Overexpression of tobacco osmotin gene leads to salt stress tolerance in strawberry (Fragaria × ananassa Duch.) plants

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Transgenic plants of strawberry tolerant to salt stress were produced using Agrobacterium mediated gene transfer technology. Leaf discs of in vitro grown plantlets of strawberry cv. Chandler were used as explants. Agrobacterium tumefaciens strain GV2260 harbouring osmotin gene under the control of CaMV 35S promoter in a binary vector system pBinAR was used in transformation experiments. Transgene integration and copy number were assessed by PCR and Southern hybridization confirming single copy as well as multiple copies of transgene integration in 10 different lines of transgenic strawberry. Expression of osmotin gene was confirmed in transgenic lines T_L3, T_L5, T_L9 using Northern hybridization, while biochemical analysis revealed enhanced levels of proline, total soluble protein and chlorophyll content as compared to the wild plants. Leaf disc assays performed using both wild-type and transgenic plants had shown that these transgenic lines were tolerant to salt stress.

Keywords: Agrobacterium, proline, stress, strawberry, transgenic plants.

Introduction

It is known for a long time that the concentration of proline increases in a large variety of plants under stress, up to 100 times the normal level\(^1\), which makes up to 80% of the total amino acid pool. This dramatic increase occurs over several hours\(^2\) or days\(^3\). Stresses, such as, cold\(^4\), heat\(^5\), salt\(^6\), drought\(^7\), UV\(^8\) and heavy metal\(^9\) cause significant increase in the proline concentration in a variety of plants. The increase in proline content under salt stress has been correlated with the increased activity of \(\Delta\)-pyrroline-5-carboxylate reductases\(^{10}\) and \(\gamma\)-glutamyl kinase\(^{11}\), and with the low activity of the degrading enzymes, viz., proline oxidase and proline dehydrogenase\(^{11}\). Proline is synthesized from glutamate by the catalytic action of enzyme \(\Delta^1\)-pyrroline-5-carboxylate synthase (P5CS) and the over-expression of this enzyme in transgenic tobacco lead to increased levels of proline and consequently improved growth, enhanced biomass production, and flower development under drought and salinity stress conditions\(^{12}\). Accumulation of proline has also been reported in transgenic tobacco over-expressing osmotin gene; hence, osmotin has been implicated in conferring tolerance to osmotic stress\(^{13,14}\). Transgenic plants that are not able to produce proline, have a significantly lower stress tolerance\(^{15,16}\).

Osmotin is a stress responsive multifunctional 24 kDa basic protein\(^{17}\) belonging to PR-5 protein family providing osmotolerance\(^{18–20}\) to plants. Osmotin could be involved in osmotic adjustment by the cells either by facilitating the accumulation or compartmentation of solutes\(^{13}\), or by being involved in metabolic or structural alterations during osmotic adjustment\(^{21}\). Besides, osmotin shows antifungal activity also\(^{22}\), which is correlated with plasma membrane permeabilization and dissipation of the membrane potential of sensitive fungi\(^{23}\). In this paper, authors communicate the development of transgenic strawberry overexpressing osmotin gene constitutively under the control of CaMV 35S promoter and its effect on proline accumulation and salt tolerance.

Materials and Methods

Kanamycin Sensitivity of Leaf Discs

To identify the lethal concentration of kanamycin for effective selection of putative transgenic plants, regeneration medium with MS salts + B\(_5\) vitamins+ 2% glucose + TDZ (4 mg/L) with different concentrations of kanamycin (0-90 mg/L) were tested.

Transformation of Strawberry Plants

Leaf discs of strawberry (Fragaria × ananassa
Duch. cv. Chandler) were transformed with Agrobacterium tumefaciens strain GV2260 containing 732 bp osmotin gene under the control of CaMV 35S promoter, in the binary vector pBinAR. npt II gene was used as a plant selectable marker.

**PCR and Southern Blot Analysis**

Genomic DNA was isolated from in vitro developed plantlets of control and transgenic plants. Integration of the transgene into the plant genome was screened by PCR using osmotin gene specific primer pair. The PCR products were then separated on 0.8% agarose gel.

For Southern blot analysis, genomic DNA (10 µg) was digested with BamH1, resolved on a 0.7% agarose gel, and blotted on a nylon membrane (Roche Diagnostics, Indianapolis). The blot was probed with digoxigenin labeled osmotin at 46ºC. The probe was labeled with digoxigenin using the DIG High Prime DNA labeling and detection starter kit (Roche Diagnostics, Indianapolis). The filter was washed and the probe detected following the manufacturer’s instructions.

**Analyses of Transgenic Plantlets for Tolerance Against Salt Stress**

**Leaf Disc Senescence Assay**

The leaves of transgenic and wild-type plantlets were removed and leaf segments of 1 cm² were floated in Petriplates having different concentrations (0, 50, 100, 150, 200 mM) of NaCl in deionized milliQ water. Chlorophyll estimation was done from the samples after 7 d incubation at 25ºC under 16-h photoperiod. Total chlorophyll content of the leaves was estimated according to the method of Hiscox and Israelstam.

**Estimation of Chlorophyll, Total Soluble Protein, Proline and Leaf Relative Water Contents in Plantlets**

Transgenic and wild-type plantlets were exposed to different concentrations (0, 50, 100, 150, 200 mM) of NaCl under in vitro conditions. The leaves of these plantlets were then collected after 1 and 2 wk. Chlorophyll, total soluble protein, proline and relative water contents were determined using the methods of Hiscox and Israelstam, Bradford, Bates et al and Barr and Weatherley, respectively. All measurements were carried out in triplicate.

**Measurement of Shoot and Root Length**

At every sampling, plantlets were taken from each treatment and separated into roots and shoots. The length of roots and shoots was measured. All measurements were carried out in triplicates.

**Results**

**Kanamycin Sensitivity of Leaf Discs**

Sensitivity of leaf discs to kanamycin was established prior to actual transformation experiments in order to determine the effective concentration for selection. In the absence of kanamycin, the leaf discs regenerated normally and produced multiple shoots on the periphery. The shoot regeneration capacity of leaf discs was inhibited even at 30 µg/mL of kanamycin, but they expanded to some extent before bleaching. However, the growth of leaf discs was considerably restricted and bleaching was complete at 50 µg/mL kanamycin within 4 wk. Hence, this concentration was used for selection of transformed cells in leaf discs. Higher kanamycin concentrations were too toxic to leaf discs and caused immediate browning.

**Transformation of Strawberry with Tobacco Osmotin Gene**

Transformation of strawberry was carried out with the osmotin gene using the construct as shown in Fig. 1. This resulted in the regeneration of several putative transgenic shoots, of which only 17 survived and the rest perished in the different stages of their growth and development. The young shoots from these 17 putative transgenic lines were analyzed for osmotin gene presence using PCR. Ten transgenic lines tested positive, whereas wild-type control shoots were negative. Transgenic plants showed 0.73 kb amplification products when amplified with osmotin gene specific primers (Fig. 2). Southern hybridization confirmed single and multiple copy integration of the transgene in different transgenic lines (Fig. 3).

**Analysis of Transgenic Strawberry Plantlets for Tolerance to Salt Stress**

The plants from transgenic line, T₃, were analysed for tolerance to NaCl stress by the leaf disc senescence assay. Leaf discs were floated in NaCl solution (0, 50, 100, 150, 200 mM) and after one wk of stress clear difference was visible as the leaf discs from transgenic plants remained green, whereas the leaf discs from wild-type plantlets showed complete senescence (Fig. 4A,B). The visual phenotype was confirmed by estimating total chlorophyll content, and it was found that transgenic seedlings had significantly higher chlorophyll than wild-type seedlings when exposed to NaCl stress (Fig. 5).
To further confirm the ability of plants from transgenic lines, T\textsubscript{L3}, T\textsubscript{L5} and T\textsubscript{L9} over-expressing osmotin gene to tolerate salt stress, the shoots of transgenic and wild-type seedlings were cultured on MS salts + B\textsubscript{5} vitamin + glucose (2%) + Kn (1 mg/L) supplemented with NaCl (0, 50, 100, 150, 200 mM) (Fig. 6). Variation in chlorophyll and total soluble protein in the leaves of such plantlets was determined at 1 and 2 wk intervals (Figs 7 & 8). As osmotin has been implicated to increase proline content, proline content was measured in the transgenic and wild-type plants. Interestingly, 4 to 6-fold increase in proline content over the wild-type plants was observed in leaves of transgenic plants without being subjected to stress (Fig. 9).
Authors observed significant differences in the growth and development between wild-type and transgenic plants. Shoot length and root length of transgenic plants was greater than that of wild-type plants even after 2 wk of exposure to salt stress (Figs 10 & 11).

Discussion

Of 2445 leaf discs that were co-cultivated with genetically engineered Agrobacterium, only 17 putative transgenic shoots could be regenerated. The young transformed shoots were analyzed for nptII gene presence using PCR. Ten transformants tested positive, whereas untransformed control shoots were negative. These findings are supported by the results obtained by James et al. who had screened 15 kanamycin resistant shoots of strawberry cultivar Rapella, of which only 10 tested positive by Southern hybridization. Of these ten transgenic lines only three lines, which showed single (T13) and double copy (T15, T19) integration, were able to show expression at metabolite levels.

Osmotin gene is induced by salt, water and low temperature stress. With regard to osmotin, numerous studies have been attempted to determine the physiological role in stress tolerance, but the mechanism of its action still remains unknown. As osmotin is a small protein of 24 kDa mol wt, it may have a role in signaling especially in the upregulation of proline biosynthetic pathway. The susceptibility in Saccharomyces cerevisiae to osmotin is controlled by
activation of a heteromeric G-protein and MAP kinase pathway\(^3\), and since a number of abiotic stress factors, such as, wounding, low temperature, high osmolarity, high salinity and reactive oxygen species, act as a signal in activating MAP kinase cascade\(^3\), osmotin could be associated with MAP kinase signaling, regulating the cellular redox state. Identification of osmotin-like protein from intercellular space of a halophyte, *Mesambryanthemum crystallnum* and association of osmotin protein with tonoplast of tobacco\(^2\,^3\,^6\) support the contention that osmotin is involved in intracellular compartmentation of Na\(^+\) ions both to the vacuole as well as to the intercellular space and, thereby, minimizing the buildup of Na\(^+\) ions in the cytoplasm or reducing the uptake of Na\(^+\) ions in the cell\(^6\).

The leaching of chlorophyll during the ‘leaf disc senescence assay’ of strawberry might be due to increased membrane permeability under high NaCl concentration\(^3\), and the difference in the extent of leaching between wild and transgenic strawberry may be due to osmotin’s role in sequestering Na\(^+\) ions both in the vacuoles as well as intercellular spaces and enhancing the stability of membranes.

In general, there was some reduction in the amount of chlorophyll of the strawberry plantlets treated with NaCl. This reduction in chlorophyll could be due to the interference of Na\(^+\) and Cl\(^-\) ions with the enzymes associated with chlorophyll biosynthetic pathway or to a disturbance in the integration of chlorophyll molecules into stable complexes. Transgenic strawberry lines overexpressing osmotin were able to maintain higher chlorophyll content under salt stress probably due to its ability in enhancing their proline content, which in turn significantly lowers the level of reactive oxygen species and alleviates salt stress induced enhancement in ribulose oxygenase activity\(^3\). Maintenance of high chlorophyll content in the leaves of transgenic strawberry plantlets even at high NaCl concentrations is of special significance, as low concentration of chlorophyll has been reported to directly limit the photosynthetic potential and primary productivity in plants\(^3\,^9,^40\).

Protein synthesis is rapidly inhibited by salinity stress\(^41\) and overall protein content is reported to decrease under severe stress, as proteins being macromolecules do not contribute much towards colligative properties like osmolarity. In wild-type strawberry the decrease in protein content can be attributed to the fact that abiotic stresses enhance the protein degradation process as a result of reactive oxygen species generation, increased protease activity and inhibited transcription, translation and polyribosome activity\(^42,^43\). However, since transgenic strawberry plantlets overexpressing osmotin show reduced level of stress even at high salinity, their ability to maintain higher protein content may be due to osmotin-induced proline accumulation, and hence preventing the buildup of proteases by ROS scavenging and membrane stabilization. Proline has been suggested to directly influence protein solvation by preventing dehydration-induced thermodynamic perturbations in proteins\(^44\), which is due to its ability to induce the formation of strong H-bonded water around the proteins\(^45\) under water stressed conditions. The higher protein content of transgenic plants under unstressed condition may be due to both reduced degradation of protein and accumulation of osmotin
protein in response to its overexpression under CaMV 35S promoter.

The results in transgenic tobacco\textsuperscript{13}, tomato\textsuperscript{46} and strawberry (present study) showing improved tolerance to salt stress, in combination with the results showing antifungal action of osmotin\textsuperscript{23,47} indicate the usefulness of osmotin in developing transgenic plants for tolerance against both abiotic and biotic stresses. In particular, transgenic strawberry plants produced in the present study with enhanced ability to grow under long period of NaCl exposure provide a way of achieving a significant yield gain in salinity affected areas.

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**References**

27. Bradford M M, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the