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Over-expression of Osmotin Induces Proline Accumulation and Confers Tolerance to Osmotic Stress in Transgenic Tobacco

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Osmotin has been implicated in conferring tolerance to drought and salt stress in plants. We have over-expressed the osmotin gene under the control of constitutive CaMV 35S promoter in transgenic tobacco, and studied involvement of the protein in imparting tolerance to salinity and drought stress. The transgenic plants exhibited retarded leaf senescence and improved germination on a medium containing 200mM NaCl. Further, the transgenics maintained higher leaf relative water content (RWC), leaf photosynthesis and free proline content than the wild type plants during water stress and after recovery from stress. When subjected to salt stress (200mM NaCl), the transgenic plants accumulated significantly more proline than the wild type plants. These results suggest the involvement of the osmotin-induced increase in proline in imparting tolerance to salinity and drought stress in transgenic plants over-expressing the osmotin gene.

Key words: osmotin, transgenic, proline, salinity, drought.

Osmotin is a basic 24 kD protein that was originally identified in tobacco cells adapted to NaCl and desiccation. The stress-induced synthesis and accumulation of the osmotin protein is correlated with osmotic adjustment in tobacco cells. The synthesis of osmotin is induced by ABA which accumulates in response to osmotic stress and subsequently plays a pivotal role in osmotic adjustment. However, the physiological function of osmotin and its possible involvement in osmotic adjustment in stress adapted cells remain unknown and need further investigation. It has been hypothesised that the osmotin protein could induce synthesis and accumulation of certain solutes, or could be involved in metabolic or structural changes in cells (1).

To gain insight into the function of this stress-induced protein, Singh *et al* (2) cloned the osmotin gene from tobacco and its expression was studied in response to several abiotic and biotic stress factors (3,4). Studies on the regulation of the osmotin gene promoter carried out using *GUS* gene showed that expression of the reporter gene increases significantly in response to osmotic stress in roots, stems and mature leaves. However, it remains to be determined whether the increased expression of the osmotin gene in terms of both mRNA and protein abundance in response to osmotic stress in plants is a general stress response or that the osmotin protein accumulation confers tolerance to osmotic stress caused by drought and salinity. To study this and to elucidate the possible functions of the osmotin protein in conferring stress tolerance, we have over-expressed the osmotin gene constitutively in transgenic tobacco under the control of CaMV 35S promoter We found that over-expression of the osmotin gene induced proline accumulation in unstressed as well as stressed plants and imparted tolerance to both salinity and drought stress.

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Materials and Methods

Construction of plasmid for transformation of tobacco — A 0.75 kb osmotin cDNA from tobacco (5) was cloned into *Bam*HI and *Xba*I site of the binary vector BinAR under the control of constitutive 35S CaMV promoter (Fig. 1). The resultant plasmid osm/BinAR was mobilized into *Agrobacterium tumefaciens* strain GV2260 by the freeze-thaw method for transformation of tobacco. The plasmid osm/BinAR contains kanamycin resistant gene *npt* II to act as an antibiotic selection marker.

Transformation of tobacco — Leaf disc method as described by Horsch *et al* (6) was used for transformation of tobacco (*Nicotiana tabacum cv* Harrison Special). Calli resistant to kanamycin (250 mg/l) were selected and plants regenerated under selection. Plants

Fig. 1. The osmotin gene construct. The gene is cloned under the control of CaMV 35S promoter in binary vector Bin AR containing *npt*II gene as selection marker.

regenerated from the same resistant callus were regarded as clones of the same line. The regenerated primary transformants were transferred to a mixture of soil and soilrite and grown in growth chambers under 16 h photoperiod at 25 ± 2°C. The putative transgenic plants were subsequently transferred to greenhouse for further molecular and physiological analysis.

Southern, PCR, northern and immunoblot analysis of transgenic plants — Integration of the transferred genes into the tobacco genome and copy number of the osmotin gene were determined by Southern hybridization using the *Hin*dIII and *Xba*I fragment containing the CaMV 35S promoter region of the gene construct shown in Fig. 1. Genomic DNA (10μg) extracted from plant leaves according to Dellaporta's method (7) was digested with *Bam*HI, fractionated on 0.8% agarose gel, and hybridised with ^{32}P- labeled CaMV 35S promoter fragment. Hybridization was carried out according to Sambrook *et al* (8). PCR was carried out using the *npt*II gene-specific primers.

Expression of the introduced osmotin gene in transgenic plants was detected by northern hybridization and SDS-PAGE immunoblots. For northern analysis, total RNA was extracted from leaves using RNAgents RNA isolation kit (Promega), and 20 μg of RNA was fractionated on 0.7% denatured agarose gel, and transferred to nitrocellulose membrane. Hybridisation was carried out using an osmotin cDNA clone according to the method described by Sambrook *et al* (8).

For western blotting, total protein was extracted and estimated by Bradford's method (9). Twenty micrograms of total proteins were electrophoresed on 12% denaturing acrylamide gel, transferred to nitrocellulose membrane using Biorad's Mini Protean transfer apparatus, and overproduction of the osmotin protein was detected by using chicken anti-osmotin antibodies and rabbit anti-chicken antibodies conjuagted to alkaline phosphatase.

Analysis of primary transformants for tolerance against salt stress — The youngest fully expanded leaves were removed from plants, washed with de-ionised water, and leaf discs of 1 cm diameter punched and floated in 200mM NaCl solution for a period of one week in petri plates kept at 25 ± 2°C under continuous illumination ($70\mu E.m^{-2}.s^{-1}$). Phenotypic differences between wild type and the transgenics (Osm2 and Osm3) were observed and estimated by measuring total chlorophyll content according to Arnon (10).

Estimation of leaf relative water content, photosynthetic rate and free proline content — Relative water content (RWC) measurements were made on a fully expanded young leaf from each plant according to the method reported earlier (11). The leaf samples were taken between 1200 to 1400 hours and 10 – 12 discs of 1cm diameter each from the middle portion of the leaf avoiding the midrib were taken. The RWC was estimated from the three potted transgenic lines and the wild type plants after 5 days of water stress and after rewatering the stressed plants. The RWC was also measured in excised leaves after 24 h drying on the laboratory bench (25 ± 2°C). Leaf photosynthetic rate was measured on third leaf from the top of each plant by using a Infrared Gas Analyzer (LICOR, USA). Free proline content in leaves and seeds was estimated by following the protocol of Bates *et al* (12). All measurements were carried out in triplicate.

Seed germination and seedling growth in kanamycin and NaCl supplemented media — To determine the effects of salt stress on seed germination and seedling growth, twenty seeds from wild-type plants, and from each of the transgenic lines were allowed to germinate on NaCl plates as described below.

The seeds were first washed under running tap water in an autoclaved flask with Triton X-100 (0.02% w/v) for 5 min to remove any dust, debris, accumulated particles, etc. The seeds were then surface sterilized in 70% alcohol for 3 min followed by sodium hypochlorite (20% w/v) for 5 min. After several washings with sterile water, seeds were placed in petri dishes containing half strength MS medium (13) supplemented with various amounts of NaCl to give final concentrations of 100 and 200 mM and solidified with 0.7% agarose. Kanamycin was added at a concentration of 250 mg/l. The plates were incubated at 4°C for 48 h in darkness for synchronization of germination. Then they were incubated at 22°C in growth chambers, with daily illumination for 16 h at $70\mu E.m^{-2}s^{-1}$.

Results

Transformation of tobacco with osmotin gene — Transformation of tobacco was carried out with the osmotin gene using the construct as shown in Fig. 1. From about 50 calli, nine primary transformants (T0) that were resistant to kanamycin at 300 mg/l were obtained. Based on Southern analysis (Fig. 2A) that revealed stable integration and single copy insertion of the osmotin gene, four independent antibiotic resistant T0 plants designated as Osm1, Osm2, Osm3 and Osm4 were selected and selfed to generate T1 seeds. The T1 seeds were then germinated on kanamycin enriched basal half strength MS medium (Table 1) and homozygous plants were selected. These homozygous transgenic lines were subjected to further experimentation. Presence of the osmotin gene in the transgenic plants was also detected by PCR amplification of the *npt* II gene using gene-specific primers. An amplified product of about 700 bp was detected (Fig. 2B).

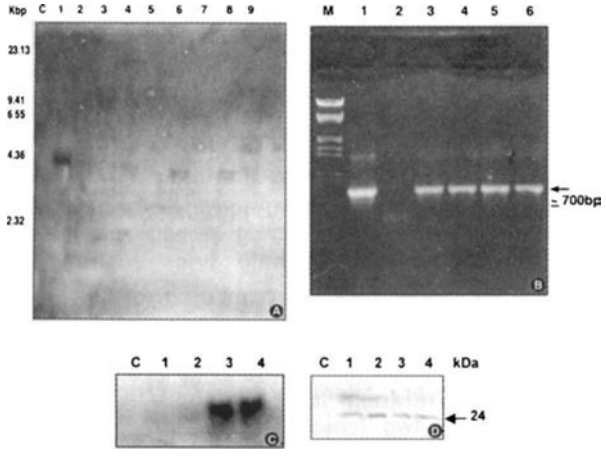

Fig. 2. Molecular analysis of transgenic tobacco plants. **(A)** Southern analysis of transgenic plants. The genomic DNA was digested with *Bam*HI and probed with CaMV 35S promoter fragment. C : wild type control, Lanes 1–9: putative transgenics. **(B)** PCR analysis of plants using *npt* II gene-specific primers. A fragment of about 700 bp corresponding to the coding region of the *npt* II gene was amplified in all transgenics. M:DNA molecular weight marker (λDNA cut with *Hind*III & *Eco*RI), Lane1: positive control – the osmotin plasmid DNA, Lane 2: negative control – wild type DNA, Lanes 3–6 transgenics Osm 1–4. **(C)** Northern analysis of transgenic plants. Total RNA was extracted from leaves and hybridized with osmotin cDNA C: wild type control, Lanes1–4: transgenics Osm 1–4. **(D)** Immunoblot analysis of transgenic plants. Total protein was extracted from leaves, electrophoresed on SDS-PAGE and overproduction of osmotin was detected by anti-osmotin antibodies. C: wild type control, Lanes 1 – 4: transgenic plants Osm 1–4.

Table 1. Germination of transgenic T1 seeds in medium containing 250mg/l kanamycin

Genotype	Total seeds sown	Seeds germinated	Seeds not germinated	Ratio
Osm 1	36	27	9	3.0 :1
Osm 2	37	29	8	3.6 :1
Osm 3	44	34	10	3.4 :1

Over-expression of the osmotin gene was confirmed by RNA blot hybridization of the total RNA isolated from the leaves using the osmotin cDNA as a probe. Northern analysis revealed that osmotin transcripts accumulated in varying amounts in the transgenic Osm lines (Fig. 2C). The osmotin transcripts could not be detected from the endogenous gene in unstressed wild type plants (Fig. 2C), probably due to the fact that the osmotin gene promoter is stress inducible.

Overproduction of the osmotin protein in transgenic plants was detected by western blotting using primary antibodies raised in chicken against the tobacco osmotin protein and presence of the 24 kD protein was confirmed in all four transgenic tobacco lines (Fig. 2D). Quantity of the osmotin protein in transgenics in relation to total soluble proteins could not be determined.

Analysis of transgenic tobacco plants for tolerance against salt stress — The primary transgenic plants (T0) were analysed for tolerance to NaCl stress by the leaf disc assay. Five leaf discs punched from the youngest fully expanded leaves were floated in 200 mM NaCl solution. After one week of stress, the leaf discs from transgenic plants remained green whereas, the discs from wild type plant showed almost complete senescence (Fig. 3). The visual phenotype as shown in Fig. 3 was confirmed by estimating total chlorophyll. The transgenic plants had significantly higher total chlorophyll content than the wild type plants when exposed to 200 mM NaCl stress (Fig. 4). These results confirm the role of osmotin in imparting tolerance to salt stress in plants.

To further confirm the ability of transgenic plants overexpressing osmotin to tolerate salt stress, the T1 seeds, obtained after selfing the primary transgenics were germinated in the MS medium supplemented with NaCl (100 and 200 mM).

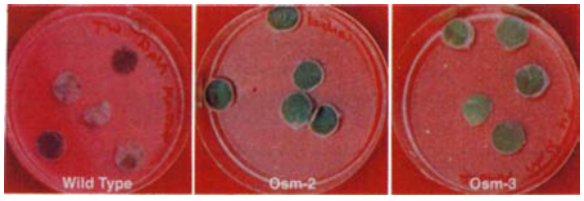

Fig. 3. Difference between wild type and transgenic plants (To) in their response to salt stress. Leaf discs, each of 10mm diameter, from wild type and transgenics Osm 2 and Osm 3 lines were floated in 200 mM NaCl solution for one week in an incubator illuminated (70 $\mu E.m^{-2}.s^{-1}$) continuously at 25±2°C.

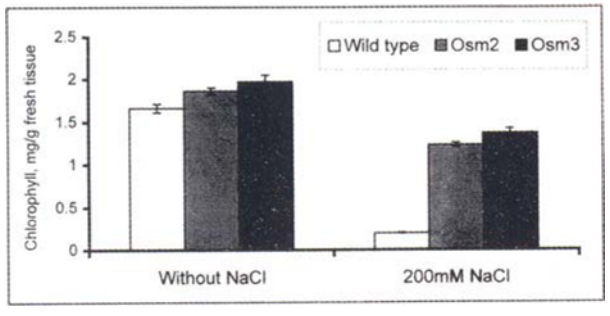

Fig. 4. Total chlorophyll content in wild type and transgenics Osm 2 and Osm 3 leaf discs subjected to salt stress, 200 mM NaCl

Fig. 5 shows the differences in germination under salt stress between the wild type and transgenic plants. While almost all seeds germinated in the Osm2 transgenic line in 100 and 200 mM NaCl, negligible germination was detected in the wild type seeds. The transgenic seeds that germinated under NaCl stress grew well and developed a good root system (data not shown). This further supports a correlation between osmotin overe-xpression and tolerance against salt stress.

Analysis of transgenic tobacco plants for tolerance against water stress — The homozygous T1 seeds of Osm1, Osm2 and Osm3 lines and wild type seeds were sown in pot culture in the green house (30°C/ 20°C, 500 - 700 $\mu E.m^{-2}.s^{-1}$ and 12 h photoperiod). Water stress was imposed in 30-d-old potted plants by withholding water supply for 5 days. In a separate treatment, the youngest fully expanded leaf was excised and dried for 24 h at the laboratory bench (25±2°C) to create dehydration stress in each of the three transgenic and wild type plants. Data on the RWC in both kinds of stressed leaves were recorded. Leaf

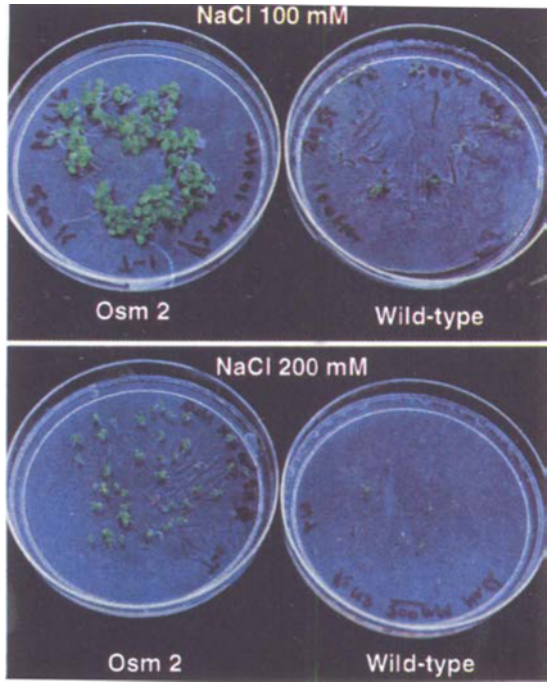

Fig. 5. Difference in germination between wild type and Osm2 transgenic seeds (T1) on MS medium containing 100 mM or 200 mM NaCl.

photosynthesis as an index of stress tolerance (14) was measured daily during stress and after stress-recovery in the potted transgenic Osm2 and the wild type plants.

All the three transgenic lines maintained a significantly higher RWC as compared to the wild type plants (Table 2). In addition, the transgenic plants recovered better (after 5 d stress) and showed a near complete recovery after rewatering of the plants. On comparing the two types of water stress treatments, it was observed that the potted stressed leaves maintained a much higher RWC than the excised 24 h dried leaves in both wild type and transgenic plants (Table 2). This suggests that higher RWC in transgenic stressed plants as compared to wild type plants could possibly be due to an osmotin-mediated increase in leaf turgor.

Fig. 6 shows the difference in the rate of leaf photosynthesis between wild type and the transgenic Osm2 lines during water stress and recovery from stress. A steep decline occurred in leaf photosynthesis just 2 days after water stress and continued until the 5^{th} day of stress in the wild type plants. However, the transgenic Osm 2 plants exhibited no such decrease (Fig. 6). On rewatering the stressed plants, the leaf

Table 2. Effect of water stress on relative water content (%) in leaves of wild type and transgenic tobacco

Treatment	Genotypes			
	Wild type	Osm 1	Osm 2	Osm 3
Control (excised leaf)	81.6 ± 0.70	85.7 ± 0.80	91.7 ± 0.70	91.5 ± 0.33
24 h bench drying	40.8 ± 0.43	59.8 ± 0.42	63.6 ± 0.60	68.1 ± 0.54
Water stress (5 days)	60.9 ± 0.36	83.1 ± 0.23	86.0 ± 0.04	89.6 ± 0.29
Stress recovery	74.6 ± 0.28	88.3 ± 0.16	91.6 ± 0.20	91.7 ± 0.04

Note : Values shown here represent the mean ± S.E of three independent measurements.

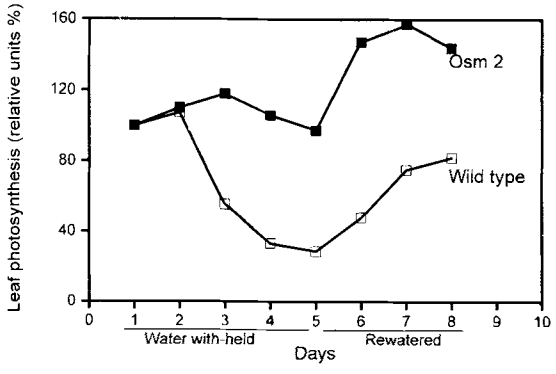

Fig 6. Leaf photosynthetic rate in wild type and Osm2 transgenic plants (T1) measured during water stress and after re-watering the stressed plants.

photosynthesis picked up in both wild type as well as transgenic plants. However, interestingly, the photosynthesis increased much more in the transgenic Osm 2 lines and even attained a rate higher than the first day value in unstressed wild type or transgenic plants (Fig. 6). These results provide some evidence to a unique physiological role of osmotin in defence against water stress.

Over-expression of osmotin and proline accumulation — To gain insight into the physiological function of osmotin in imparting stress tolerance, we decided to measure proline content in the transgenic tobacco lines (T1) over-expressing osmotin, since proline is known to improve plants' ability to withstand drought or salt stress (15-17). Interestingly, 2 to 6 fold increase in proline content, over the wild type plants, was observed in transgenic plant leaves as well as seeds without being subjected to stress (Fig. 7). Among the

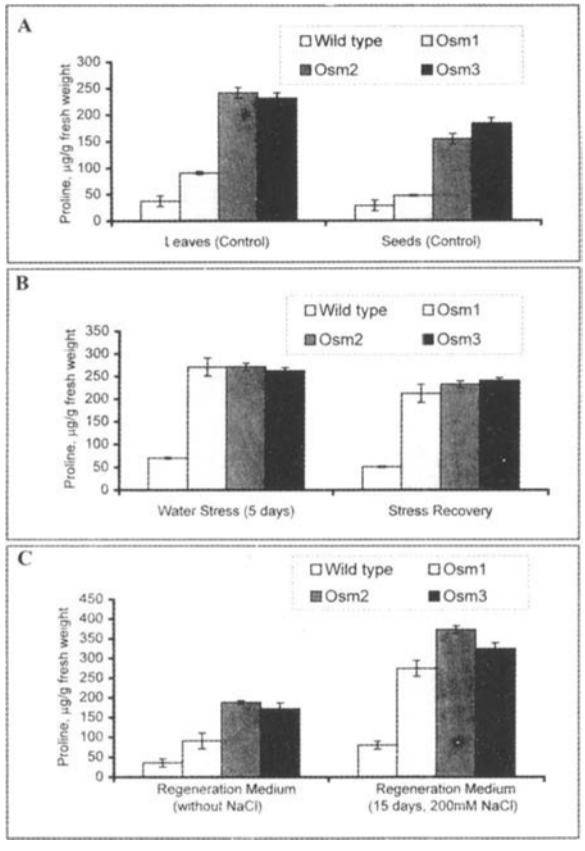

Fig. 7. Free proline content in wild type and transgenics Osm1-3 plants. **(A)** Proline content in unstressed control leaves and seeds, **(B)** proline content after 5 days of water stress and after recovery from stress, **(C)** proline content in leaves from *in-vitro* plants raised without and with 200mM NaCl.

transgenics, the Osm2 and Osm3 accumulated relatively higher proline content. The proline content in these two transgenic lines increased only marginally when subjected to water stress (5 days of no watering); however, the wild type plants and the Osm1 line exhibited 2 to 3 fold increase in response to water stress. On rewatering

the stressed plants, the proline content remained at a higher level in all the transgenic lines as against the wild type plants, in which a reduction was noticed. In contrast to the increase observed in water stressed transgenic plants, the free proline content increased significantly more in the transgenic lines subjected to salt stress (15 days on 200 mM NaCl). This resulted in more accumulation of proline in salt stressed than in the water stressed transgenics; maximum accumulation being noticed in the Osm 2 transgenic line. While the reasons for this high accumulation of proline in salt stressed transgenics remain unknown, it is, however, evident from these results that there is a constitutive osmotin-induced build up of free proline content in the transgenic plants.

Discussion

Osmotin protein has been shown to be associated with tolerance to drought and salinity stress in plants by numerous studies (1-4). However, a direct evidence to show that osmotin is involved in imparting stress tolerance is lacking. Further, physiological function of this basic stress protein remains unknown. Here, we report through transgenic approach the direct involvement of osmotin in conferring tolerance to salinity and drought stress in transgenic tobacco over-expressing the osmotin gene. We also report for the first time that overproduction of osmotin induces free proline accumulation in unstressed as well as stressed plants which presumably leads to enhanced stress tolerance in the transgenic tobacco.

The transgenic approach serves dual purpose of conferring stress tolerance in plants and assessing the physiological function(s) of the over-expressed protein. Synthesis of osmotin is induced by ABA which is involved in adaptation of cells to salt stress and is instrumental in osmotic adjustment (18). It has been hypothesised that osmotin could be involved in osmotic adjustment of plants under stress either by facilitating the accumulation or compartmentation of solutes (1). Our results support this hypothesis and points out that the solute that accumulates in response to overproduction of osmotin in plant cells is proline. It is at the moment, however, not clear that what could be the mechanism(s) of this osmotin-induced proline accumulation. Possibilities could be that osmotin induces proline biosynthesis by activating the Δ^1pyrroline-5-carboxylate synthetase (P5CS) enzyme that catalyses the rate limiting step in proline biosynthesis (15,19), by repressing the proline catabolic pathway, or by suppressing feed back inhibition of proline biosynthesis (20). Further, it may be pointed out that osmotin is a proline-rich protein (1) and its degradation could also possibly lead to increased accumulation of proline, at least under conditions when the protein is over-produced, as observed in the present study. Furthermore, if the osmotin protein has no function to perform in normal unstressed plants because its expression is stress-induced, there is a possibility of its degradation. However, it remains to be determined whether the osmotin protein is maintained in our tobacco transgenic plants at high levels throughout the plant growth or gets degraded to further increase the proline content. Interestingly, two bean cell wall proteins, p33 and p36, found recently to accumulate in response to water stress are proline-rich (21). We know that proline accumulates to high levels in water stressed plants and besides *de novo* synthesis, proline levels could also increase in stressed plants due to the degradation of these proline-rich proteins. The hypothesis that proline could accumulate in plant cells due to the degradation of proline-rich proteins finds support from studies where it has been shown that proline accumulation in developing grapevine fruits is independent of and not associated with either increases in steady-state levels of P5CS mRNA or proteins or a decrease in steady-state levels of proline dehydrogenase protein (22). Results reported in this study have clearly suggested that increase in proline levels in unstressed plants could be due to some unknown physiological mechanisms that are different than the ones operative during the stress-induced proline accumulation. We thus hypothesize based on over-production of the proline-rich protein osmotin (this study) and accumulation of proline-rich, p33 and p36 proteins during water deficit (21) that increase in proline in plants could be due to degradation of these proteins. However, more experiments are clearly needed to determine the exact role of these proline-rich proteins and free proline in stress tolerance.

The slow rate of senescence, better chlorophyll stability and improved germination of T1 transgenic tobacco over-expressing osmotin (Figs. 3-5) are probably due to the osmotic adjustment effect of proline in addition to other unknown effects of osmotin and its role in conferring stress tolerance to plants. Proline has been earlier shown to act as a compatible osmolyte and its increased production confers osmotolerance in transgenic plants (15-17). With regard to osmotin,

numerous other studies have attempted to determine its physiological function in stress tolerance (3,4). It could act as storage protein or as molecular chaperones. It might function in water retention and ion sequestration as well. Since our transgenic tobacco was able to exhibit increased photosynthesis as well as higher RWC during water stress (Fig. 6, Table 2), it is possible that the osmotin protein is involved in stabilizing PSII activity, maintaining high stomatal conductance and / or better root development. That osmotin protein could play a role in the development of well developed root system through increased proline content, gets support from the work of Kavikishor *et al* (15) wherein transgenic tobacco over-expressing P5CS for increased proline production had better root growth than the wild type plants. Clearly, more experiments are needed to confirm this and to determine precisely the role of osmotin in conferring tolerance to drought and salinity stress in plants. Experiments are in progress in our laboratory with more transgenic crops over-expressing osmotin to elucidate mechanism and the physiological function of this stress-induced protein in stress tolerance.

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References

1 **Singh NK, Bracker CA, Hasegawa PM, Handa AK, Buckel S, Hermodson MA, Pfankoch E, Regnier FE & Bressan RA,** *Plant Physiol,* **85** (1987) 529.

2 **Singh NK, Nelson DE, Kuhn D, Hasegawa PM & Bressan RA,** *Plant Physiol,* **90** (1989) 1096.

3 **LaRosa PC, Chen Z, Nelson De, Singh NK, Hasegawa PM & Bressan RA,** *Plant Physiol,* **100** (1992) 409.

4 **Kononowiez AK, Nelsen DE, Singh NK, Hasegawa PM & Bressan RA,** *Plant Cell,* **4** (1992) 513.

5 **Kumar V & Spencer ME,** *Plant Mol Biol,* **18** (1992) 621.

6 **Horsch RB, Fry JE, Hoff Mann. NL, Eichhollz D, Rogers SE & Fraley RT,** *Science,* **227** (1985) 1229.

7 **Dellaporta SL, Wood J & Hicks JB,** *Plant Mol Biol Rep,* **1** (1983) 19.

8 **Sambrook J, Fritsch EF, Maniatis T,** *Molecular cloning : A laboratory manual,* Cold Spring Harbor Laboratory Press, NY (1989).

9 **Bradford MM,** *Anal Biochem,* **72** (1976) 248.

10 **Arnon DI,** *Plant Physiol,* **24** (1994) 1

11 **Bansal KC & Nagarajan S,** *Potato Res,* **30** (1987) 497.

12 **Bates LS, Waldeen RP & Teare ID,** *Plant Soil,* **39** (1973) 205.

13 **Murashige T & Skoog F,** *Physiol Plant,* **15** (1962) 473.

14 **Bansal KC, Nagarajan S & Gambhir PN,** Xth International Congress on Photosynthesis, Montpellier, France, August 1995 (Abstract).

15 **Kavikishor PB, Hong Z, Miao GH, Hu CAA & Verma DPS,** *Plant Physiol,* **108** (1995) 1387.

16 **Zhu B, Su J, Chang M, Verma DPS, Fan YL & Wu R,** *Plant Sci,* **139** (1998) 4.

17 **Nanjo T, Kobayashi M, Yoshiba Y, Sanada Y, Wada K, Tsukaya A, Kakubari Y, Yamaguchi-Shinozaki K & Shinozaki K,** *Plant J,* **18** (1999) 185.

18 **LaRosa PC, Handa AK, Hasegawa PM & Bressan RA,** *Plant Physiol,* **79** (1985) 138.

19 **Delauney AJ & Verma DPS,** *Plant J,* **4** (1993) 215.

20 **Phutela A, Jain V, Dhawan K & Nainawatee HS,** *J Plant Biochem Biotech,* **9** (2000) 35.

21 **Garcia-Gomez BI, Campos F, Hernandez M & Covarrubias AA,** *Plant J,* **22** (2000) 277

22 **Stines AP, Naylor DJ, Hoj PB & Heeswijack R,** *Plant Physiol,* **120** (1999) 923.