Chapter 2

Review of Literature

The improved production of sweet potato is highly desirable since it is a significant industrial material and new energy resource crop as well as food throughout the world. Since sweet potato is a vegetative propagated plant, the molecular breeding based genetic transformation is an important option to improve this crop species. The successful genetic transformation cannot be achieved unless efficient plant regeneration system has been established. A reliable, efficient, robust and reproducible method is the key for efficient production of plants in tissue culture. Genetic engineering offers a great potential for improving disease, pest or stress resistance as well as the nutritional quality of sweet potato. Genetic transformation is a promising tool that can be used in the augmentation and advancement of sweet potato by enabling the introduction of enviable and commercially significant traits into known genotypes, without altering their existing, highly selected genetic background. Significant progress has been made in regeneration, somatic hybridization, gene cloning, genetic transformation and molecular markers in sweet potato. However an efficient plant regeneration and transformation system is very important and highly desirable for the successful application of genetic engineering to sweet potato improvement as it is a hexaploid, highly male sterile and self incompatible crop [157].

2.1 Traditional breading of sweet potato: Problems, strategies, and achievements

An enormous amount of investigation has been carried out to improve overall sweet potato production. However, regardless of the remarkable improvements in sweet potato, incredibly small progress has been made in terms of yield when compared to other crops. Sweet potato includes pre- and postharvest losses from insects, nematodes, and diseases, as well as a whole host of other factors globally [158]. Extensive genetic diversity is observed in sweet potato; therefore, it is difficult to improve by conventional breeding because of being hexaploid ($2n=6x=90$), cross incompatible genes, and having low seed set [159]. Additionally, in many cases it is impossible to cross different lines due to cross incompatibility and frailty of hybrid progeny. In sweet potato, conventional breeding methods have revealed limited advancement regarding the genetic improvement for, resistance to diseases, nematodes, insects, and improvement of nutritional quality, especially in terms of protein contents [160]. The efficacy of *in vitro* regeneration system is the key to the success of plant genetic manipulation. However, substantial studies have been conducted in sweet potato tissue culture, but the lack of a reliable

regeneration system has limited the application of biotechnology [155, 159, 161-163]. Thus, there is a critical need for screening of a large number of genotypes to test their regeneration potential and for their subsequent use in transformation studies.

2.2 Regeneration in sweet potato

Sweet potato is found to be relatively easy to micro-propagate, however it is quite recalcitrant to transform [155]. The regeneration frequency in sweet potato, ranging from 0 to 85% in tested cultivars, is observed to be genotype-dependent [164-171]. It also shows a high genetic diversity and its major anthocyanins and polyphenolic compounds are genotype dependent which could explains, along with other factors, its genotype dependent responses to *in vitro* manipulation [37, 170]. In addition, **Aloufa (2002)** has also reported the cultivar specific regeneration behavioral difference between two distinct sweet potato cultivars [172]. Nevertheless, in most of the cases, plant regeneration at a high frequency has been restricted to one or a few genotypes. Therefore there is a great amount of justification in exploring the possibility towards developing an efficient system of plant regeneration for a wide range of sweet potato genotypes.

2.2.1 Regeneration via different explant sources

There have been various reports of regeneration from a variety of methods using different explant sources and genotypes but with varying degrees of success. Stem internodes, leaves and roots has been used for shoot organogenesis [173, 174]. Some reports are also there concerning leaf segment culture for embryogenic callus formation, and plant regeneration in sweet potato [168, 175] as well as in wild species of sweet potato [176]. A regeneration protocol was established recently using leaf lobe of sweet potato as a source of explant and the effect of 2,4-D on callus induction was investigated [177]. However, reproducibility of these methods was always under question since all of them could not be repeated with plant regeneration. Therefore, a practical and detailed procedure is needed to bridge the blank of leaf segment culture, as it is very simple and valuable culture system for propagation and gene transformation in sweet potato. Regeneration has also been induced directly from adventitious roots from shoot cultures [178], and from de novo roots formed in tissue culture [173, 179].

2.2.2 Somatic embryogenesis: As a route to regeneration

Somatic embryogenesis as a route to regeneration in sweet potato has also been well documented and can be induced from explants such as axillary bud shoot tips [164, 180-183], apical bud meristems, lateral buds and nodal segments of sweet potato and also from stem, petiole, leaf, and root tissues [172, 184, 185]. Regeneration from storage roots has also been demonstrated [186, 187]. In addition to it, several methods have been so far established, concerning the shoot apex culture [175, 188], petiole protoplast culture [189], ovule culture, mesophyll and cell suspension protoplast culture [175, 190] and embryogenic suspension cultures [191] in sweet potato.

2.2.2.1 Regeneration via suspension culture

A protocol of cell suspension culture for regeneration in sweet potato was developed recently by **Sultana and Rahman (2011)** [192]. However, the production of responsive embryogenic tissues is a complicated task that requires substantial time. Once produced, high quality embryogenic tissues must be proliferated and maintained by frequent subculture without loss of their morphogenetic potential [185, 191, 193]. In addition, production of suitable somatic embryogenic tissues is usually cultivar-specific and many sweet potato cultivars were found to be quite recalcitrant [193, 194]. Embryogenic suspension cultures were developed successfully in sweet potato cv. White Star [185, 195, 196]. Further, these strategies were improved and advanced as well as efficient embryogenic suspension culture and plant regeneration methods for a wide range of genotypes, especially for commercial cultivars have been developed [191, 197, 198]. This embryogenic suspension culture system has been extended to over 40 commercial cultivars and exceptionally high frequencies of plant regeneration ranging from 96.8-100% was obtained [199].

2.2.2.2 Regeneration via organogenesis and somatic embryogenesis

Several researchers have reported plant regeneration in different tissues via organogenesis and/or somatic embryogenesis [200-202] nonetheless, the frequency of embryogenic callus formation remained extremely low in the majority of genotypes. **Gong et al. (1998)** had established a plant regeneration procedure through organogenesis and somatic embryogenesis for sweet potato using different explants such as meristem tips, lamina segments,

stems, petioles, storage roots, anthers and ovaries. This method again suffered the same problem of low regeneration frequency [203]. Further, *in vitro* propagation of sweet potato has been developed through organogenesis with different combinations of auxins and cytokinins. The regeneration efficiency from different explants of sweet potato was further improved by using the ethylene inhibitor silver nitrate [204, 205]. Regeneration of three sweet potato accessions was established using meristem and nodal cuttings [206]. An efficient and reproducible method for the regeneration of a South African sweet potato cv. Blesbok is reported by using different hormone combinations and different explants [207].

2.2.3 Regeneration from protoplasts and somatic hybrids

The advancement of protoplast isolation and culture techniques perhaps suggest the most promising approach for developing novel plant hybrids and cultivars, in vegetatively propagated plants like sweet potato in particular [208]. There are limited reports on the development of productive sweet potato plants from protoplasts. First report regarding the successful isolation of protoplasts and formation of callus was by **Wu and Ma (1979)** [209]. A range of tissues including petioles, stems [208, 210, 211], and calli have been used to isolate the protoplasts [212]. However, low regeneration frequency was observed in few cases of regenerated plants from sweet potato protoplast [213, 214]. Consequently, there is a need for improvement of the existing methods to make them applicable across the genotypes.

Some intra and inter-specific somatic hybrids have also been reported [214-216]. The successful application of somatic hybridization to crop improvement is highly dependent upon efficient plant regeneration from protoplasts. Regeneration of sweet potato is also possible from protoplasts as evident from various reports [157, 189, 190, 208, 211, 215], but all these studies reported a low frequency of plant regeneration from protoplast-derived callus. However, high frequency plant regeneration had been achieved in protoplast cultures of its relatives such as *I. triloba*, *I. lacunosa*, and *I. cairica* [213, 217, 218]. The interspecific somatic hybrid of sweet potato cv. Kokei No.14 and *I. triloba* had been produced by fusing petiole protoplasts of two species using the polyethylene glycol (PEG) method [219]. Interspecific somatic hybrids from the fused petiole protoplasts of sweet potato with *I. lacunosa* and *I. triloba*, respectively using similar methods have also been obtained [220, 221].

2.2.4 Anther culture

Production of consistent homozygous lines in a limited time is the major advantage of using haploids in a breeding program. Much advancement has not been witnessed so far regarding the true haploids in sweet potato, but there have been numerous efforts using anthers as the source for generation of callus tissue from which regenerations were developed. Nonetheless, the developed plantlets were not found to be true haploid after downstream analysis, but callus tissues were obtained which were derived from anthers of sweet potato flowers [222-225].

2.3 Sweet potato: A potential candidate for transgene introduction

To develop transgenic plants, it is essential to have reliable methods for efficient production of the plants in tissue culture. An efficient system of embryogenic suspension cultures was successfully developed for a wide range of sweet potato genotypes especially for commercial cultivars but the methods were time consuming and quite difficult to reproduce [191]. There are many approaches hitherto used, to produce transgenic sweet potato especially the electroporation of protoplasts [226, 227] and particle bombardment [228, 229]. In particular, the *Agrobacterium tumefaciens*-mediated transformation system has been widely used because of its efficiency, simplicity and stability of the introduced gene. The regeneration potential and scenario of different methods has been dealt in depth in the following text.

2.3.1 Electroporation

Electroporation offers a unique prospect to introduce DNA, which enters the cells through reversible pores formed in the cell membrane by the action of short electrical pulses. Approach for obtaining transgenic plants via electroporation of naked plasmid DNA in protoplasts/cells has also been used in sweet potato [230-232]. Electroporation has been used to achieve transient as well as stable transformation in protoplasts, and to generate transgenic microcalli [233-235]. Nevertheless, obtaining transformants from these microcalli was proved to be a futile effort [226]. **Dhir et al. (1998)** had transformed successfully and obtained transient gene expression in sweet potato petiole protoplast [157]. Transgenic sweet potato lines expressing the coat protein gene of sweet potato feathery mottle virus (SPFMV) were obtained

electroporation of protoplasts. The transgenic lines were found to be extremely resistant not only to the primary but also to the secondary infection of SPFMV [236].

2.3.2 Particle Bombardment

The biolistic method of particle bombardment has been a major development in direct gene transfer of DNA which has allowed the transformation of several plant species, which were not feasible to *Agrobacterium* or protoplast-based gene transfer techniques. The process is considered to be independent of genotype and the tissue [237]. Earlier sweet potato cv. Jewel and TIS- 70357 were successfully transformed with *GUS* gene by this method. However, efforts to generate somatic embryos or to promote shooting via these transgenic calli have become unsuccessful [163, 228] used this means to transform sweet potato with *β-glucuronidase* gene (*gus*A) and herbicide-resistant gene (*bar*) and a successful transformation events were reported [238]. Furthermore, genes of CuZn superoxide dismutase (CuZnSOD) and ascorbate peroxidase (APX) under the control of an oxidative stress-inducible *SWAP2* promoter was transformed in the chloroplasts of sweet potato plants by biolistic method. This further imparted tolerance to methyl viologen-mediated oxidative stress and chilling [239]. Moreover, green-fluorescent protein (*GFP*) gene was introduced in sweet potato tissues by electroporation as well as particle bombardment using leaf and petiole segments from young *in vitro*-raised plantlets [235].

2.3.3 *Agrobacterium*-mediated transformation

The first successful *Agrobacterium*-mediated transformation protocol for sweet potato was based on the formation of hairy roots using leaf discs as explants by *Agrobacterium rhizogesnes* [240, 241]. However, morphological abnormalities shown by regenerated transgenic plants remain a big question mark for the method. Nevertheless, *A. tumefaciens* mediated transformation in sweet potato has also been well established by several workers [159, 161, 242-244]. Different explants, for instance leaves, petioles, stems, storage roots, and embryogenic calli have been used for *A. tumefaciens*-mediated transformation of sweet potato. However, in general, these procedures have been very genotype-dependant having lower transformation efficiency [245-250], and often difficult to reproduce [159]. Comparatively, apical meristem-derived embryogenic calli can give superior transformation efficiency in sweet potato [251, 252]. However, these are not readily accessible target tissues for most of cultivars owing to the lower

frequency of the formation of embryogenic callus in apical meristem cultures [169]. Consequently, substantial efforts are still required to develop an efficient transformation system in sweet potato. In this regard, *Agrobacterium*-mediated transformation in sweet potato has also been well applied for regeneration via somatic embryogenesis using somatic embryos or organs as explants in the last decade by several workers [187, 250-252]. A simple and efficient plant regeneration system via somatic embryogenesis using leaf segments was established to transform *GUS* and an apomixis-specific gene *ASG*-1 [253]. Embryogenic suspension cultures of sweet potato have also been used to establish an efficient *Agrobacterium tumefaciens*-mediated transformation system [254-256]. **Yu et al. (2007)** transformed sweet potato with *gusA* and hygromycin phosphotransferase II gene (*hpt II*) genes using embryogenic suspension cultures [254]. Transformation of *hpt* and *uidA* gene in 13 elite sweet potato cultivars using suspension cultures by **Yang et al. (2011)** was the extended version of this work [257]. GUS-positive transformants were obtained from embryogenic suspension cultures of sweet potato cv. Lizixiang using *A. tumefaciens* [258]. By *A. tumefaciens* mediated transformation of *oryzacystatin-I* and *nptII* transgenic plants have also been produced via embryogenic suspension subcultures [259]. Also a *de novo* (via callus) rapid genetic transformation system via organogenesis has been developed from sweet potato leaves. Through, an efficient and rapid transformation method has been developed by *A. tumefaciens* mediated transformation using *nptII* and *dihydrodipicolinate synthase* (*dhdps*-r1) gene, coding for a lysine insensitive mutant of the tobacco *dhdps* gene [250]. Earlier, a MADS-box protein cDNA (*SRD1*), isolated from an early stage storage root cDNA library has also been introduced via *A. tumefaciens*-mediated transformation of embryogenic calli, induced from sweet potato cv. Yulmi. *SRD1* mRNA was mainly found in the actively dividing cells, including the vascular and cambium cells of the young storage root. A concomitant increase in endogenous auxins with the transcript level of *SRD1* has been observed, suggesting an involvement of *SRD1* during the initial stages of tuber development in an auxin-dependent manner [2].

2.4 Development of transgenic sweet potato with agronomic importance

Transgenic sweet potatoes have been developed mostly for the nutritional improvements, stress tolerance and herbicides resistance of this crop, to increase their overall agronomic value.

The development of transgenic sweet potato with agronomic importance can be categorized under following subheads:

2.4.1 Transgenic for stress tolerance

Sweet potato is susceptible for various biotic and abiotic stresses. A significant amount of work is done so far for its improvement.

2.4.1.1 Biotic stress

Several transgenic plants have been produced to endure biotic stresses, such as insect [187, 260], weed [247], fungus, virus and nematode resistance [236]. In the field, production of sweet potato herbicides is effective for weed control. **Otani et al. (2003)** introduced the phosphinothricin acetyltransferase (*pat*, *bar*) gene for herbicide resistance. Transgenic sweet potato plants exhibited bialaphos resistance and also showed resistance to the commercial herbicide [247]. The major sweet potato insect pest worldwide, especially in developing countries is sweet potato weevil. Cowpea trypsin inhibitor and snowdrop lectin genes have been introduced into sweet potato cv. 'Jewel' to confer resistance to insect pests such as sweet potato weevil and sweet potato butterfly [187]. Nevertheless, the report did not confirm the insect tolerance of regenerated transgenic plants. Sweet potato cv. 'Jewel' transformed with *cryIIIA*, was found to be less affected by weevils than the wild type control plants in field trials [260]. A major constraint in sweet potato production worldwide is Sweet Potato Feathery Mosaic Virus (SPFMV). Coat protein (CP) and *hpt* genes under the control of CaMV 35 S promoters were introduced in sweet potato and the transgenic lines were observed to be extremely resistant to SPFMV infection [236]. Lately, transgenic lines of sweet potato cv. CPT560 were also transformed with SPFMV *CP* gene was evaluated in the field conditions to evaluate virus resistance, yield and other agronomic characteristics [261]. Furthermore, RNA silencing method has also been used to investigate the manifestation of transitive RNA silencing by graft experiments on SPFMV infection [262]. Further, **Okada and Saito (2008)** also developed transgenic sweet potato using SPFMV *CP* genes, which had shown considerable resistance to SPFMV [263]. Sweet Potato Chlorotic Stunt Virus (SPCSV) is another most important pathogen of sweet potato. As no ways of true resistance to SPCSV are offered in sweet potato germplasm, a pathogen-derived transgenic resistance approach was used as an alternative solution. The

replicase encoding sequences of SPCSV and SPFMV were transformed in sweet potato cv. Huachano and accumulation of transgene-specific siRNA was detected in most of transgenic lines. Nonetheless, none of the transgenic events were immune to SPCSV, but few of them exhibited mild or no symptoms following infection, and accumulation of SPCSV was significantly reduced [264]. Recently, **Sivparsad and Gubba (2014)** has developed transgenic sweet potato (cv. Blesbok) with broad virus resistance in order to address the problem of multiple virus infections and synergism of Sweet Potato Virus G (SPVG), Sweet Potato Mild Mottle Virus (SPMMV), SPFMV and SPCSV. For this coat protein gene segments of SPFMV, SPCSV, SPVG and SPMMV were used to induce gene silencing in transgenic sweet potato [265].

Besides this, several efforts have also been made for antimicrobial and antifungal resistance. Antimicrobial peptides from various organisms have broad range activities of killing bacteria, mycobacteria, and fungi [266]. Plant thionin peptide manifested anti-fungal activities; therefore, a gene for barley α-hordothionin (αHT) was positioned downstream of strong constitutive promoters of E12Ω and a tuber specific ß-amylase promoter of a sweet potato, and introduced into sweet potato cv. Kokei. Transformants showed elevated expression of αHT mRNA in E12Ω:αHT transformants and sucrose-inducible expression in β-Amy:αHT in leaves as well as in tubers [267]. The effort for enhanced stem nematode resistance of transgenic sweet potato was achieved using Oryzacystatin-I (*OCI*) gene by **Gao et al. (2011)** as already described in earlier section.

2.4.1.2 Abiotic stress

Abiotic stresses such as dehydration, salt, low temperature and early frost are also a severe problem globally for sweet potato production. Introduction of fatty-acid desaturase gene to increase the content of unsaturated fatty acid for low temperature tolerance remains an exemplary endeavor in this direction [245]. Since, oxidative stress is one of the most important factors owing to the damage to plants exposed to environmental stresses, manipulation of anti-oxidants can to some extent recompense the damage from multiple environmental stresses. Alleviation of multiple environmental stresses in transgenic sweet potato plants expressing the genes of both *CuZn-superoxide dismutase* (*CuZnSOD*) and *ascorbate peroxidase* (*APX*), as described in earlier, is an effort to mitigate the yield penalty due to abiotic stress in sweet potato

especially via the tolerance to methyl viologen-mediated oxidative stress and chilling [239]. These transgenic lines were also found to be drought tolerant in a separate study [268]. Additionally, these transgenic sweet potato lines were further assessed for their resistance to salt stress by **Wang et al. (2012)** [269]. Isolation and overexpression of the late embryogenesis abundant 14 (*LEA14*) from an EST library of dehydration-treated fibrous roots of sweet potato displayed enhanced tolerance to drought and salt stress, whereas RNAi calli exhibited increased stress sensitivity. In addition, under standard culture conditions, lignin contents increased in *IbLEA14*-overexpressing calli as a consequence of the increased expression of a range of monolignol biosynthesis-associated genes. Stress treatments elicited higher expression levels of the gene encoding cinnamyl alcohol dehydrogenase in *IbLEA14*-overexpressing lines than in control or RNAi lines [270]. Moreover, overexpression of soybean cold-inducible zinc finger protein (SCOF-1) under control of an oxidative stress-inducible peroxidase (*SWPA2*) promoter enhanced tolerance to different low-temperature treatments [271].

In order to increase β-carotene content of sweet potato by the inhibition of further hydroxylation of β-carotene, a partial cDNA encoding β-carotene hydroxylase (*CHY-β*) was cloned from the storage roots of orange-fleshed sweet potato to generate RNAi construct *IbCHY*-β and introduced into cultured cells of white-fleshed cv. Yulmi. The downregulation of *IbCHY-ß* increased β-carotene and total carotenoid contents in transgenic plants, eventually elevating the antioxidant potential [272]. In addition, the impact(s) of the overexpression of an *Orange (Or)* gene, isolated from storage roots of orange-fleshed sweet potato, responsible for the accumulation of carotenoids in plants was evaluated. Besides the increased level of carotenoids, the transgenic calli exhibited better antioxidant activity and increased tolerance to salt stress than that of untransformed calli. Also, the downregulation of the *lycopene ε-cyclase* gene increases carotenoid synthesis via the β-branch-specific pathway and enhances salt-stress tolerance in sweet potato transgenic calli [273, 274].

2.4.2 Transgenic for herbicide resistance

The use of *in vitro* genetic manipulation to generate germplasms with herbicide resistance is an obvious choice since conventional breeding for herbicide-resistant varieties is not possible in sweet potato due to the lack of knowledge of herbicide resistant germplasm [266]. Further, herbicide resistant sweet potato with *bar* gene using a hygromycin selection system has been

reported by **Otani et al. (2003**). Herbicide-resistant sweet potato plants were produced through biolistics of embryogenic calli derived from shoot apical meristems as well as from *Agrobacterium*-mediated transformation system. **Yi et al. (2007)** reported successful introduction of the *bar* gene by particle bombardment that confer herbicide resistance in transgenic sweet potato [275]. *Agrobacterium*-mediated genetic transformation of *bar* gene has also been reported [276]. In order to establish a transformation system for a diverse group of sweet potato genotypes, as well as for herbicide tolerance, an *Agrobacterium tumefaciens* transformation system was used for the introduction of mammalian cytochrome P450 genes in five cultivars of sweet potato [277].

2.4.3 Transgenic for nutritional improvements

Starch is one of the most important products of sweet potato and offers important food processing and industrial materials and the ratio of amylase to amylopectin is an important factor in the textural characteristics of starch. Introduction of full-length sense cDNA of *GBSSI* resulted into the amylase content of transgenic lines [246]. Inhibition of *GBSSI* gene expression through RNA interference has been used more effectively to obtain the amylose-free transgenic sweet potato plants [247]. In recent times, double-stranded RNA-mediated gene silencing has been found to be a useful methodology in the genetic improvement of crops as well as in functional genomic studies. Transgenic sweet potato plants were obtained by RNAi of starch branching enzyme II (SBEII) which had a starch with higher amylose content than that of wild type counterparts [249]. Even amylose-free transgenic sweet potato plants were also produced by inhibiting sweet potato *GBSSI* gene expression through RNA interference [278]. The physiological effects of reducing the activity of starch synthase (SSII) in transgenic plants with reduced expressions of the *SSII* gene have been evaluated. The pasting temperatures of transgenic plants were found to be lower than in wild-type plants [279]. **Tanaka et al. (2009)** characterized the functions of the sweet potato *SRF1* gene, which encodes a Dof zinc finger transcriptional factor expressed preferentially in the storage roots. Transgenic plants overexpressing SRF1 showed considerably superior storage root dry matter content as compared to the wild type, and decreased activity of soluble acid invertase, an enzyme involved in the sugar metabolism suggesting the modulation of carbohydrate metabolism in the storage roots

[280]. Furthermore, a gene encoding a hyperthermophilic α-amylase from *Thermotoga maritima* has also been introduced in sweet potato, with the capacity to self-process the starch [281].

Introduction of alien DNA associated to fatty acid metabolism to modify the fatty acid composition of the lipids, expecting thereby a functional or nutritional improvement is still a naive area. Earlier, a tobacco microsomal ω-3 fatty acid desaturase gene (*NtFAD3*) under the control of the CaMV 35S promoter or an improved CaMV 35S promoter (El2Ω) was introduced into sweet potato. Further analysis of transgenic plants revealed that the expression of *NtFAD3* gene under the control of El2Ω promoter was more robust as compared to the CaMV 35S promoter, thus increasing the linolenic acid content in the transgenic sweet potato plants [247].

Sweet potato is not a rich source of protein, and the quality of the protein is also not high, particularly the content of tryptophan and sulfur amino acids. However, there are very few reports available regarding the improvements in terms of protein. The introduction of *asp-1* gene coding for the ASP-1 protein to increase the protein quality and quantity in sweet potato has been achieved [118]. Nonetheless, the safety of the purified ASP-1 protein and regulatory assessment of the potential toxicity and allergenicity of transgenic proteins is still not available for its potential use [282]. However, two sweet potato lines with low trypsin inhibitor and high protein levels in roots have been developed by conventional means for animal feed and human food applications [283]. Nonetheless, a sustained effort is still required for the nutritional improvement of sweet potato in terms of protein. Sweet potato has several health benefits which are already described earlier. In this regard a mouse adiponectin cDNA was used for developing transgenic sweet potato plants *via Agrobacterium*-mediated transformation. Since adiponectin is exclusively produced and secreted from adipocytes and has a role in obesity, insulin resistance, as well as type-2 diabetes it further strengthen its pharmaceutical applications [284].

A brief account of published studies on the development of transgenic sweet potato is given in **Table 2.1**.

Table 2.1. Review of sweet potato transformations

S.No.	Cultivar	Explant/target tissue	Gene name	Method	Reference
1	Blesbok	Apical shoots	Coat protein gene segments of SPFMV, SPCSV, SPVG and SPMMV	*A. tumefaciens* (LBA4404)	Sivparsad and Gubba, 2014
2	Yulmi	Non-embryogenic calli induced from shoot meristems	*Or gene* (*IbOr*)	*A. tumefaciens* (strain EHA105)	Kim et al., 2013a
3.	Yulmi	Non-embryogenic calli induced from shoot meristems	*LCY-ε*	*A. tumefaciens* (strain EHA105)	Kim et al., 2013b
4.	Miyazakibeni	callus formed from the leaf segments	*ASG*-1 & *GUS* gene	*A. tumefaciens* (strain GV3101/PMP9)	Chen et al., 2013
5.	Shinhwangmi, Yulmi	Tuber, calli	*ß-cartoene hydroxylase*	*A. tumefaciens* (strain EHA105)	Kim et al., 2012
6.	Yulmi	Leaves	*Cu-Zn SOD, APX*	*A. tumefaciens*	Wang et al., 2012
7.	Kokei 14	Leaf and tuber	*α-Hordothionin*	*A. tumefaciens*	Muramoto et al., 2012
8.	Ayamurasaki, Sushu, Wanslu, Xushu	Embryogenic calli induced from apical or axillary buds	*NptII & GUS*	*A. tumefaciens*	Yang et al., 2011
9.	White star	Non-embryogenic calli induced from shoot meristems	*LEA14*	*A. tumefaciens* (strain EHA105)	Park et al., 2011
10.	Jewel	Leaves (lamina	*α-amylase*	*A. tumefaciens*	Santa-Maria

		with petiole)		(EHA 105)	et al., 2011
11.	Yulmi	Leaves	*SCOF1*	*A. tumefaciens*	Kim et al., 2011
12.	Lizixiang	Cell aggregates from embryogenic suspension cultures	*Oryzacystain (OCI)*	*A. tumefaciens* (EHA 105)	Gao et al., 2011
13	5 cultivars	Leaves, petiole, roots, internodes and apical meristems	*Mammalian cytochrome P450*	*A. tumefaciens* (LBA4404)	Anwar et al., 2011
14	Jinhongmi	Embryogenic calli from shoot epical meristems	*MADS-box protein cDNA (SRD1)*	*A. tumefaciens*	Noh et al., 2010
15	Quick sweet		*starch synthase (SSII)*	*A. tumefaciens*	Takahata et al., 2010
16	Kokei 14	Leaves	*Dof zinc fingerTranscription Factor*	*A. tumefaciens*	Tanaka et al,. 2009
17	Huachano	Leaves	*Replicase of SPCSV*	*A. tumefaciens*	Kreuze et al., 2008
18	116 different cultivars		*CP of SPFMV*	Electroporation	Okada and Saito 2008
19	Xu55-2	Embryonic suspension culture from top buds	*SBD2 & NPT II*	*A. tumefaciens* (EHA 105)	Xing et al., 2008
20	Yulmi	Embryogenic callus from shoot meristems	*gusA, CuZuSOD, APX*	Particle bombardment	Lim et al., 2007
21	Kokei 14	Embryogenic callus from shoot	*hpt, GBSSI RNAi*	*A. tumefaciens* (EHA 101)	Otani et al., 2007

		meristems			
22	Lizixiang	Embryogenic callus from shoot apical meristems	*hptII, gusA*	*A. tumefaciens* (EHA 105)	Yu et al., 2007
23	Yulmi	Embryogenic callus shoot apical meristems	*gusA, bar*	Particle bombardment	Yi et al., 2007
24	Yulm	Embryogenic callus from shoot apex meristem	*gusA, bar/pat*	*A.tumefaciens* (EHA 105)	Choi et al., 2007
25	Kokei 14	Embryogenic callus from shoot meristems	*hpt, FSPD1*	*A.tumefaciens* (EHA 101)	Kasukabe et al., 2006
26	Kokei 14	Embryogenic callus from shoot meristems	*hpt, SBEII RNAi*	*A.tumefaciens* (EHA 101)	Shimada et al., 2006
27	Kokei 14	Embryogenic callus from shoot meristems	*hpt, mouse adiponectin cDNA*	*A.tumefaciens* (EHA101)	Berberich et al., 2005
28	Yulmi	Embryogenic callus	*nptII, SOD, APX*	Particle bombardment	Lim et al., 2004
29	Beniazuma	Stem	*nptII, hpt, gusA*	*A.tumefaciens* (EHA 105)	Song et al., 2004
30	Lizixiang	Embryogenic callus from shoot apices	*nptII, OCI*	*A. tumefaciens* (LBA4404)	Jiang et al., 2004
31	Kokei 14	Embryogenic callus from shoot meristems	*hpt, bar*	*A.tumefaciens* (EHA101)	Otani et al., 2003
32	Lizixiang	Embryogenic	*nptII, gusA*	*A. tumefaciens*	Zhai and Liu

		callus			2003
33	Mary Anne	Cell culture	*gusA*	*A.tumefaciens* (LBA4404 EHA105), particle bombardment	Deroles et al., 2002
34	Nanging 51-93	Embryogenic cell suspension culture	*hpt, SPFMV-S CP*	Particle bombardment	Okada et al., 2002
35	Kokei 14	Embryogenic callus from shoot meristems	*hpt, NtFAD3*	*A.tumefaciens* (EHA101)	Wakita et al., 2001
36	Chikei 682-11	Mesophyll protoplasts	*gusA, hpt, SPFMV-S CP*	Electroporation	Okada et al., 2001
37	Beauregard	Protoplast, leaf segments	*GFP*	Electroporation, Particle bombardment	Winfield et al., 2001
38	Kokei 14	Embryogenic callus from the apical meristem	*GBSSI*	*A.tumefaciens* (EHA101)	Kimura et al., 2001
39	Beauregard	Leaves and petioles	*GFP*	Particle bombardment, Electroporation	Lawton et al., 2000
40	Kokei 14	Embryogenic callus from shoot meristems	*hpt, gusA*	*A.tumefaciens* (EHA101)	Otani et al., 1998
41	Jewel	Leaf derived embryogenic callus	*nptII, gusA*	Electroporation	Mitchell et al., 1998
42	Jewel	Leaves	*nptII, cryIIIA*	*A. tumefaciens* (C58C1)	Moran et al., 1998
43	White Star	Embryogenic callus	*nptII, gus*	*A.tumefaciens* (EHA101)	Gama et al., 1996
44	Jewel	Storage roots	*nptII, gusA, cowpea trysin inhibitor, snow-*	*A.tumefaciens* (LBA4404)	Newell et al., 1995

			drop lectin		
45	5 cultivars	Leaves	*nptII, gusA*	*A. rhizogenes* (15834)	Otani et al., 1993
46	Jewel, TIS-70357	Leaves and petioles	*nptII, gusA*	Particle bombardment	Prakash and Varadarajan 1992
47		*In vitro* whole plant	*Synthetic sequence*	*A. rhizogenes*	Dodds et al., 1991

APX-ascorbate peroxidase; cryIIIA – Bacillus thuringiensis endotoxin gene; CuZuSOD- CuZu superoxide dismutase; FSPD1 - Spermidine synthase; GBSS- Granule-bound starch synthase; gus - β-glucuronidase; hpt - hygromycin phosphor nptII - neomycin phosphotransferase; OCI - oryzacystain-I, NtFAD3 - fatty acid desaturase gene [gfp – green fluorescent protein gene; pat/ bar-phosphinothricin N-acetyltransferase; php-, luc–firefly luciferase gene; SBEII – starch branching enzyme; SPFMV-S CP – Sweet potato feathery mottle virus coat protein gene; swpa4-peroxidase SBD2 (starch binding domain 2); SCOF1- Soybean cold-inducible zinc finger protein; Or gene (IbOr)- sweet potato orange gene; LCY-ε- Lycopene ε-cyclase; ASG-1 - Apomixis-specific gene.

2.5 Overexpression of AmA1: An overview

As an initiative in the direction of developing transgenic plants with balanced protein composition, a full-length cDNA that encodes a 35-kDa protein, rich in essential amino acids from *Amaranthus hypochondriacus* was cloned and characterized. It has a single major open reading frame corresponding to a 304-amino acid polypeptide. AmAl is synthesized during early embryogenesis, reaching a maximum by midmaturation and expressed until maturation. Seed storage proteins are localized in protein bodies and are often glycosylated but unlikely AmAl is found to be localized in the cytosol [127]. Since 102 bp of 3' UTR (untranslated region) of *AmA1* was found to be essential for its higher expression therefore, *AmA1* coding sequence along with 102 bp of 3' UTR (untranslated region) was cloned under the control of CaMV *35S* promoter and *GBSS* promoter in order to express it in constitutive as well as in a tuber specific manner respectively. The successful regeneration and transformation with both the constructs in Potato (*Solanum tuberosum* L. cv. A16) was achieved and the transgenic potato revealed an increased

amino acid profile as well as 35–45% increase in total protein. In addition, 3.0- to 3.5-fold increase in tuber yield in terms of fresh weight has also been observed [112]. Expression of AmA1 in *Saccharomyces cerevisiae*, a safe organism with a long history of use for the production of biomass rich in high quality proteins and vitamins was an endeavor which offers the possibility of further improving food and animal feed additives [285]. With an objective of finding an effective way of expressing *AmA1* in yeast, the gene was cloned into an episomal shuttle vector and four diverse promoters were used *i.e.* the glyceraldehyde-3-phosphate dehydrogenase promoter, galactose dehydrogenase 10 promoter, alcohol dehydrogenase II promoter, and a hybrid ADH2-GPD promoter. The recombinant *AmA1* genes were then introduced into the yeast *Saccharomyces cerevisiae* 2805. The outcome of amino acid composition analysis showed no significant increase in essential amino acids in the recombinant yeast, as compared with the wild type. It is likely due to extremely low level of expression of the *AmA1* gene in the recombinant yeasts to improve the overall nutritional quality of the yeasts [285]. In another effort, the transcriptional process of *AmA1* in *Schizosaccharomyces pombe* was investigated. Although, *AmA1* intron does not have any sequence similarity to the transcription termination elements of *S.pombe* , intriguingly the expression of *AmA1* produced a truncated mRNA that extended ahead of the 5′-splice site but terminated within the intronic sequence. This indicates that the transcription machinery of *S.pombe* specifically recognizes the putative poly(A) signals located within the *AmA1* intronic sequence and a detailed investigation of upstream regulatory elements revealed that multiple *cis*-acting sequence elements, including two upstream sequences, the AAUAAA motif, and the sequences downstream of the poly(A) addition site are responsible for 3′-end processing and thereby responsible for its premature termination [286]. Further, the expression of *AmA1* genomic clone in *S. pombe* has been investigated to analyze the splicing of a plant intron [287]. Additionally, introduction of AmA1 in bread wheat (*Triticum aestivum* L.) cv. Cadenza resulted into accumulation of the recombinant protein in the endosperm tissue during seed development thereby resulted into the augmentation of essential amino acid content as well as total protein. However, total protein content showed little difference compared to the protein content of the control flour samples but the content of essential amino acid showed a remarkable increase [288]. For the development of transgenic potatoes with enhanced nutritive value by tuber-specific expression of a seed protein, AmA1, in seven genotypic backgrounds suitable for cultivation in different agro-climatic regions. Analyses

of the transgenic tubers revealed up to 60% increase in total protein content. In addition, the concentrations of several essential amino acids were increased significantly in transgenic tubers. Moreover, the transgenics also exhibited enhanced photosynthetic activity with a concomitant increase in total biomass. These results are outstanding as this genetic manipulation also resulted in a moderate increase in tuber yield. The comparative protein profiling suggests that the proteome rebalancing might cause increased protein content in transgenic tubers. Furthermore, the data on field performance and safety evaluation indicated that the transgenic potatoes are suitable for commercial cultivation. *In vitro* and *in vivo* studies on experimental animals demonstrate that the transgenic tubers are also safe for human consumption [112, 113]. Recently, a comparative proteomics approach has been applied to elucidate the AmA1-regulated molecular mechanism affecting increased protein synthesis, reserve accumulation, and enhanced growth, across the tuber life-cycle between wild-type and AmA1 transgenic potato. A comprehensive proteomic as well metabolomic analysis revealed its role in cell differentiation, regulating diverse functions, *viz.*, protein biogenesis and storage, bioenergy and metabolism, and cell signaling. Metabolome study indicated upregulation of amino acids that further substantiated the proteomics analysis [121].

2.6 Assessment of transgenic crops using 'omic' profiling techniques

In most of the cases introduction of exogenous DNA sequences into the plant genome is a random process, leading to physical disruption in the genome (e.g., insertional mutation) or gene regulation (pleiotropic effect) and possible inactivation of endogenous genes. Therefore, genetic modifications generally represent a double-edged sword. Apart from instigating the preferred traits, modifications in a plant genome might result in inadvertent effects, which may affect human health and/or the environment [289, 290]. The comparison between transgenic and their wild type counterparts frequently include agronomic/phenotypic features, feed performance studies and crop composition. Molecular profiling may facilitate a more complete, holistic comparative analysis. This approach includes several technologies, like DNA microarrays, proteomics, mRNA profiling and metabolomic profiling, which are now accessible as complementary methods for the safety evaluation of transgenic crops. The combination of these nontargeted approaches is considered to facilitate a more comprehensive approach than the targeted methods and, thus, to provide additional opportunities for identifying unintended effects.

2.6.1 Transcriptome profiling to compare transgenic crops *vis-à-vis* to wild type

Transcriptome profiling has been used to characterize several transgenic crops, including maize, barley and rice [291-294]. Transcriptome profiling may possibly be exploited to examine the changes in gene expression in transgenic crops and has potential to perceive inadvertent effects. However, changes in a transcriptome do not inevitably lead to changes in a proteome or metabolome; therefore do not necessarily envisage changes in food composition and quality, consequently transcriptomic profiling is inadequate in evaluating unintended effects [290].

2.6.2 Proteomics as a tool to compare transgenic crops

Besides transcriptomic techniques, proteomic and metabolomic methods are two complementary tools for evaluating transgenic crops. The application of proteomics to investigate differences in the plant proteome due to genetic engineering was explored using *A. thaliana* as a model organism [295]. A similar 2DE-based proteomics approach was utilized to compare the protein profiles of transgenic tomato [296-298], potato [121, 299], rice [300, 301], maize [289, 302-304], soybean [305, 306], tobacco [307, 308] and wheat [309, 310] lines with their nontransgenic counterparts. As a model for testing this kind of approach, **Corpillo et al. (2004)** compared protein expression of two types of tomato plants, having the same genetic background, except for a virus resistance trait introduced by genetic engineering. When proteins extracted from seedlings of the both the types were analyzed by two-dimensional electrophoresis, no significant differences, either qualitative or quantitative, were detected, indicating that in this case the expression of major proteins was unmodified by the genetic manipulation [296]. In potato the effects of transformation on the proteome were considerably less pronounced as compared with the natural variation observed in the non-GM samples. Indeed, statistical analysis showed no clear differences between the protein patterns of the GM lines and their controls. No new proteins unique to individual GM lines were observed. The study reveals that there was no evidence for any major changes in protein pattern in the GM lines tested. On the basis of the changes detected for the proteins surveyed, the genetic modification of *Arabidopsis* using three different genes and three different promoters did not result in any phenotypic change or seed proteome differences exceeding the natural variation other than the intended differences due to the introduction of the transgene [295]. The process of transformation seems not to have caused insertional or pleiotropic changes to the analyzed seed

proteome. Not much change was seen here that would inform a safety assessment. Differences in spot quantity between transgenic and nontransgenic lines fell in the range of natural variation. Any protein changes due to genetic modification were not observed. This study demonstrated that 2-DE can be utilized to reliably analyze intended and/or unintended effects in the transgenic crops.

The proteomics approach described for potato, tomato and *A. thaliana* promises to be useful for the analysis of the proteome in other plant species including crop plants and the genetically modified plants as well, in order to assess the transgene-mediated changes, if any. The method could be applied to classification of ecotypes. In the context of safety assessment it could be used as a screen for global changes in protein profiles that could be taken as a signal for further investigation.

2.6.3 Metabolomics as tool to compare transgenic crops to their wild type counterparts

The most universal testing strategy has been to compare the transgenic line to its nearest isogenic version, *i.e.* the same wild type variety lacking only the transgene insertion. It has been established that the effects of the transgene on the plant tend to be small for the traits which is prevalently in use, with the exception of parameters related to the intended engineered trait. Indeed, evaluation over several years at multiple sites has shown that environmental effects on general metabolic variation within a particular line can be much greater than the variation due to the transgene itself [311]. Metabolomic analysis has become prevalent during recent years due to its capability for analyzing a large number of metabolites and reducing the cost of analysis for each analyte. Metabolomics has been considered a replacement for conventional compositional analysis [312, 313]. A comprehensive comparison of total metabolites was carried out in field-grown GM and conventional potato tubers where significant differences were investigated by using a hierarchical approach initiating with rapid metabolome ‘‘fingerprinting’’ to guide more detailed profiling of metabolites [312]. To test whether or not 14-3-3 protein expression affects plant phenotype and metabolism, transgenic potato plants either overexpressing *Cucurbita pepo* 14-3-3 or underexpressing endogenous 14-3-3 isoforms were analysed using metabolome profiling approach [314]. Furthermore, by using GC-MS approach a method of comparative analysis of metabolome to evaluate comprehensive phenotyping of genetically or environmentally modified plant systems was established [315]. The unintended effect of three

transgenic wheat expressing additional high-molecular-weight subunit genes and the corresponding parental lines was examined using metabolite profiling of samples grown in replicate field trials [316]. The results clearly demonstrate that the environment affects the metabolome and that any differences between the control and transgenic lines are generally within the same range as the differences observed between the control lines grown on different sites and in different years. **Mattoo et al. (2006)** have carried out a nuclear magnetic resonance spectroscopy-based metabolite profiling analyses of transgenic tomato (*Solanum lycopersicum*) fruit engineered to accumulate the higher polyamines spermidine (Spd) and spermine (Spm) to bring an insight into the metabolic processes that Spd/Spm regulate in plants [317]. Recently, genetically modified soybean was assessed in relation to natural variation in the soybean seed metabolome and it has been shown that the metabolome of transgenic lines had no significant deviation from natural variation within the soybean metabolome, with the exception of changes in the targeted engineered pathway [311]. However, this method is simply capable of measuring hundreds of metabolites, not the thousands of metabolites in a plant. Furthermore, differences in metabolomic methodologies, data analysis, and statistical analysis have resulted in less reproducibility. Therefore, metabolomics may not be that much useful for safety assessment. Proteins are key players in gene function and are directly involved in metabolism and cellular development, thus forming the central bridge between the transcriptome and metabolome. Besides, proteins have roles as toxins, antinutrients, or allergens, which have great impact on human health. Therefore, proteomic studies would provide important information for understanding changes in biological processes after genetic modification and are important for evaluating biological safety of GM crops [290]. The widespread application of quantitative proteomic techniques in combination with sophisticated imaging techniques for the identification and mapping of PTMs is expected to provide detailed understanding of protein regulation in complex biological networks [318]. Therefore, combination of these techniques will provide more detailed information on possible changes than can be obtained from single-compound analysis. Once differences have been identified, further safety evaluation of the observed differences may be needed by specific *in vitro* and/or *in vivo* testing.

2.7 Sweet potato proteomics: An unexplored area

Although sweet potato is one of the most important tuberous crops, its proteomics study is still in its early stages. Earlier the optimum conditions for protein extraction from sweet potato leaves and its functional properties were studied [319]. Initially, a methodology for extracting proteins from sweet potato peels was optimized chemically [320]. The extraction procedure involved mixing peel with saline solvent to dissolve proteins and then precipitating with $CaCl_2$. A proteomic approach was used for the analysis of responses to storage stress in sweet potato. For this the carbohydrate contents and the total activity of amylase in cv. Xushu18 were estimated during storage period. To elucidate the molecular changes caused by store-stress, the proteins of tuberous roots at two storage stages were analyzed by proteomic approach using 2-DE and MALDI-TOF-TOF/MS [321]. Furthermore, using the same strategy differentially expressed proteins between young and mature leaves of sweet potato were identified. This study led to the identification of 25 differentially regulated proteins between young and mature leaves. Rubisco activase was found to be one of the differentially regulated proteins was cloned and characterized further [321]. In a similar study comparative proteomic analysis between tuberous roots of light orange and purple-fleshed sweet potato cultivars revealed upregulation of 35 protein spots and out of which 23 and 12 were expressed in light orange and purple-fleshed cultivar respectively [322].