Gene cloning and transgenic development in Indian mustard (*Brassica juncea* L. Czern) for improved tolerance to abiotic stresses

K.C. Bansal, Deepti Tayal, Monica Dalal, V. Chinnusamy, AK Singh, Shiv Shanker

National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute, New Delhi 110012, India
Email : kailashbansal@hotmail.com; kcbansal2001@yahoo.com

Abstract

Abiotic stresses are the major environmental factors that adversely affect crop production. Mustard (*Brassica juncea*) in India is grown mostly on marginal lands and is subjected to intermittent drought stress and also by salinity stress in some areas. Consequently, a severe reduction in seed yield, and oil quantity and quality occurs. We have taken up a programme to genetically engineer the crop with enhanced tolerance to a range of abiotic stresses. We have cloned several abiotic stress-related genes and promoters from different *Brassica* species and other plant species, and prepared appropriate gene constructs to develop transgenic plants. Several gene constructs are being validated in model species before deploying them in transgenic mustard development. However, transgenic mustard with osmotin gene has been developed and has undergone physiological testing in response to drought and salinity stresses under standard phytotron conditions. The transgenics performed significantly better than the wild type plants in terms of improved plant growth, chlorophyll retention and relative leaf water content. Recently, we have optimized plastid transformation system in *B. juncea,* and transplastomic plants have been developed with *ots*BA operon cloned from *E. coli* for improved tolerance to abiotic stresses. We describe in this paper only the results obtained with transgenic mustard over-expressing osmotin.

Key words: Indian mustard, *Brassica juncea*, Transgenic, abiotic stress, plastid transformation

Introduction

Abiotic stresses such as salinity, drought, and low and high temperatures severely affect crop productivity. Recently, transgenic technology has come of age and is being currently utilized for developing stress tolerant transgenic crops (Pareek-Singla et al., 2001, Tayal et al., 2004). Indian mustard (*Brasssica juncea)* is an important oilseed crop and covers a large area under cultivation. However, the crop productivity in terms of both seed yield and oil recovery is adversely affected due to salinity and drought stresses. The crop is grown in different agro-ecological regions and is inevitably subjected to different abiotic stresses at different stages of growth and development.

A number of genes control the complex trait of abiotic stress tolerance (Shinozaki et al., 2002). Different categories of genes, including genes encoding for enzymes involved in osmolyte biosynthesis such as proline, mannitol, glycine betaine and trehalose, genes encoding ROS detoxifying enzymes such as superoxide dismutase, ascorbate peroxidase and catalase, and genes encoding stress-induced proteins such as late embryogenesis abundant proteins, antifreeze protein, etc. have been effectively utilized in generating transgenic plants. In addition, genes encoding protein kinases such as calcium dependent protein kinases and transacting factors such as C-repeat binding factors have also been used for developing transgenic plants tolerant against multiple stresses (Tayal et al., 2004, Hazen et al., 2003). Among stress-induced proteins, osmotin was originally identified in NaCl-cultured tobacco cells (Singh et al., 1987). Tobacco cultured cells adapted to 428mM NaCl were found to contain 4 to 30 times higher level of osmotin protein as compared to the unadapted cells, depending on the growth stage. In general, osmotin accumulates to about 10-12% of the total soluble protein and has been found to be associated with salinity tolerance.

The function and mode of action of *osmotin* gene is well documented in providing protection against the fungus, *Phytopthora infestans* that causes late blight in potato (Liu et al., 1994, Zhu et al., 1996). On the contrary, analysis of freezing tolerance in transgenic potato plants expressing sense and antisense genes did not reveal any appreciable role for osmotin. However, transformation of potato with a cDNA encoding osmotin-like protein suggested a role for osmotin in salt tolerance (Evers et al., 1999). We reported previously that transgenic tobacco over expressing osmotin were tolerant to both salt and water stress (Barthakur et al., 2001). In the present paper, we report production of transgenic Indian mustard over expressing the *osmotin* gene. The transgenic plants were analyzed for tolerance to salt, water stresses at different stages of plant growth and development. The transgenics exhibited marked tolerance to salt stress and moderate tolerance to water stress. Our results suggest that stress tolerance in transgenic mustard might be due to the high level of osmotin-induced praline accumulation, which in turn, is helpful in scavenging ROS generated during these abiotic stresses.

Material and Methods

Generation of transgenic plants: The plasmid, pOsm containing tobacco osmotin cDNA driven by the constitutive CaMV35S promoter was used for generating transgenic plants of Brassica juncea (Indian mustard cv. Pusa Jaikisan), through Agrobacterium-mediated transformation system.

Molecular characterization of transgenic plants: Integration of the osmotin gene in transgenic plants was analyzed by Southern analysis. Expression of the osmotin gene was analyzed by RNA gel blot analysis.

Physiological analysis of T1 plants for tolerance to salt and water stresses: The response was analyzed physiologically at seedling stage in aquaculture and at whole plant stages in pot culture under standard Phytotron conditions. The stresses were imposed at seedling stage only on kanamycin resistant four-week-old uniform seedlings, and the wild type plant. Salt stress was imposed by exposing the seedlings to 0.1X Hoagland solution containing 0, 100, 150 and 200mM NaCl. After 6 d of treatment, fresh weight of the seedlings was determined. For creating water stress, the seedlings were exposed to 0.1X Hoagland solution supplemented with 20% (w/v) PEG-6000. After 3 d of stress treatment, fresh weight of the seedlings was recorded. To examine the low temperature stress tolerance, four-week-old uniform seedlings were exposed to 4°C for one week at 70 $\mu E.m^{-2}s^{-1.}$

The response of transgenics to different stresses at whole plant level was studied in three representative samples from each T1 line. Salt stress was created by applying 0.1X Hoagland solution containing 200 mM NaCl to the plants every third day. 18 d after the treatment, the plants were flooded with distilled water twice at a gap of 72 h and normal Hoagland solution was applied there after until maturity of the plants. Chlorophyll fluorescence emission from the third uppermost leaf from the stressed as well as non-stressed plants was measured using PAM-2100 Walz fluorometer (Effeltrich, Germany). Also, total chlorophyll and free proline content was measured. Water stress at whole plant level was imposed by withholding water supply to plants for 5 d. Photosynthetic rate was measured on 3rd upper most leaf of each plant by using an Infrared Gas Analyzer (LiCOR, USA) in water stressed as well control plants that were watered regularly. Relative water content of the stressed leaves from each plant was estimated as described (Bansal and Nagarajan, 1987).

Analysis of T1 plants for tolerance to oxidative stress: The transgenic plants were analyzed for tolerance to oxidative stress caused by methyl viologen and hydrogen peroxide through leaf discs assay. For H_2O_2-mediated stress, leaf discs of 1cm diameter were punched from the youngest fully expanded leaves of the OmB16-7 and wild type plant, and floated on 0.1 mM and 1 mM H_2O_2 under continuous illumination (120 $\mu E.m^{-2}s^{-1}$) for one week. For methyl viologen-induced oxidative stress, leaf discs were treated with 0.1 and 1 µM methyl viologen containing 0.1% (v/v) Tween 20 as surfactant and incubated in dark for 16 h followed by exposure to light (120 $\mu E.m^{-2}.s^{-1}$) for 26 h. Visible differences between the transgenics and wild type were recorded after the stress treatment.

Results

Production of transgenic plants and their molecular analyses: An osmotin cDNA driven by CaMV 35S promoter was introduced and over-expressed in B. juncea cv. Pusa Jaikisan through Agrobacterium-mediated transformation. Fig. 1 shows RNA hybridization of ten selected lines. Significantly high level of expression was, however, observed in three transgenic lines, OmB5, OmB16 and OmB18 as compared to rest of the transgenics. The inheritance of the osmotin gene was analyzed in T1 plants. The number of loci of T-DNA integration ranged from one to three in independent transgenic lines. DNA from wild type plant did not show any hybridization signal. DNA hybridization pattern of T1 transgenic plants, OmB1-2, OmB5-7, OmB16-6, OmB18-3, OmB24-1, OmB25-3, and OmB26-5 confirmed single copy gene integration as well as inheritance to the next generation (Fig. 1a). Expression pattern of only three transgenic lines, OmB5, OmB16 and OmB18, which showed higher expression at T0 level, was again estimated at T1 stage by RNA hybridization (Fig. 1b). The results reaffirmed high level of osmotin expression in these lines in T1 generation. These three lines were chosen for subsequent physiological analyses.

Response of transgenics to salt stress: To test the ability of the T1 seedlings to withstand salinity, stress was imposed by allowing the kanamycin resistant progeny seedlings of the transgenics to grow in Hoagland solution containing varying concentration of NaCl (100, 150 and 200 mM). At 100 mM NaCl, there was no significant difference in growth of wild type and transgenic seedlings, whereas at 150 mM and 200 mM NaCl, growth of the transgenic seedlings was significantly better as reflected by differences in their fresh weight. At 200 mM NaCl, there was approximately 52% decrement in the weight of wild type seedlings whereas in case of transgenics, this decrease ranged from 28-41%. The tolerance of transgenics to salt stress was further confirmed at whole plant level. When 200 mM NaCl was supplied to the whole plants for 18 d, the wild type plants were severely affected by the salt stress and showed necrosis. On the contrary, the transgenic plants were comparatively healthy with negligible necrotic symptoms (Fig. 2).

Response of transgenics to water stress: The response of whole plant to water stress was studied by withholding water supply for 5 d. After 5 d of water stress, leaves of the wild type plants wilted and started drooping, whereas most of the progeny lines of transgenic plants remained relatively healthy (Fig. 2). Transgenic plants maintained significantly higher RWC than the wild type plant. Measurement of leaf photosynthesis rate also supported these results. The rate of leaf photosynthesis on an average was 34% higher in transgenic plants under stress conditions except in OmB18. The proline content in transgenics varied between 15 to 30% as compared to the wild type plants under water stress conditions.

Response of transgenics to oxidative stress: Oxidative stress was induced by incubating the leaf discs from OmB16-7 as well as wild type plant with either methyl viologen or hydrogen peroxide. After one week of 1.0 mM H2O2-mediated oxidative stress, leaf discs from the transgenic plant remained green and healthy, whereas the wild type leaf discs turned yellowish, indicating damage to the photosynthetic machinery. Similar results were obtained when the leaf discs were exposed to 1.0 µM methyl viologen. No significant visible difference was observed between the leaf discs of wild type and transgenic plants treated with 0.1 mM H2O2 or 0.1 µM methyl viologen (data not shown).

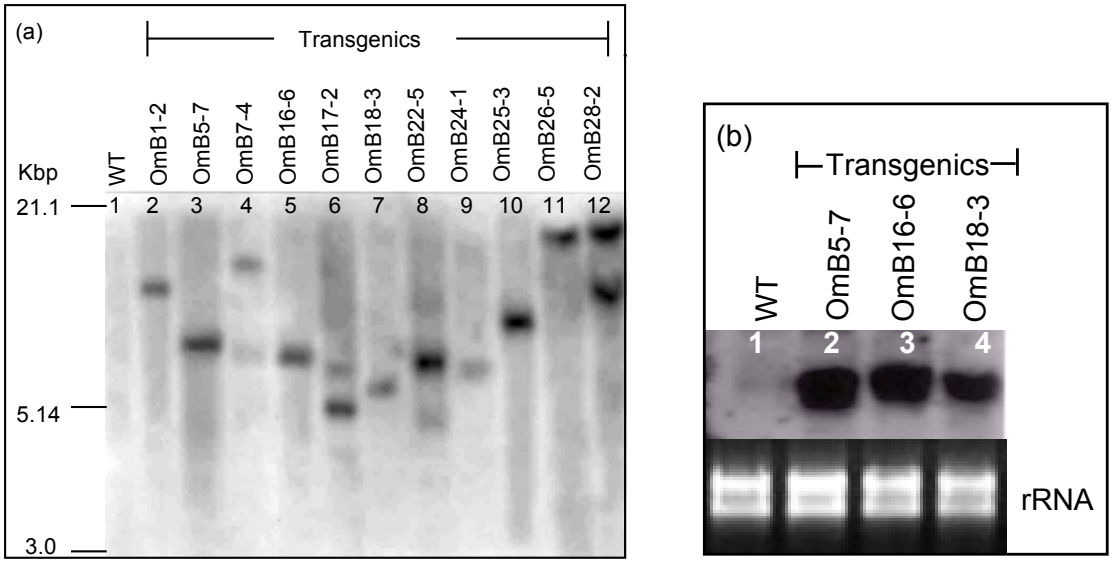

Fig. 1. Molecular analysis of selected T1 transgenic lines. **(a)** Southern blot analysis using osmotin cDNA as a probe. Lane 1 : Wild type, Lanes 2-12: Transgenic showing transgene integration and inheritance, **(b)** Expression analysis by northern hybridization in three selected T1 lines using osmotin cDNA as a probe. Lane 1 : Wild type, Lane 2 : OmB5-7, Lane 3 : OmB16-6, Lane 4 : OmB18-3

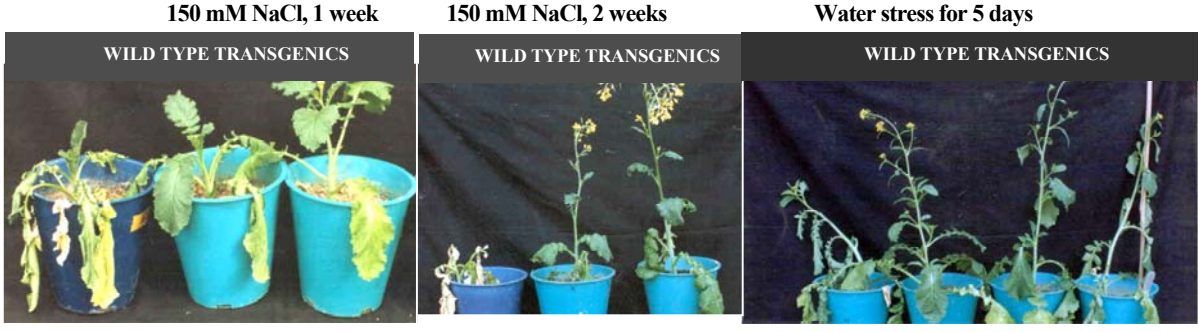

Fig. 2. Response of transgenic osmotin mustard to salt (150mM NaCl) and water stress (5 days of no watering)

Discussion

We have developed transgenic lines of B. juncea (cv. Pusa Jaikisan) that over express osmotin under the control of CaMV35S promoter, and have assessed their tolerance to salt, water and oxidative stresses. Comparative analysis revealed that the transgenics were relatively more robust and performed better under salt stress as compared to water stress. Out of the three independent lines tested, OmB16 showed maximum tolerance at all stages under salt stress.

In general, young seedlings of Brassica juncea are highly susceptible to NaCl stress (Alia et al., 1992). Osmotin transgenic seedlings exhibited significantly better growth as compared to the wild type above 100mM NaCl over a period of one week, which are otherwise inhibitory to wild type seedlings. Exposure of plants to 200mM NaCl at whole plant stage maintained the capability of transgenic plants to photosynthesize by maintaining the chlorophyll content and PSII integrity (data not shown). The osmotin gene has been reported to be induced by salt, water and low temperature stress (Zhu et al., 1993). Our earlier studies revealed that the over expression of osmotin in tobacco enabled the transgenics to perform significantly better than the wild type plants when subjected to either salt or water stress (Barthakur et al., 2001). On the contrary, Zhu et al. (1996) failed to detect any tolerance against freezing stress in transgenic potato developed with osmotin gene. In our previous report, we have shown significantly higher accumulation of free proline in transgenic tobacco plants over expressing osmotin gene as compared to wild type plants both under control and stress conditions. Similar increase in free proline was observed in the osmotin transgenic Indian mustard in the present study (data not shown). Previous studies have shown enhanced proline accumulation in transgenic tomato ectopically expressing Arabidopsis CBF1 transcription factor gene (Hseih et al., 2002] and in transgenic Arabidopsis over expressing DREB1A (CBF3) gene (Gilmour et al., 2000). It appears that similar to CBF1 and CBF3, osmotin plays a role directly or indirectly in inducing the expression of genes encoding enzymes of proline biosynthesis or repressing the genes responsible for proline degradation.

Further analysis of osmotin transgenic plants will help elucidate the mechanism of osmotin action in providing tolerance to salt and water stress. Nevertheless, transgenic mustard plants produced in this study with enhanced ability to grow under long periods of NaCl stress treatment provide a way of achieving significant yield gains in the salinity affected areas.

References

1. Pareek-Singla S.L., Reddy M.K., Sopory S.K. (2001). Transgenic approach towards developing abiotic stress tolerance in plants, Proceedings of Indian National Science Academy **5**, 265-284.
2. Tayal D., Srivastava P.S., Bansal K.C. (2004). Transgenic Crop for Abiotic Stress Tolerance, in: Srivastava P.S., Narula A., Srivastava S., (Eds.), Plant Biotechnology and Molecular Markers, Kluwer Academic Publishers. , 346-365.
3. Shinozaki K.Y., Kasuga M., Liu Q., Nakashima K., Sakuma Y., Abe H., Shinwari Z.K., Seki M., Shinozaki K. (2002). Biological mechanisms of drought stress, JIRCAS working report, 1-8.
4. Hazen S.P., Wu Y., Kreps J.A. (2003). Gene expression profiling of plant responses to abiotic stress, Functional and Integrated Genetics **3,** 105-111.
5. Singh N.K., Bracker C.A., Hasegawa P.M., Handa A.K., Buckel S., Hermondson M.A., Pfenkoch E., Regnier F.E., Bressan R.A. (1987). Characterization of osmotin: A thaumatin-like protein associated with osmotic adaptation in plant cells, Plant Physiology **85**, 529-536.
6. Liu D., Ragothama K.G., Hasegawa P.M., Bressan R.A. (1994). Osmotin over expression in potato delays development of disease symptoms, Proceedings of National Academy of Sciences U.S.A. **91**, 1888-1892.
7. Zhu B., Chen T.H.H., Li P.H. (1996). Analysis of late-blight disease resistance and freezing tolerance in transgenic potato plants expressing sense and antisense genes for an osmotin like protein, Planta **198,** 70-77.
8. Evers D., Overney S., Simon P., Greppin S., Hausman J.F. (1999) Salt tolerance of *Solanum tuberosum* L. overexpressing a heterologous osmotin-like protein, Biologia Plantarum **42**, 105-112.
9. Barthakur S., Babu V., Bansal K.C. (2001). Over-expression of osmotin induces proline accumulation and confers tolerance to osmotic stress in transgenic tobacco, Journal of Plant Biochemistry and Biotechnology **10**, 31-37.
10. Bansal K.C., Nagarajan S. (1987). Reduction of leaf growth by water stress and its recovery in relation to transcription and stomatal conductance in some potato (*Solanum tuberosum* L.) genotypes, Potato Research **30**, 497-506.
11. Alia, Mohanty P., Saradhi P.P. (1992). Effect of sodium chloride on primary photochemical activities in cotyledonary leaves of *Brassica juncea*, Biochem. Physiol. Pflan., **188, 1**-12.