Biotechnological interventions for genetic amelioration of Actinidia deliciosa var. deliciosa (kiwifruit) plant

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Kiwifruit (Actinidia deliciosa var. deliciosa) is a recent introduction among the promising fruit crops. Popularity of its nutritious fruit berries has created a substantial demand for quality planting material, which needs to be planted in certain proportions of female and male plants for Actinidia is dioecious. The large-scale propagation of planting material can be achieved through micropropagation; whereas, its quality and clonal nature need to be ensured, preferably, through molecular markers. With further popularity of this fruit crop, tailor-made genotypes will be required, which can be made possible through biotechnological interventions. As A. deliciosa is limited in its adaptation to a very narrow range of agro-climatic conditions, there is also a need to develop cultivars endowed with traits of wider adaptation. So that this fruit crop can be cultivated in larger range of geographical areas. Development of micropropagation protocols, identification of gender at seedling stage, identification of molecular markers to test genetic/clonal fidelity and incorporation of genes for genetic amelioration are the issues that need immediate consideration. Some of these issues have been addressed in the present paper.

Keywords: Actinidia, conservation, genetic transformation, in vitro techniques, kiwifruit, molecular markers

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Introduction
The genus Actinidia is a perennial vine, bearing berry fruits. It contains about 60 species, distributed in the temperate and subtropical regions of the Asian continent. Of these, A. deliciosa var. deliciosa and A. chinensis are the only two species bearing edible fruits. Further, A. deliciosa (A.Chev.) C.F. Liang et A.R. Ferguson, commonly known as kiwifruit, is the only species cultivated all over the world. The first commercial cultivation of kiwifruit began in New Zealand and, thereafter mostly during 1970s, it spread to other countries. Presently, New Zealand has the best-developed kiwifruit industry with 12,000 ha of land under its cultivation, out of total world cultivation of 22,900 ha. The land under kiwifruit plantations in New Zealand has further increased to 15,000 ha in 1992, producing almost 230,000 tons fresh fruit.

In India, kiwifruit was introduced in 1960s in parts of the Himalayan region. Where it has been grown successfully. Initially, only seven cultivars of A. deliciosa var. deliciosa were imported from New Zealand and USA. However, the commercial potential and suitability in the mid and foothills of Himachal Pradesh further led to their large-scale propagation. During the last three decades, success in marketing of selected cultivars of A. deliciosa has also initiated breeding programmes aimed at developing novel commercial cultivars from a large range of variation available in this genus. Under such programmes, traditional breeding along with molecular technologies is expected to result in genetically improved new cultivars. Efforts have also been made in the field of its molecular biology, such as detection of molecular markers and genetic transformation, to ameliorate the genetic make up of this novel fruit crop. However, dioecy and long juvenile period in kiwifruit are the other equally important constraints of breeding programmes.

Taxonomy/Evolution
The genus Actinidia, containing about 60 species, can be further divided into varieties and forms. The two edible species, A. deliciosa and A. chinensis are
considered closely related. *A. deliciosa* is hexaploid and is probably derived from *A. chinensis*, which is diploid or tetraploid\(^3\). Other species with economic potential are *A. arguta*, *A. eriantha*, *A. latifolia*, *A. chrysanth a*, *A. melandra*, *A. kolomikta* and *A. polygama*. However, they are not commercial fruit crops as yet.

The origin of kiwifruit plant in New Zealand can be traced back to two female and one male plants, introduced in 1904 from China in the form of seeds\(^3\). In 1920s, grafted plants of known sex were sold there and selections for high quality fruit were made\(^6\). As a result, the well-known cultivars of today, viz. ‘Abbot’, ‘Allison’, ‘Bruno’, ‘Monty’, ‘Hayward’ and ‘Gracie’, originated as seedling selections in New Zealand. ‘Hayward’ has now attained pistillate cultivar of commercial importance, for its better shape, size, and long storability of fruits, not only in New Zealand but also in the rest of world\(^6\). Breeding programmes for *Actinidia*, which currently has sizeable commercial industry, are being set-up in New Zealand and Italy.

**Propagation**

Propagation by seed can easily be carried out in kiwifruit but it results in a highly variable progeny and sex of the seedlings remains unidentifiable till flowering. Therefore, vegetative propagation is considered superior to maintain uniformity and genetic purity of the cultivars. Kiwifruit is generally propagated by grafting, budding, softwood and hardwood cuttings, etc. However, large-scale planting materials are needed to meet the commercial requirements. Hence, a simpler, quicker and economically viable technique has to be needed for mass propagation. Several investigators have suggested micropropagation as the best alternative for kiwifruit propagation\(^7\)-\(^11\). Micropropagation and plant regeneration from callus was first tried in kiwifruit over a quarter century ago\(^12\),\(^13\). Now, plants can be regenerated from a variety of explants, such as apical meristem\(^13\), apical parts of shoots\(^14\), auxillary buds\(^15\), bark\(^16\), cotyledons\(^17\), endosperm\(^18\),\(^19\), internodes\(^20\), leaf segments\(^21\), nodes and petioles\(^22\), stamens\(^23\), and stem segments\(^24\). Whereas, nodal segments or shoot tips are the most commonly used initial explants. Also, for *in vitro* multiplication of kiwifruit, MS\(^25\), Cheng’s\(^26\) and Gambog B5\(^27\) media have so far been used.

Harada\(^12\) was the first to develop a protocol for micropropagation of kiwifruit, which was later improved by others\(^28\),\(^29\). Further, Standard\(^28\), and Kumar and Sharma\(^11\) described in detail the important features of micropropagation, i.e. the choice of explant, establishment of aseptic cultures, proliferation, rooting and hardening. Micropropagation by axillary bud stimulation has successfully been employed for production of a large number of uniform kiwifruit plants from a single explant in a short period of time. Successful shoot-tip culture of mature plants has also been applied for vegetative propagation of staminate and pistillate plants\(^38\).

**Organogenesis and Somatic Embryogenesis**

The regenerative potential of kiwifruit cells has in general been found to be fairly high irrespective of the tissue source. Leaf segments, stem pieces, petioles and root tissue have been reported to be amenable for callus formation and subsequent plant regeneration. Cultivar ‘Hayward’ clones had higher proliferation rates than those of the cultivar ‘Tomuri’. Root cultures of these two cultivars have also shown high regenerative potential\(^30\). Endosperm tissue has also been successfully cultured to produce triploid plants (2n=3x=87)\(^31\). Another species, *A. arguta* also exhibited high morphogenetic potential from various explants such as internode, mature and immature leaves, petioles, immature seed and fruit, and *in vitro* differentiated roots\(^32\). Internodal segments of *A. kolomikta*\(^33\) and petiolar sections of *A. deliciosa* cv. ‘Hayward’ showed high morphogenetic potential\(^34\). Apart from these normal tissues as explant, plants were also successfully regenerated from the callus derived from fruit galls of *A. polygama*\(^35\). The root tips of such regenerants showed 2n=58 as the chromosome number, which was the same as that of the mother plant.

Marino and Battistini, while comparing pH effects of the culture media, observed that though pH above 5.7 increased the callus growth yet it inhibited the plant regeneration\(^36\). The morphogenetic responses of the explants also vary with the type, combination and concentration of growth regulators added to the basal medium. Canhoto and Cruz cultured pieces of young leaves from female and male plants of *A. chinensis*\(^7\). The explants were cultured on MS medium with auxin (NAA) and cytokinin (BAP). The buds arose from swellings on the cut ends of the leaf veins and differentiated into shoots. MS medium with NAA (0.1-0.5 mg/l) and BAP (2 mg/l) gave the best results. The shoots rooted well in MS medium with 1 mg/l IBA and produced normal plants. Only minor differences in the morphogenetic potential among
explants of different origins were observed. Similar observations, that nature of the explant did not affect plant regeneration from callus, were also reported in cv. ‘Hayward’.

Somatic embryogenesis can also be used for rapid clonal propagation of homogeneous transgenic plants. However, inconsistency in embryo induction in kiwifruit was reported due to diversity of the genotype, explant sources and culture conditions. A number of attempts have been made to induce somatic embryogenesis from different tissues of different Actinidia species. In most cases, only globular stages of somatic embryos have been obtained and regeneration has been restricted to few genotypes and explants only. On the other hand, A. chinensis var. chinensis is reported to be highly responsive to embryogenesis. Different explants tested for somatic embryogenesis showed different behaviour. Root, stem and stamen filaments gave rise to only embryos. Embryoids formed from endosperm tissue allowed regeneration of some plants; whereas, embryoids differentiated from anthers, further developed into heart-shaped embryos. The calli from kiwifruit have been found to be recalcitrant to differentiate into somatic embryos in spite of the fact that their organogenetic potential is high.

**In vitro Selection and Somaclonal Variation**

Ability to regenerate plants from kiwifruit callus led to exploitation of somaclonal genetic variations with an aim to increase their fruit size and drought tolerance. Following in vitro culture, somaclonal variations have been observed in different Actinidia explants, such as stem, leaf, ovary, anther filament and mature endosperm.

Marino and Battistini attempted to regenerate shoots from leaves, taken from in vitro raised plants of kiwifruit cvs. ‘Hayward’ and ‘Tomuri’, on a medium with pH ranging from 5.7 to 8.5. Leaf callus grew better at pH 7.0 and 7.5 but shoot regeneration and survival were greatly reduced at pH more than 5.7. All regenerated clones exhibited somaclonal variations for at least one trait.

Variation in leaf morphology and chromosome number was also observed when the plants obtained from protoplast-derived kiwifruit callus were characterized. A significant change in chromosome number in the somaclones was observed in callus-derived plants of cv. ‘Hayward’. In which some clones were found to be 12C for DNA content. The mean length of their stomatal guard cells was significantly greater than those of hexaploid somaclones. Using callus cultures of cv. ‘Hayward’, somaclones were regenerated with increased tolerance to high pH as compared to the control. These clones also retained the pH tolerance under field conditions. Further, testing of these materials under field conditions resulted in the development of alkaline resistant ‘Hayward’ and ‘Tomuri’. In the same cultivar, somaclonal variation was also reported for node formation and apical dominance.

With anther culture, good results were obtained in ploidy manipulation. In parthenocarpic triploid from cv. ‘Hayward’, the doubling of chromosome number was reported by colchicine and oryzaline treatments.

**Genetic Transformation**

In the early 90s, infectivity of virulent strains of A. tumefaciens was demonstrated in kiwifruit seedlings. Since then a large number of reports have demonstrated that kiwifruit is an amenable system for genetic transformation using direct as well as indirect gene delivery systems. Transient expression of the CAT gene was demonstrated by using most promising polyethylene glycol (PEG) mediated transfection in kiwifruit protoplasts. These methods are suitable to species not susceptible to Agrobacterium infection. In kiwifruit, however, different types of explants, such as leaf segments, petiolar tissue, stem pieces etc., have been found amenable for Agrobacterium mediated gene transfer. A. tumefaciens and A. rhizogenes are reported to mediate genetic transformation in different cultivars of A. deliciosa. In most of such studies, gus and npt-II have been used as the reporter and marker genes.

Agrobacterium-mediated gene transfer has been used for transformation and isolation of transgenic plants in Actinidia. It involves transfer of T-DNA segment of Ti or Ri plasmid into the nuclear genome of the plant. Healthy and actively growing tissue source, ability of Agrobacterium to efficiently transform the cells, and appropriate bacterial strains as binary vector strongly influence the frequency of transformation. The most commonly used strains of A. tumefaciens are LBA4404, A281, EHA 101, C58, GV 3101, whereas in A. rhizogenes, NCPPB-1855, NIAES 1724, ArM123, IFO 14555, A5, A13 and G746 are the strains of choice. Co-cultivation period (24-36 hrs), use of wounding agents, such as carborundum granules, and antibiotics, like cefotaxime or carbenicillin, to inhibit bacterial growth without affecting the regeneration process were used.
reported to be the important factors. The expression of npt-II and gus genes was demonstrated in the transgenic plants of kiwifruit using A. tumefaciens mediated transformation system\textsuperscript{53}. Inoculation of A. deliciosa hypocotyls with wild type strain of A. rhizogenes gave rise to adventitious roots on MS medium without growth regulators\textsuperscript{58}. The plants regenerated from these roots had shorter internodes, darker and wrinkled leaves and high degree of root formation. Further, detailed studies were performed to optimise co-cultivation conditions for leaf discs and Agrobacterium so as to develop an efficient and reproducible gene transfer system in A. deliciosa\textsuperscript{59}. It was observed that health of the explant, Agrobacterium strain and the vector were important factors. Preculture of the leaf discs inhibited gene transfer at the cut edge; while placing the explants on moist filter paper during co-cultivation gave increased frequency of gene transfer.

A. rhizogenes mediated transformation of kiwifruit was achieved by direct formation of adventitious buds on infected petioles\textsuperscript{60}. In this study, leaf explants of four cultivars, viz., ‘Hayward’, ‘Mastsua’, ‘Abbott’ and ‘Bruno’ were co-cultivated with four Japanese wild type strains of A. rhizogenes. Transformed buds were obtained in ‘Hayward’ and ‘Bruno’, which were confirmed by opine assays and Southern blotting using a 7.5 kb Eco RI fragment of T-DNA. Also, transgenics in cultivars ‘Hayward’ and GTS (a staminate genotype) were successfully obtained using A. tumefaciens strain LBA 4404, harbouring binary vector Bin 19 containing rol ABC and rol B genes\textsuperscript{61}. The transformation frequencies were sufficiently high and hundreds of transgenic shoots were recovered. Addition of cycloheximide to the medium did not increase the transformation frequencies; however, at 10, 100 and 1000 mg/l, it induced 68, 82 and 100% rooting. The plants transformed with rol B gene were morphologically similar to the controls but were more sensitive to auxin. Rol ABC transformants maintained a hairy root phenotype for over 6 years and showed increased lateral root formation. Biochemical assays revealed that the transformed buds had a lower concentration of conjugated polyamines than free polyamines. Moreover, about 50% of the progeny from a cross between transgenic GTS plants and wild type ‘Hayward’ had the hairy root phenotype. It was observed that GTS derived transgenic plants had some potentially useful agronomic characteristics, such as reduced leaf size and an extensive root system.

A rice homeobox, containing gene, was introduced into kiwifruit so as to elucidate the mechanism of morphological changes due to specific genes\textsuperscript{62}. The transgenic plant showed morphological changes including dissected leaf margin and dwarfism. Some correlations between gene expression and the resultant morphological changes were also established. Further, transgenics in four genotypes of A. chinensis were obtained using A. tumefaciens strains A281 and C58, both carrying the binary vector pKIWI 105\textsuperscript{53}. In these experiments A. chinensis was compared with A. deliciosa for its amenability to transformation and regeneration procedures. Although some differences were observed among genotypes within A. chinensis and between the two species, yet overall procedures were similar. The strains of A. tumefaciens, which were effective for gene transfer in A. chinensis, also proved effective to transform A. deliciosa. The transgenic nature of the regenerated plants in both the species was confirmed by PCR amplification of the transferred genes.

Besides A. chinensis and A. deliciosa, other species of Actinidia have also responded well for genetic transformation. An efficient transformation and plant regeneration method for A. kolomikta was carried out using leaf and internodal explants from in vitro grown plants. A. tumefaciens mediated transformation system could be successfully used to transfer npt-II and gus genes, which were confirmed in the transformed cell lines by PCR amplification of the transferred DNA\textsuperscript{56}.

With these developments in kiwifruit, it is now possible that genes of interest can be transferred to the target genotypes for altering its traits with a choice. The genes for disease and pest resistance, increased shelf life and tolerance to abiotic stress conditions are expected to be exploited in due course.

Gender Identification Using Molecular Markers

Dioecy in kiwifruit represents an important constraint in breeding programmes aimed at developing new cultivars. Hence, identification of male and female genotypes is needed as the first step for the correct classification of kiwifruit germplasm. After the early work of Hirsch and Fortune\textsuperscript{64}, who used peroxidase isoenzymes for sex identification, considerable progress has been achieved in this area in recent years. Molecular markers associated with sex- were found in A. chinensis sibling family by bulked segregant analysis using RAPD-PCR.
technology. The parents, siblings and plants from related families were screened for the polymorphism and a pattern of inheritance was established. These results along with data obtained from crossing experiments led to the development of a model for genetics of sex determination in Actinidia, which indicates that an XY chromosome system operates with male being the heterogametic sex. Molecular approaches have also been used to explore the nature of dioecy in Actinidia. It was observed that pistil development was inhibited in the initial stages of development in the staminate flowers; whereas, anther developed normally after meiosis in pistillates but subsequently deteriorated. It appears that there are two genes fundamental to the process; one to suppress pistil development in staminate and the other to initiate pollen death in pistillate phenotypes. Later, it was shown in A. delicosa that females and males have XXXXX and XXXXY chromosomes, respectively and the presence of a single Y chromosome actually allows the retention of dioecism. Since the chromosomes in this genus are very small in size, the X and Y chromosomes cannot be distinguished morphologically. Further, two male specific markers and six female specific markers in A. delicosa var. delicosa were identified, using RAPD-PCR techniques, within the limited number of male and female genotypes. These two male sex related DNA markers were OPC-05350 and OPN-01600. (Fig. 1a) and the six female associated markers were OPA-011031, OPA-08700, OPA-112800, OPA-163000, OPB-012000, OPB-022000 (Fig. 1b).

To construct the sex test primers, sex linked RAPD bands were isolated from agarose gel. It was purified and sequenced to enable the designing of long primers, which would give a more specific sequence characterised amplified region (SCAR). Gill et al isolated and sequenced the male and female bands and compared the nucleotide sequences. Using sex-linked RAPD marker of 800 bp, they confirmed that sex-determining genes are located on a pair of chromosome functioning like XX/XY system with male being heterogametic. Bulk segregant and individual analyses of a sibling family of diploid A. chinensis were also carried out and one each male exclusive and female exclusive band was identified using AFLP technology.

Isozyme polymorphism has also been used in the identification of sex in kiwifruit. A peroxidase test has been developed for screening sex in young kiwifruit seedlings. It was opined that peroxidase is involved in the hormonal sex control in dioecious plants. Later, peroxidase and catalase isozyme systems were detected for identification of gender in kiwifruit. A band of peroxidase with RM 0.60 was found associated with female genotypes and a catalase band with RM 0.85 was consistently observed in the male genotypes.

Cultivar Identification by Molecular Markers

Authentic identification of cultivars is of paramount importance for programmes aimed at genetic improvement. Additionally, it is prudent to verify the material imported from various sources, which may have got mistaken identity during labelling or propagation. To verify progeny from controlled crosses followed by selection, DNA fingerprinting can be used. For this purpose, isozymes, RFLP, RAPD and microsatellites are the few approaches being used. It was reported that isozymes, viz. aspartate amino transferase (AAT), phosphogluco isomerase (PGI) and phosphogluco mutase (PGM) showed considerable variation among the cultivars. Later, malate dehydrogenase (MDH), peroxidase (PER), aspartate amino transferase (AAT), phosphogluco isomerase (PGI) and esterase (EST) were identified as possible markers to differentiate the cultivars of A. delicosa. However, RFLP technique was used to determine the origin of A. delicosa var. delicosa, which is considered to be closely related to A. chinensis.
For typing of kiwifruit cultivars, RAPD-PCR technique has been found suitable. The high informativeness of RAPD primers in *Actinidia* is mainly due to the high genetic polymorphism in these species, which in turn is due to their out crossing. RAPD technique is one of the easiest approaches to the molecular analysis of genomes in the absence of any knowledge of nucleotide sequence. Cipriani *et al.* screened 80 primers and selected only 20 for fingerprinting of 13 cultivars of *A. delicosa* 77. These primers grouped kiwifruit cultivars into 2 to 11 clusters and separated cultivar 'Blake' from the rest of them. The most informative primer was OPQ-20, which amplified 6 polymorphic fragments allowing 10 groups to be created from the twelve cultivars. Further, OPA-07, OPA-16, OPD-05 and OPU-01 were identified as to be the informative markers, which were able to distinguish between seven cultivars of kiwifruit 78. Sex-linked RAPD markers were also detected in a sibling family of diploid *A. chinensis* using bulked segregant analysis 86. Five hundred random primers were screened and only two sex-linked markers were found, which were inherited from the male parent of the cross.

For fingerprinting, c-DNA libraries were constructed from female and male buds of a sibling family of *A. chinensis* at pre and post meiotic pollen stage 79. Harvey *et al.*, while concentrating on microsatellites, showed that di- and tri-nucleotide repeats gave banding patterns, which were highly polymorphic in diploid and tetraploid *A. chinensis* and hexaploid *A. delicosa* 80. Microsatellite repeats of different types, such as (CA)n, (CT)n, or (CTT)n; (CT)2 CC (CT)8, (CCT)4 CCTT CAT (CTT)2, or (CT)10 (CA)5 (AC)3 have been cloned in *Actinidia* 81. Eighteen primer pairs were designed according to the DNA sequence of 35 hybridising clones and, using their primer pairs, they successfully fingerprinted a range of closely related varieties in *A. delicosa*. Further, 263 microsatellite containing clones were isolated and sequenced from *A. chinensis* with a high degree of polymorphism 82. Several polymorphic microsatellites have been combined to form a fluorescent fingerprinting kit, which can be used to identify important genotypes used in *Actinidia* breeding programme 83. The application of novel molecular techniques has, thus, supplemented the conventional approaches for improving genetic make up of this newly domesticated fruit crop. Besides, these techniques have been found of great importance in deciphering varietal identity and gender of the vines.

**Chloroplast Inheritance**

In *Actinidia*, DNA based molecular markers have established that chloroplasts are inherited from the male parent, contrary to the general rule of maternal inheritance of plastids in other higher plants. RFLP’s of PCR amplified fragments were studied to trace the pattern of plastid DNA inheritance in this genus 84. Using a total of 51 progeny originating from interspecific crosses between three *A. arguta* cultivars and *A. delicosa* and 12 progeny originating from the cross between *A. kolomikta* and *A. chinensis* along with their parents, it was shown in all cases that chloroplast inheritance was paternal without exception. Further studies in this genus have confirmed that while mitochondrial DNA (mt DNA) is maternally inherited, chloroplast DNA (cp DNA) is paternally inherited 85,86. It has also been shown that the cp DNA of *A. delicosa* was inherited from *A. chinensis* and that the latter species must be the paternal, if not the only progenitor of *A. delicosa*.

**Germplasm Conservation**

Within the genus *Actinidia*, a wide range of germplasms has favourably responded for cryoconservation as subsequent retrieval has shown fairly high survival rates. The stem segments of *A. chinensis* when stored in liquid nitrogen (-196°C) for 120 days, approximately 77% callus induction frequency was recorded after thawing, which was similar to that in the fresh stem segments 87. Later, Hakozaki *et al.* reported a protocol for successful conservation of calli of *A. delicosa* cv. ‘Hayward’ in liquid nitrogen 88. The calli were cultured in a medium containing 24% or 41% sucrose for two days followed by dehydration twice, first for 22 minutes with 60% PVS2 solution (30% glycerol, 15% DMSO, 15% ethylene glycol and 13.7% sucrose) and then for 23 minutes with 100% PVS2 solution. Following these steps, the calli were stored in liquid nitrogen. Such calli could later be successfully retrieved by warming in water at 37°C, immediately after removal from the liquid nitrogen.

In a comparative study, it was observed that growth stages of shoot tips of kiwifruit had a pronounced effect on survival of calli after cryostorage 89; before cryoconservation in alginate encapsulation condition, the shoot tips were cold hardened for 4-6 weeks at 5°C and precultured on agar media containing high sucrose concentration. While shoot tips at two leaf
stage recorded high survival (83.3%), shoot tips from young seedlings did not survive. In the subsequent studies, effects of ascorbic acid and proline on viability of cryopreserved kiwifruit shoot tips were compared\textsuperscript{90}. Another species, A. arguta has also responded well to cryopreservation. Excised lateral buds from in vitro culture derived shoots of A. arguta were subjected to pretreatments in freezing solutions and cold acclimation before their cryopreservation in liquid nitrogen\textsuperscript{91}. Variation in the treatments resulted in variable survival of the buds after retrieval. Besides shoot tips and calli, pollen grains have also been successfully cryoconserved\textsuperscript{92}.

Cryopreservation has open up possibilities of conserving tremendous amount of genes for posterity in a small space. With passage of time, it is likely that a germplasm repository representing major genes of interest is not only conserved in more sophisticated gene libraries but also in the form of pollen, seed and vegetative propagules in liquid nitrogen.

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