the transgenic plants was much

lower than those from the control (NT, non- transgenic) plants exposed to same salt treatment. The control plants, which were not exposed to salt stress (top line in the table), gave a measure of ion leakage (1143±49) due to cutting and incubation of leaf discs.

Table 1. Growth performance of transgenic rice plants in soil under salt-stress conditions[a]

Plant line	Recovery after 24 hrs			Recovery after 8 days		
	Plant ht.[b]	Fresh wt.[c]	Dry wt.[d]	Plant ht.[b]	Fresh wt.[c]	Dry wt.[d]
NT	39.4±2.8	1.56±0.8	0.38±0.1	47.2±2.7	2.05±0.6	0.56±0.1
6	48.0±4.5	2.95±0.5	0.71±0.2	59.7±4.0	4.08±0.7	1.06±0.2
8	46.7±3.5	1.82±0.6	0.46±0.1	59.3±3.0	2.84±0.4	0.74±0.1
9	43.6±2.5	2.77±0.8	0.67±0.2	59.4±4.8	3.25±0.7	0.81±0.1

[a]Seven-week-old plants were treated with 150 mM NaCl for 4 days, followed by watering for seven days for recovery; then, a second cycle of salt stress was given before taking the readings. Mean ± S.E. represents the average of four plants.

[b]Plant height was measured in cm.

[c]Plant fresh and dry weights were measured in grams per plant.

Table 2. Ion leakage from leaves of R2 rice plants[a] after salt stress[b]

Plant line	Electrical conductivity (micromho/mg leaf tissue)
NT (no stress)	1143±49
NT	3135±118
6	1869±121
7	1718±32
8	1683±29

[a]Four hygromycin-resistant R2 plants from each line were analyzed and mean values are shown.

[b]150 mM NaCl solution was supplied to plants for 6 days before measuring the electrical conductivity.

The above results show that *AtMYB2* transgene driven by an ABA-inducible promoter confers increased tolerance against salt stress. The transgenic plants clearly exhibited less leakage of ions, which supports our finding of better growth in terms of plant height, and fresh and dry weights. The reduced ion leakage indicates that the cell membranes in the leaves of transgenic rice plants are protected from damage. In summary, we showed that over-expression of *AtMYB2* cDNA driven by a composite stress-inducible promoter increased salt-stress tolerance in transgenic rice plants.

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