*Chapter 7*

# *Summary*

Sweet potato is a dicotyledonous species belonging to the morning glory family, Convolvulaceae. It is a perennial crop which serves as one of the major sources of food, animal feed and industrial raw materials. It has a significant contribution as energy supplement and phytochemical source of nutrition. It is widely cultivated in the tropics, subtropics and even in some temperate zones of the developing world. Contribution of sweet potato towards health is acknowledged due to high food values content and its anti-carcinogenic and cardiovascular disease preventing properties. Almost all cultivars of sweet potato are excellent source of vitamin C, $B_2$, $B_6$ and E, as well as dietary fiber, potassium, copper, manganese and iron, and are low in fat and cholesterol. However, protein content of most of sweet potato genotypes is reported to be low. Sweet potato is the seventh most important crop in terms of global production. Regardless of the profound breeding emphasis on enhancing productivity and imparting resistance to diseases and insect pests, advancement through conventional breeding is impeded due to the major constrains like incompatibility and sterility associated with hexaploid nature of this crop. In recent years, much attention has given on its genetic improvement(s) due to its lower protein content. Therefore, it is pertinent to use the genetic engineering approach which is a better alternative to guarantee a sufficient supply of quality protein in sweet potato. This intervention will help in meeting the ever-increasing demand for sweet potato, as a healthy food and for revenue generation.

During the past decade, several potential candidate genes associated with nutrient acquisition including AmA1 have been targeted for the nutritional improvement in crops, particularly the protein. However, except *AmA1*, introduction of these genes in target plants has often resulted in an increase in one amino acids at the expense of others, leading to an imbalance of the amino acid profile in transgenic crops. Interestingly, AmA1 has great agricultural importance because it is a well balanced protein in terms of amino acid composition, possessing even better values than recommended by the World Health Organization for a nutritionally rich protein. Sweet potato proteins are deficient in tryptophan and sulphur amino acids whereas, AmA1 is rich in all essential amino acids. Therefore, AmA1 is selected as a promising candidate for nutritional improvement of sweet potato by genetic engineering. This improvement strategy may prove to be more acceptable to the general public than currently used genetically modified crops since AmA1 is an edible crop derived sequence. Our aim was to introduce *AmA1* gene into sweet potato in such a way so that expression will be affected in both constitutive and tuber-

specific manner. For constitutive expression AmA1 was cloned in pSB8 construct under the control of *CaMV-35S* promoter and for tuber-specific expression, *CaMV-35S* promoter in pSB8 was replaced by native *β-amylase* promoter of sweet potato and the construct thus resulted was named as pSB8ß.

An efficient plant regeneration and transformation system is very important and highly desirable for the successful application of genetic engineering. Sweet potato is found to be relatively easy to micro-propagate; however, it is quite recalcitrant to transform. In the present investigation *in vitro* regeneration experiments were carried out via *Agrobacterium tumefaciens* mediated transformation system using leaf, petiole, roots and internodal explants. Different genotypes of sweet potato were also tested for their ability for regeneration and genetic transformation. In the present study to establish a simple, robust, efficient and genotype independent regeneration and transformation system initially different source of explants and genotypes were considered with varying media and hormone combinations. Based on the responses of different genotypes in various culture conditions internodal explants as well as Gamborg's B-5 basal medium supplemented with plant hormone NAA was proved to be a better choice. Further, two genotypes were selected for transformation of *GUS* with internodal explants and *in vitro* regeneration experiments were carried out. A stable transformation system was developed without the addition of exogenous signaling compound, considering the predominant presence of phenolic compounds in sweet potato. In our study we found the induction of root as well as the shoot from a single regenerative act. Furthermore, for the transformation of *AmA1* constructs we selected cv. SP-6 (cv. OFSP-6) due to its better efficacy for genetic transformations. In addition, the transcript accumulation and enzyme activity was also higher in cv. SP-6. While, from pSB8, 18 putative transformants (COE) were obtained, 15 putative transformants were obtained (TOE) in case of pSB8ß on kanamycin selection.

The kanamycin selected putative transformants were examined for successful integration and expression of transgene at genome, transcript and protein level. PCR, northern, qRT-PCR and immunoblot analyses was performed to examine the successful integration as well as the expression of transgene in the transformants. These analyses revealed the successful integration and expression of transgene. Based on PCR as well as immunoblot analysis 13 COE and 15 TOE lines were found to be positive. Transformation efficiency measured as percentage of confirmed

transgenic plants out of total number of plants regenerated. PCR analysis revealed 72% and 60% transformation efficiency in cv. SP-6 and SP-17, respectively which is quite higher than the earlier reports of 20% and 30.8%. Based on the transgene expression at protein level by immunoblot analysis, all the transgenic lines were categorized in three categories *i.e.* low expression, moderate expression and high expression. For further analysis six lines were considered from COE as well as from TOE lines, representing all the three categories. Moreover, reference gene was standardized for the accurate qRT-PCR normalization considering three housekeeping genes *tubulin, GAPDH* and *actin*. *Actin* was found to be the ideal reference gene for the accurate normalization. Further analysis at transcript level revealed a range of 3- to 12-fold higher expression in different COE and TOE lines. To study the developmentally regulated expression of *AmAl* in sweet potato as well as the stability of its expression, a comparative analysis was conducted to check transcript abundance in developing and mature tubers from COE and TOE lines. Both independent lines were considered on the basis of high expression of transgene. Northern analysis revealed higher transcript abundance in COE line than that of TOE lines. Additionally, the expression of *AmA1* was invariably more stable in COE line. This was further correlated by qRT-PCR analysis which revealed no leaky expression in the aerial portion of TOE line. Quantification of the copy number of the *AmA1* gene by absolute quantification in qRT-PCR revealed a low copy number of the transgene per genome. The results of this quantification revealed the presence of a single copy of the transgene in most of the transgenic events with very few having two to three copies. To check transgene effect(s) vis-à-vis to the increase in total protein content at molecular level, a comparative proteomic approach was used via 1-DE as well as 2-DE analyses. 1-DE analysis showed no change in overall protein profile in both the transgenic lines. However, change in total contents of proteins was evident in both the COE and TOE lines than that of wild type. The comparative proteomic analysis of WT and COE lines unraveled several conserved as well as unique expression of proteins, suggesting that subtle changes in the genome might lead to the distinct proteome.

A comparative analysis of metabolites and agrophysiological characterization of transgenic lines vis-à-vis to the wild type was performed to perceive the extent of change(s) in metabolites as well as the agrophysiological aspects of transgenic events. To negate the possibility of natural variations and/or somaclonal variations amongst different transgenic lines, another wild type variety (WFSP-WTThat was a close white-fleshed (WFSP) relative of cv. SP-6

and designated as WFSP-WT. All the transgenic events were grown in parallel with the wild types under the same experimental conditions to investigate the extent of miscellany between them. The purpose of this study was to examine the key question pertaining to any metabolic shift due to the transgene introduction. All the transgenic lines were raised in the genetic background of cv. SP-6 (OFSP-6). No morphological differences were found between both the OFSP-6 and the transgenic events. However, genetic differences between OFSP-6 and WFSP-WT were evident at morphological level. Most of the transgenic events showed a greater yield than that of the wild types which was closely associated with the photosynthetic carbon metabolism. The photosynthetic efficiency of transgenic events showed superior efficacy and a strong correlation was found between the increased leaf areas, photosynthetic rate. Transgene introduction in sweet potato resulted into the increase in total proteins in transgenic tubers as revealed by micro-kjeldahl analysis. Higher content of the total free amino acids as well as individual amino acids in transgenic events further corroborated the increase in total protein. In addition, the increase in amino acids was not limited to essential amino acids which contradicts several of the previous reports wherein introduction of genes encoding seed storage proteins in target plants has often resulted in an increase in amino acids at the expense of others, leading to an imbalance of the amino acid profile. The proximate and biochemical analysis imparted commonalities as well as diversities between the wild type and transgenic lines. The proximate analysis of the wild types and transformants revealed no significant differences in case of moisture, ash and fiber contents. Nonetheless, no considerable change in total carbohydrate content was observed across the transgenic lines. Intriguingly, the reducing sugar content was remarkably higher in wild type counterpart cv. OFSP-6 whereas starch content was higher in the transgenic events. Virtually a similar trend was observed in case of OFSP-6 where the reducing sugar content was considerably lower but the starch content was quite higher when compared to WFSP-WT. This further necessitated the analysis of the degradation pattern of starch in wild types and transgenic lines. Degradation pattern of starch as well as the cellulose was monitored. The analyses revealed that the transgenic lines maintained a constant high starch and cellulose content during the storage stress. The degradation of starch and cellulose altogether indicates the better adaptability of the transformants during storage stress. The overexpression of AmA1 may possibly providing a better adaptability to sustain the basal metabolism in transgenic tubers in such a way that the degradation of stored carbohydrate source (starch and/or cellulose) is lesser

in demand. Moreover, the structure of starch revealed no sign of alteration starch which was further corroborated by FT-IR analysis. In addition, the XRD pattern also advocated in support of SEM and FT-IR analysis.

Metabolomics is becoming an extensively used technology to assess global metabolite levels. With respect to the functional genomics, the non-targeted profiling of metabolites in biological samples is at present considered as a possible complementation to protein and transcript profiling technologies. Transgenic or mutant plants in combination with metabolomics provide an excellent means to look at changes in metabolic networks through the specific perturbation of a gene of interest and vice-versa. A detailed analysis of secondary metabolites in this study revealed a significantly higher content of total phenolics and flavonoids in transgenic lines and that too some extent followed the trend of the expression of *AmA1*. However, elevation in total carotenoids was found to be restricted to higher transgene expressing lines. Nonetheless, total anthocyanin and carotenoids were remarkably higher in cv. OFSP-6 when compared with WFSP-WT. Higher contents of anthocyanin and carotenoids substantiated its higher 'a' and 'b' color space values. Polyphenols are synthesized from the phenylalanine produced by the shikimic acid pathway, and flavonoids are produced through a bifurcation of this pathway. Therefore, increase in overall amino acid content including phenylalanine, in particular may be anticipated as the increased flux of phytophenols. These findings were in agreement with the higher expression of flavonoid pathway genes in the transgenic lines. In addition, metabolome profiling of transgenic tubers in parallel to its wild type counterpart was performed by GC-MS to assess any transgene mediated metabolic shift(s). A detailed comparative analysis of metabolites in transgenic events vis-à-vis to their wild type counterparts revealed no major change in the metabolome.

In conclusion, introduction of AmA1, a seed storage protein has profound effect on the overall nutrient acquisition of sweet potato. The introduction of AmA1 has not only improved the total protein content but also its agrophysiological and biochemical characteristics without a major metabolic shift. Nonetheless, the total carbohydrate content was not affected but the slower degradation of starch and cellulose increased the post-harvest durability of the transgenic tubers thereby increasing its higher consumer acceptability.