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Applications of Biotechnology in Kiwifruit (*Actinidia*)

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1. Introduction

Actinidia is a genus of 55 species and about 76 taxa native to central China and with a wide geographic distribution throughout China and South Eastern Asia (X. Li et al., 2009). Palaeobiological studies estimate *Actinidia* to be at least 20–26 million years old (Qian & Yu, 1991). *Actinidia* species are vigorous and long-lived perennial vines, producing oblong or spherical berries that vary considerably in shape and colour (Fig. 1). *Actinidia* are normally dioecious, but occasional plants have perfect flowers (A. R. Ferguson, 1984). The basic chromosome number in *Actinidia* is *X*=29, with a diploid number of 58. During evolution a chromosome may have duplicated (McNeilage & Considine, 1989), followed by an aneuploid event, such as breakage of a centromere, to give an additional chromosome (He et al., 2005). The genus has a reticulate polyploidy structure, with diploids, tetraploids, hexaploids and octaploids occurring in diminishing frequency (A. R. Ferguson et al., 1997). The genus has unusual inter- and intra-taxal variation in ploidy (A. R. Ferguson & Huang, 2007; A. R. Ferguson et al., 1997), with, for example, *A. chinensis* found as both diploid and tetraploid and *A. arguta* as usually tetraploid, but also found as diploid, hexaploid or octaploid. In this chapter, we will describe advances in *Actinidia* plant tissue culture and molecular biology and the present and future applications of these biotechnology techniques in kiwifruit breeding and germplasm improvement.

2. Global significance of kiwifruit

Actinidia species were introduced to Europe, the U.S.A., and New Zealand in the late 19th and early 20th century (A.R. Ferguson & Bollard, 1990). New Zealand was largely responsible for the initial development and commercial growing of kiwifruit, with the first commercial orchards established in the 1930s. Domestication and breeding of firstly A*ctinidia deliciosa,* and more recently, *A. chinensis,* from wild germplasm has resulted in varieties now cultivated commercially in a number of continents. The inherent qualities of novel appearance, attractive flesh colour, texture and flavour, high vitamin C content and favourable handling and storage characteristics make kiwifruit a widely acceptable and popular fruit crop for producers and consumers.

Commercial kiwifruit growing areas have expanded rapidly and consistently since the 1990s. By 2010, the global kiwifruit planting area had reached over 150,000 ha. China (70,000 ha), Italy (27,000 ha), New Zealand (14,000 ha) and Chile (14,000 ha) account for about 83%

of world kiwifruit plantings, and global kiwifruit production represents about 0.22% of total production for major fruit crops, with the majority of kiwifruit consumed as fresh fruit. Science has made a significant contribution to the success of the New Zealand kiwifruit industry, particularly in developing excellent breeding programmes and technologies for optimal plant growth, orchard management, fruit handling and storage, and transport to the global market, to ensure high quality premium fruit reach the consumer.

Fig. 1. Fruit of the *Actinidia* genus showing variation in flesh colour, size and shape

Kiwifruit have a reputation for being a highly nutritious food. A typical commercial *A. deliciosa* 'Hayward' kiwifruit contains about 85 mg/100 g fresh weight of vitamin C, which is 50% more than an orange, or 10 times that of an apple (A. R. Ferguson & Ferguson, 2003). The fruit of some *Actinidia* species, such as *A. latifolia, A. eriantha* and *A. kolomikta,* have in excess of 1000 mg of vitamin C per 100 g fresh weight (A. R. Ferguson, 1990; A. R. Ferguson & MacRae, 1992). Kiwifruit are also an excellent source of potassium, folate and vitamin E (Ferguson & Ferguson, 2003), and are high amongst fruit for their antioxidant capacity (H. Wang et al., 1996).

2.1 Breeding and commercial cultivars

The extensive *Actinidia* germplasm resources, with tremendous genetic and phenotypic diversity at both the inter- and intra-specific levels, offer kiwifruit breeders infinite opportunities for developing new products. Since its development in the 1920s, *A. deliciosa* 'Hayward' has continued to perform extraordinarily well on the global market in terms of production and sales; it remains the dominant commercial kiwifruit cultivar. Advances in *Actinidia* breeding have seen the appearance of a number of new commercial kiwifruit varieties. In 1999 an *A. chinensis* cultivar named 'Hort16A', developed in New Zealand by HortResearch (now Plant & Food Research), entered the international market, with fruit sold under the name of ZESPRI® GOLD Kiwifruit, reflecting the distinctive golden-yellow fruit flesh. 'Hort16A' fruit are sweet tasting and the vine is more subtropical than 'Hayward'. Subsequently, a range of new cultivars were commercialised in China and Japan, some of which have become significant internationally. Jintao®, a yellow-fleshed cultivar selected in

Wuhan, China (H.W. Huang et al., 2002b), is now widely planted in Italy (Ferguson & Huang, 2007) and more recently, the *A. chinensis* cultivar 'Hongyang' selected in China, and with a distinctive yellow-fleshed fruit with brilliant red around the central core, is widely cultivated for the export market, particularly Japan (M. Wang et al., 2003). Most cultivars to date have been selected from *A. chinensis* and *A. deliciosa;* however, *A. arguta* are now commercially cultivated in USA, Chile and New Zealand (Ferguson & Huang, 2007). The fruit of *A. arguta* are small, smooth-skinned, with a rich and sweet flavour, and can be eaten whole (Williams et al., 2003). Internationally, kiwifruit breeding programmes are directed primarily at producing varieties mainly from *A. deliciosa* and *A. chinensis,* with large fruit size, good flavour, novel flesh colour, variations in harvest period, improved yield and growth habit, hermaphroditism, tolerance to adverse conditions and resistance to disease (A. R. Ferguson et al., 1996). Although kiwifruit cultivars currently on the commercial market have been developed using traditional breeding techniques (MacRae, 2007), the expansion of genetic, physiological and biochemical knowledge and the application of biotechnology tools are being used increasingly to assist breeders in the development of novel cultivars.

3. Tissue culture and crop improvement

Although the genetic diversity of *Actinidia* provides tremendous potential for cultivar improvement, there are features (including the vigorous nature of climbing vines, the 3- to 5-year juvenile period, the dioecious nature and the reticulate polyploidy structure) that make *Actinidia* less amenable to achieving certain breeding goals, compared with many other agronomic crops. Plant tissue culture, the *in vitro* manipulation of plant cells, tissues and organs, is an important technique for plant biotechnology, and a number of plant tissue culture techniques have been employed to overcome some of the limitations that *Actinidia* presents to classical breeding.

3.1 Multiplications

Plant tissue culture for kiwifruit propagation was first reported by Harada (1975), followed by numerous reports using a range of explant types and genotypes (Gui, 1979; M. Kim et al., 2007; Kumar & Sharma, 2002; Q.L. Lin et al., 1994; Monette, 1986). Murashige & Skoog (MS) basal salts are the most widely used media for shoot regeneration and callus formation. However, other media have been used successfully, including Gamborg B_5 medium (Barbieri & Morini, 1987) and N_6 medium (Q.L. Lin et al., 1994).

Multiplication protocols essentially follow three steps: (1) surface sterilization of explants with 0.5–1.5% sodium hypochlorite; (2) shoot multiplication from explants (e.g. buds, nodal sections or young leaves) on MS medium, supplemented with 2–3% sucrose, 0.1–1.0 mg/l zeatin and 0.01–0.1 mg/l naphthalene acetic acid (NAA), solidified with 0.7% agar, at pH 5.8; and (3) rooting on half strength MS medium containing 0.5–1.0 mg/l indole-3-butyric acid (IBA). Generally, cultures are incubated at 24±2^{o}C under a 16 h photoperiod (20–30 μmol/m^2/s of light intensity applied). Shoot proliferation rates vary depending upon species, cultivar, explant type, plant growth regulator combinations and culture conditions. Standardi & Catalano (1984) achieved a multiplication rate of 5.3 shoots per bud explant using a 30-day subculture period, and 90% of shoots rooted after three weeks, developing

into 150–200 mm high plantlets, with 6–10 leaves within 60 days. A multiplication rate of 2.61 at seven weeks was achieved using 800 μm or 1200 μm transversal micro-cross section (MCS) of *A. deliciosa* 'Hayward' explants, cultured on ½ MS medium supplemented with 3% (w/v) sucrose, 4.5 x 10^{-3} μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 4.6 x 10^{-1} μM zeatin in 0.8% agar (w/v), pH 5.8 (Kim et al., 2007).

3.2 Protoplast culture and somatic hybridization

As dioecy and polyploidy of *Actinidia* can often restrict breeding possibilities, somatic hybridization provides an approach to combine different genetic backgrounds of the same gender or to overcome inter-specific incompatibility, to produce valuable material with desirable traits from two species. Somatic hybridization is generally achieved through protoplast fusion, and methods of protoplast isolation from callus, suspension cultures, leaf mesophyll and cotyledons of various *Actinidia* genotypes and species have been developed. Tsai (1988) isolated protoplasts from calli derived from *A. deliciosa* leaves and stems and used TCCM medium with 0.23 μM 2,4-D, 0.44 μM 6-benzylaminopurine (BAP), 2% coconut milk, 10 g/l sucrose, 1 g/l glucose, 0.3 M mannitol and 0.1 M sorbitol, for preconditioning. Enzymatic degradation of cell walls was achieved in 2% Cellulase Onozuka R-10, 0.5% Macerozyme R-10, 0.5 M mannitol and 3 mM MES. *A. eriantha* protoplasts were isolated from newly growing leaves of *in vitro* culture seedlings, by preconditioning in MS liquid (without NH_4NO_3), supplemented with 1.0 mg/l 2,4-D and 0.4 M glucose and isolated using 1% Cellulase R-10, 0.5% Macerozyme R-10, 0.05% Pectolyase Y-23 and 3 mM MES (Y.J. Zhang et al., 1998). Plating efficiency after 3 weeks of culture was 19.4%, and calli subsequently recovered and regenerated shoots when cultured on MS media containing 2.28 μM zeatin and 0.57 μM indole-3-acetic acid (IAA).

Xiao & Han (1997) reported successful protoplast fusion of *A. chinensis* and *A. deliciosa*, demonstrating the potential of using this technique to aid breeding programmes. Isolated protoplasts from cotyledon-derived calli for *A. chinensis* ($2n = 2x = 58$) and *A. deliciosa* ($2n = 6x = 174$) were fused, using a PEG (polyethylene glycol) method and plantlets were regenerated from the fused calli. Xiao et al. (2004), in an attempt to introduce the chilling tolerance characteristics of *A. kolomikta* into *A. chinensis*, fused protoplasts isolated from cotyledon-derived calli of *A. chinensis* ($2n = 2x = 58$) and the mesophyll cells of *A. kolomikta* ($2n = 2x = 58$). A number of techniques were employed to confirm that the regenerated plantlets were an inter-specific somatic hybrid ($2n = 4x = 116$) and assessment of the chilling tolerance of *in vitro* leaves suggested that the somatic hybrid was more similar to *A. kolomikta*, with a higher capacity of cold resistance than *A. chinensis*.

3.3 Other culture techniques

Embryo culture techniques, for embryo rescue were developed to recover hybrids from inter-specific crosses in *Actinidia*. From an *A. chinensis* ($2x$) × *A. melanandra* ($4x$) cross, embryo rescue was used successfully to transfer hybrid embryos to *in vitro* culture at an early stage of their development (Mu et al., 1990). Nutrient and hormone requirements were dependent on the stage of embryo development and the endosperm, and nursing tissue was beneficial when globular embryos were cultured. Embryo size and their genetic background are major factors in determining the success of the procedure (Harvey et al., 1995; Kin et al.,

1990). Hirsch et al. (2001) carried out inter-specific hybridizations of different *Actinidia* species and ploidy races, using embryo rescue to obtain hybrid plantlets of *A. kolomikta X A. chinensis, A. polygama X A. valvata, A. arguta X A. polygama* and *A. kolomikta X A. deliciosa*. When optimal media were used, the immature embryos that reached the torpedo stage could be rescued. A series of culture media were developed, which performed as the hybrid embryo's deficient endosperm to ensure embryo survival at the globular and heart stages. Ovule culture has been used also to obtain hybrid plantlets from the inter-specific cross of *A. chinensis X A. kolomikta* (X. Chen et al., 2006).

Endosperm culture is another approach to generating *Actinidia* inter-specific hybrids. Endosperms from F_1 and F_2 seeds from three inter-specific hybrids (*A. chinensis X A. melanandra; A. arguta X A. melanandra*; and an open pollinated *A. arguta X A. deliciosa*) were induced to form calli, from which plants were recovered by induction of organogenesis or embryogenesis. Media for callus induction and differentiation varied with genotype, and chromosome counts showed evidence of extensive mixoploidy in all hybrids (Mu et al., 1990).

Recently, *in vitro* chromosome doubling using colchicine treatment was reported (J. Wu et al., 2009; 2011). Petiole segments of five diploid *A. chinensis* genotypes, including 'Hort16A', were cultured on half-strength MT basal salt medium, supplemented with 3.0 mg/l BAP, 0.4 mg/l zeatin and 0.5 mg/l IBA for four weeks. Resulting microshoots were treated with 0.05–0.1% colchicine, and over one-third of the regenerated shoots were confirmed as tetraploid by flow cytometry, with orchard-grown autotetraploid 'Hort16A' plants showing polyploid characteristics such as thicker leaves and flatter flowers, and some plants producing fruit almost double the weight of the original diploid 'Hort16A' fruit (J. Wu et al., 2009).

Cryopreservation is an excellent means of preserving germplasm for long-term storage, and various techniques and methods have been investigated for *Actinidia* germplasm (Bachiri et al., 2001; Hakozaki et al., 1996; Jian & Sun, 1989; Y. Wu et al., 2001; X. Xu et al., 2006; Zhai et al., 2003). Shoot tips from *in vitro* culture of a dwarf *A. chinensis* genotype were pre-cultured in MS medium containing 5% dimethyl sulfoxide (DMSO) and 5% sucrose for four days, followed by dehydration with PVP_2 solution (30% glycerol, 15% DMSO, 15% PEG and 13.7% sucrose) for 40 min at 0°C, and then transferred to liquid nitrogen for storage, with a survival rate of 56.7% upon defrosting shoots (X. Xu et al., 2006). Encapsulation-dehydration protocols used for the preservation of *in vitro* cultured hybrids of *A. arguta X A. deliciosa*, *A. chinensis* and *A. eriantha* gave even higher survival rates, of 85–95% (Bachiri et al., 2001; Y. Wu et al., 2001).

4. Transformation systems

Since the first report of a transgenic *Actinidia* plant two decades ago (Matsuta et al., 1990), six *Actinidia* species having been transformed, almost exclusively by *Agrobacterium*-mediated transformation. Initially, the development of *Actinidia* transformation focused on the integration into the plant genome of reporter and selectable marker genes (Fraser et al., 1995; Janssen & Gardner, 1993; Uematsu et al., 1991), but transformation of various heterologous genes has followed. These include: *A. rhizogenes rol* genes (Rugini et al., 1991); a soybean β-1,3 endoglucanase cDNA (Nakamura et al., 1999); a rice *OSH1* homeobox gene (Kusaba et al., 1999), and an *Arabidopsis* Na^+/H^+ antiporter gene (Tian et al., 2011), in attempts to improve kiwifruit disease resistance or drought tolerance; a synthetic gene encoding human epidermal growth factor (Kobayashi et al., 1996); and a grape stilbene synthase (Kobayashi et al., 2000), in

attempts to accumulate bioactive compounds; citrus geranylgeranyl diphosphate synthase, phytoene desaturase, β-carotene desaturase, β-carotene hydroxylase and phytoene synthase, to modify the lutein or β-carotene content of kiwifruit (MiSun Kim et al., 2010) and the *A. tumefaciens* isopentyl transferase (*ipt*) gene, to alter vine architecture (Honda et al., 2011).

4.1 *Agrobacterium*-mediated transformation

Agrobacterium-mediated transformation of *Actinidia* is a component of the Plant & Food Research functional genomics platform and has been used to introduce over 100 *Actinidia* genes into various *Actinidia* species. In general, Plant & Food Research *Actinidia* transformation protocols are as follows: Orchard-grown winter mature and dormant canes are maintained at 4°C for 4–6 weeks. To initiate bud break, one-third of a 40 cm cane (with >3 nodes) is immersed in water, and maintained at room temperature under normal light conditions. After four weeks, newly initiated shoots are removed from the canes and shoot sections with a single node (1–2 cm) are soaked in 70% ethanol for 30 s, then surface sterilized with 25% (v/v) commercial bleach (5% active chlorine). After a sterile water rinse, the node sections are cultured on MS media, supplemented with 0.1 mg/l IBA at 24°C ± 2, 16 h photoperiod, with cool white fluorescent light (40 µmol/m²/s). Young leaves harvested from *in vitro* grown shoots are cut into 2 x 5 mm leaf strips. *Agrobacterium tumefaciens* EHA105, harbouring a pART27-derived binary vector (Gleave, 1992), is cultured in 50 ml MGL medium (Tingay et al., 1997) containing 100 mg/l spectomycin dihydrochloride, for 16–20 h at 28°C, with shaking at 250 rpm. At an $OD_{600\ nm}$ =1.0-1.5, the bacterial cells are pelleted by centrifugation (5000 g for 10 min) and re-suspended in 10 ml MS media, supplemented 100 µM acetosyringone. Leaf strips are immersed in the *A. tumefaciens* suspension culture for 10 min, blotted dry with sterile filter paper and transferred onto co-cultivation media (MS supplemented with 3.0 mg/l zeatin, 0.1 mg/l naphthaleneacetic acid (NAA) and 50 µM of AS). After two days of co-cultivation, the leaf strips are transferred to regeneration and selection medium (MS supplemented with 3.0 mg/l zeatin, 0.1 mg/l NAA, 150 mg/l kanamycin sulphate, 300 mg/l timentin, 30 g/l sucrose and 2.5 g/l Phytagel). The leaf strips produce calli along the cut edges at about four weeks and excised calli are transplanted onto fresh regeneration and selection media. Adventitious buds regenerated from the calli are excised individually and transferred to shoot elongation medium (MS supplemented with 0.1 mg/l IBA, 100 mg/l kanamycin sulphate and 300 mg/l timentin). When shoots reach 1–2 cm in height, they are transplanted onto rooting medium (½ MS basal salts and vitamins supplemented with 1.0 mg/l IBA, 150 mg/l timentin, 50 mg/l kanamycin sulphate, 20 g/l sucrose and 7 g/l agar). Rooted transgenic plants are potted in a ½-litre pot and placed in a containment glasshouse facility. The utility of a plant transformation system is very much dependent upon its efficiency, and several factors that affect *Actinidia* transformation efficiency are discussed below.

4.1.1 Agrobacterium tumefaciens strains

A. tumefaciens strains are defined by their chromosomal background and resident Ti plasmid, and exhibit differences in their capacity to transfer T-DNA to various plant species (Godwin et al., 1991). *A. tumefaciens* LBA4404, A281, C58, EHA101 and EHA105 are the strains commonly used in *Actinidia* transformation. Fraser et al. (1995) reported no marked difference in efficiency of *A. chinensis* transformation between strains A281 (a virulent L,L-

succinamopine strain) and C58 (a virulent strain carrying the nopaline Ti plasmid pTiC58), which both harbour the binary vector pKIWI105. However, Janssen and Gardner (1993) showed A281 produced slightly higher rates of gene transfer than C58 and EHA101 in *A. deliciosa* transformation, and noted that because of source material variability, strain comparisons need to be repeated several times. Strain A281 harbours a tumour-inducing plasmid pTiBo542 (Hood et al., 1986) and an extra copy of the transcription activator of virulence (*vir*) genes, which may account for the higher transformation efficiency.

Comparison of *A. chinensis* callus formation using *A. tumefaciens* A281, GV3101, EHA105 and LBA4404, all harbouring the pART27-10 binary vector, revealed that 27% of leaf strips produced calli using A281, compared with 22.2%, 18.1% or 13.9% when using EHA105, LBA4404 and GV3101, respectively (T. Wang et al., 2007). Both A281 and its non-oncogenic derivative, EHA105, have the Ti-plasmid pTiBo542 in a C58 chromosomal background (Hood et al., 1993; 1986), and have been shown to be superior in gene transfer in other plant species, e.g. apple (Bondt et al., 1994). However, high rates of callus formation do not necessarily mean high efficiency of transgenic plant production, and Wang et al. (2007) also found differences in shoot regeneration related to whether co-cultivation had been with strains harbouring an oncogenic Ti plasmid (A281) or a non-oncogenic Ti plasmid (EHA105). Transformants derived from the use of the disarmed strains EHA105, LBA4404 and GV3101 had callus and regeneration patterns similar to those of control explants, not co-cultivated with *A. tumefaciens*, whereas the use of A281 tended to result in large calli and take about two weeks longer to initiate adventitious buds. Less than 20% of the calli derived from A281 co-cultivation had subsequent shoot and root development, whereas over 70% of calli derived from EHA105, GV3101 and LBA4404 co-cultivation regenerated shoots and roots. Over-proliferation of calli derived from A281 co-cultivation was even more severe in *A. eriantha* and no regenerated shoots were obtained (T. Wang et al., 2006). It is likely that high callus formation and poor adventitious bud and root initiation from the A281 co-cultivated tissue is related to the co-integration into plant genome of the oncogenes.

4.1.2 Species

Most *Actinidia* transformation systems have been developed for *A. chinensis* and *A. deliciosa,* though transformation of *A. arguta, A. eriantha, A. kolomikta* and *A. latifolia* has been reported. All *Actinidia* genotypes tested have been found to be responsive to a range of tissue culture conditions, and relatively amenable to regeneration protocols (Fraser et al., 1995). Compared with other woody species, e.g. apple (James et al., 1989), relatively high *A. deliciosa* transformation and regeneration rates have been achieved (Uematsu et al., 1991), and *A. chinensis* transformation efficiencies of up to 27.8% have been reported (T. Wang et al., 2007). However, *A. arguta* transformation was less successful when applying the transformation protocols developed for *A. chinensis* or *A. eriantha*, with co-cultivated explants suffering considerable browning and necrosis during callus induction and shoot regeneration stages. Minimizing the extent of explant browning and necrosis was achieved through reducing the basal salt concentration to ½ MS medium, combined with lower light intensity (3.4 $\mu mol/m^2/s$) during the callus induction and regeneration stages. This resulted in adventitious shoot development and an efficient and reproducible *Agrobacterium*-mediated transformation system for *A. arguta* (Han et al., 2010).

From the production of over 1000 transgenic *Actinidia* plants at Plant & Food Research, the salient features in comparing the transformation of four species are three-fold. *A. arguta* displays a relatively, low transformation efficiency of 1–10% compared with the 5–20% for *A. deliciosa* and *A. eriantha* and 5–30% for *A. chinensis*; the induction of *A. eriantha* callus is relatively high compared with other species; but the regeneration of *A. eriantha* kanamycin-resistant shoots takes much longer than with the other three species.

4.1.3 Co-cultivation conditions

Agrobacterium-mediated DNA delivery to plant cells is initiated through a series of chemical signals exchanged between the host and pathogen, which may activate *vir* genes to signal the bacterium to enter virulence mode. Phenolics, sugars, temperature and pH can affect *Agrobacterium* virulence and presumably its capacity to transform plant cells (Alt-Moerbe et al., 1988). However, the degree to which these factors influence transformation efficiency varies with species and reports. Acetosyringone (AS), one of the phenolic compounds released by wounded plant tissue, and a signal molecule to ensure effective *vir*-induction and T-DNA transfer (Stachel et al., 1985; 1986), has been widely used to increase transformation efficiency in various crops (James et al., 1993; H. Wu et al., 2003). Janssen and Gardner (1993) found the addition of 20 μM AS to the *A. tumefaciens* growth and co-cultivation medium increased DNA transfer approximately 2-fold in *A. deliciosa* leaf pieces, whereas highest levels of *A. latifolia* transformation were achieved using 200 μM in the co-cultivation medium (Gao et al., 2007). Wang et al. (2006; 2007) used 100 μM AS in bacterial cultures for co-cultivation to improve the efficiency of *A. chinensis* and *A. eriantha* transformation. The inclusion of a suspension cell feeder layer during co-cultivation, separated from the explants by a layer of filter paper, has been used to improve *Actinidia* transformation frequency (Janssen & Gardner, 1993). In addition, as mentioned earlier, light intensity plays a role in the efficiency of *A. arguta* transformation (Han et al., 2010).

4.1.4 Plant regeneration

Selecting plant cell types or explants that have the ability to differentiate into whole plants is an essential step for the successful production of transgenic plants. Fortunately, *A. deliciosa* and *A. chinensis* callus induction and adventitious bud initiation are relatively straightforward after establishment in tissue culture if appropriate explant material is used. Young leaves, petioles and stem segments have been used successfully for *Actinidia* transformation, and, as with most other crops, the younger the explants, the easier regeneration will be. However, *A. arguta* transformation is one exception to this, as necrosis or browning occurs after *A. tumefaciens* co-cultivation if the explants used are too young (Han et al., 2010).

To maintain *Actinidia* explants in active and amenable condition for co-cultivation with *A. tumefaciens*, it is essential to subculture *in vitro* shoots at 3- to 4-week intervals (Fraser et al., 1995; Wang et al., 2006). MS basal medium has been used successfully for callus induction as well as regeneration in *Actinidia* (Kumar and Sharma 2002). However, optimum application of auxins and cytokinins, and combinations thereof, vary depending on the condition of the explant material used. Fraser et al. (1995) found that for *A. chinensis* regeneration, thidiazuron (TDZ) and kinetin, (0.1 and 10 mg/l) were clearly inferior to other cytokinins.

Differences between NAA and IAA (indole-3-acetic acid) were insignificant. The most satisfactory combination of growth regulator additives was found to be 5 mg/l zeatin combined with 0.1 mg/l of NAA, or 1 mg/l zeatin and 0.5 mg/l BAP combined with 0.1 mg/l of NAA. Zeatin was clearly superior to BAP, when either was used as the sole cytokinin, but a combination of the two cytokinins gave the best overall result, in terms of the numbers of normal-looking shoots produced. Wang et al. (2006) made similar observations with *A. eriantha* where the highest shoot regeneration rates were obtained using medium containing a combination of 2 mg/l zeatin and 3 mg/l BAP. Uematsu et al. (1991) reported that the regeneration frequency varied with the basal medium used, and B5 basal medium containing zeatin was most suitable for obtaining transformed *A. deliciosa* shoots. Using *A. deliciosa* MCS explants for transformation, Kim et al. (2010) used half-strength MS medium containing 0.001 mg/l 2,4-D and 0.1 gm/l zeatin, for callus induction and shoot regeneration. Calli formed on the surface of MCS segments after two weeks of culturing on selection medium and shoots were regenerated after four weeks. The transformation efficiencies ranged from 2.9 to 22.1% depending on the gene being transformed into the cells. The high degree of callus formation and shoot regeneration of *Actinidia* material from tissue culture makes it possible to obtain transformed shoots at a reasonably high frequency, although it is desirable to minimize callus development and maximize shoot development, to minimize the occurrence of somaclonal variation during these processes.

4.2 Particle bombardment

As opposed to the biological *Agrobacterium*-mediated transformation process, particle bombardment is a purely physical method for DNA delivery, using DNA-coated microscopic metal particles accelerated towards a target tissue. Qiu et al. (2002) used particle bombardment of *A. deliciosa* suspension cells, with a CaMV 35S transcribed maize *DHN1* gene (induced in response to abiotic stress) fused to the green fluorescent protein (GFP) reporter gene. GFP expression was localized within the cell nucleus after 10 h and was visualized in the cytoplasm (mainly around the plasma membranes) in response to increased osmotic stress (Qiu et al., 2002).

4.3 Other DNA transfer methods

Although *Agrobacterium*- and particle bombardment-mediated DNA transfer are the most commonly used systems of gene transfer to plants, a polyethylene glycol (PEG)-mediated approach was frequently used in the early 1980s to deliver DNA into protoplasts. Oliveira et al. (1991) used the chloramphenicol acetyl transferase (CAT) gene as a reporter to optimize the conditions for PEG-mediated transfection of *Actinidia* protoplasts, finding that the greatest CAT activity was obtained using 30% PEG 4000 and submitting protoplasts to a 5-min 45°C heat shock, prior to transfection. Using *in vitro* cultured *A. deliciosa* leaves, Raquel & Oliveira (1996) found protoplasts originating from the epidermis and leaf veins had cell division and regeneration ability, and displayed transient expression of a GUS gene introduced by PEG-mediated DNA transfer. Zhu et al. (2003) successfully transferred a GFP gene into *A. arguta* protoplasts by PEG-mediated transfer, with transient GFP expression detected in calli generated from the protoplasts. The physiological conditions of the protoplasts, the PEG concentration, and the time of heat stimulus are factors affecting the efficiency of DNA transfer using this approach. Because of the low yields of transformants

and the inability of many species to be regenerated from protoplasts into viable plants, direct DNA uptake methods of transformation are much less frequently adopted than *Agrobacterium*-mediated transformation. However, the successful regeneration of whole plants from *A. chinensis, A. deliciosa* and *A. eriantha* protoplasts has been published (see earlier). Future development of new commercial cultivars produced directly or indirectly via genetic manipulation may see a resurgence in direct DNA uptake methods and protoplast regeneration, as these approaches may be more amenable to some genetic manipulation technologies, such as Zinc finger nuclease targeted site-directed mutagenesis.

5. *Actinidia* molecular biology

Initial molecular studies of *Actinidia* concentrated on fruit tissue, with an emphasis on genes involved in ethylene biosynthesis, cell wall modification, and carbohydrate metabolism (Atkinson & MacRae, 2007 and references therein). The cloning and/or expression of 1-aminocyclopropane-1 carboxylic acid (ACC) oxidase, *S*-adenosyl-*L*-methionine (SAM) synthase and ACC synthase identified some of the key genes involved in ethylene biosynthesis, a control point of fruit ripening. Molecular studies on genes encoding key enzymes in carbohydrate metabolism have included: polygalacturanase; xyloglucan endotransglycosylase/hydrolase; polygalacturonase inhibitor protein; sucrose phosphate synthase; and sucrose synthase. The most widely studied genes in these early forays into *Actinidia* molecular biology were those encoding the cysteine protease, actinidin, which can account for up to 50% of fruit soluble protein. Actinidin genes have been cloned, expressed in transgenic tobacco, the promoter sequenced, and studied in transgenic petunia.

5.1 Expressed sequence tag (EST) databases

A significant watershed in advancing *Actinidia* molecular biology was the generation of a database of 132,577 expressed sequence tags (EST), from a variety of *Actinidia* species, (Crowhurst et al., 2008). This provided a significant increase in the availability of *Actinidia* transcriptomic data, which prior to this publication were represented by 511 sequences in GenBank (dbEST Jan. 2008). This genetic resource, derived primarily from four species (*A. chinensis*, *A. deliciosa*, *A. arguta* and *A. eriantha*), included a range of tissues and developmental time points (Table 1). The average sequence length of these EST sequences was 503 bases. As expected, a high frequency of redundancy was observed within the *Actinidia* EST dataset and clustering at a 95% threshold, resulting in 23,788 sequences remaining as singletons and 18,070 tentative consensus (TC) sequences, a combined total of 41,858 non-redundant clusters (NRs). Analysis revealed that 28,345 NRs had sufficient homology to *Arabidopsis* sequences ($E>1.0e^{-10}$) to be assigned a functional classification. Many of the NRs with no *Arabidopsis* homolog did however, have homologs in other crops. Crowhurst et al. (2008) also reported more specific analysis of ESTs of key genes related to distinctive features of *Actinidia* including flavour and aroma, colour, health-beneficial compounds, allergens, and cell wall structure.

Codon usage analysis revealed that *Actinidia* shared many similarities with other dicotyledonous plants, and although codon usage was similar among three *Actinidia* species, it was not identical. A higher GC ratio was seen in coding than in non-coding regions, and this was more marked in *A. deliciosa* and *A. eriantha* than in *A. chinensis*. A modest degree of

CpG suppression was also evident in the three *Actinidia* species, with an XCG/XGG ratio of 0.68–0.71. Analysis of overlapping regions of 3,901 TCs identified 32,764 bi-allelic single nucleotide polymorphisms (SNPs), with one SNP every 417 bp, although some of the SNPs were probably the result of homeologous or paralogous sequences, rather than allelic variation. The allelic SNPs have potential for the development of molecular markers for use in genetic mapping, population genetics and linkage disequilibrium studies or for marker-assisted selection. The inter-specific SNPs, identified in orthologous loci from different *Actinidia* species represent species–species variation and have utility in kiwifruit breeding using crosses between different species. Further analysis revealed that over 30% of the *Actinidia* EST NRs had at least one SSR, with dinucleotide repeats, predominantly in the 5′ untranslated region, being twice as frequent as trinucleotide repeats, which were more evenly distributed across the gene.

***Actinidia* sp.**	**Tissue type**							
	Bud	**Fruit**	**Leaf**	**Petal**	**Root**	**Cell**	**Stem**	**Total**
A. deliciosa	34,519	13,282		9,950				57,751
A. chinensis	15,689	8,453	17,325	1,061		4,851		47,379
A. eriantha		11,259		1,388				12,647
A. arguta		5,421		1,836				7,257
A. hemsleyana					5,101			5,101
A. polygama				1,348				1,348
A. setosa							1,020	1,020
A. indochinensis				74				74
Total	50,208	38,415	17,325	15,657	5,101	4,851	1,020	132,577

Table 1. Numbers of ESTs derived from various *Actinidia* species and tissues

5.2 An *Actinidia* microarray platform

Characterizing a gene's temporal and spatial expression is critical to understanding its function. Early *Actinidia* molecular studies characterized the expression of a limited number of genes, identified as being differentially expressed during a particular developmental phase (Ledger & Gardner, 1994) or members of a particular gene family (Langenkamper et al., 1998). The *Actinidia* EST database provided a resource for more global gene expression analysis, through the development of a 17,472-feature oligonucleotide microarray of *Actinidia* genes. This microarray represented genes from a variety of species: *A. chinensis* (51%); *A. deliciosa* (38%); *A. eriantha* (6%); *A. arguta* (3%); and other *Actinidia* species (2%).

Walton et al. (2009) used the *Actinidia* microarray to examine gene expression in *A. deliciosa* meristems and buds in response to the dormancy-breaking hydrogen cyanamide (HC) chemical treatment, over two growing seasons. Although most of the genes that responded early (1–3 days) to HC treatment differed between seasons, there was a high degree of commonality between seasons of genes that showed the greatest change in expression six days post treatment, with 123 genes up-regulated and 35 genes down-regulated at day 6 in both seasons. Quantitative PCR (qPCR) of 35 selected genes validated the microarray data for 97% of up-regulated and 60% of down-regulated genes. Genes that changed in expression upon HC-treatment were classified into distinct profiles, including: i) genes that reached a peak in expression at 3 or 6 days post treatment, then returned to baseline levels

by day 15; ii) genes that reached a peak in expression at 3 or 6 days post treatment, followed by a second burst of transcription at 25–40 day post treatment, iii) genes that decreased in expression prior to meristematic activity or external bud growth. Putative function of these HC-responsive *Actinidia* genes, based on homology to other plant genes, indicated that many had been identified in other plant stress-related studies, including a number of genes that had shown similar responses in HC-treated grape, suggesting similar mechanisms in response to HC-treatment in these two crops.

Actinidia species are a climacteric fruit, showing a dramatic increase in ethylene production and a high respiration rate during fruit ripening. Generally, kiwifruit are harvested firm, and then enter a period of softening, which is followed by the onset of autocatalytic ethylene production, when fruit soften to "eating ripe" firmness and develop their characteristic flavours and aromas. The final step of the ethylene biosynthetic pathway is the conversion of 1-aminocyclopropane-1 carboxylic acid (ACC) to ethylene by ACC oxidase. Atkinson et al. (2011) examined gene expression changes during the ripening process, using an ACC oxidase-silenced transgenic *Actinidia* line, the fruit of which produce no detectable climacteric ethylene, but could be induced to undergo softening, aroma and flavour development through the application of exogenous ethylene. Using the *Actinidia* microarray, expression of 401 genes changed significantly within 168 h of ethylene treatment, with 25 genes showing a response at 4 h, 81 genes at 12 h, and 183 genes 24 h after application. These ethylene-responsive genes could be grouped into functional categories, including: metabolism; oxidative stress; photosynthesis; regulation; cell wall; hormone; starch; other; and unknown functions. The expression patterns indicated that the majority of photosynthesis- and starch-related genes were down-regulated by ethylene, whereas up- and down-regulation of genes in other functional groups were observed in response to ethylene. Validation by qPCR confirmed significant changes in gene expression of a number of genes involved in cell wall modification in response to ethylene, including a polygalacturonase, a pectin lyase, a pectin methylesterase and a xylan-degrading enzyme, as well as genes involved in fruit flavour, ethylene production and perception.

The microarray platform has provided a useful tool for genome-wide gene expression, as is evident from the studies above. However, microarrays have a limited dynamic range, lack the sensitivity required to detect subtle changes in expression, and are essentially a 'closed' platform, limited to examining the expression of only those genes represented on the array. Second-generation sequencing (2ndGS) is becoming the methodology of choice for many genome-wide expression studies (L. Wang et al., 2010), as this is an 'open' platform, capable of detecting any of the genes that are expressed within a particular tissue, organ or cell type at the time of RNA sampling. Analysis of *Actinidia* transcription has been initiated using Illumina 2ndGS, with mRNA-sequence data generated from a range of *A. chinensis* tissues and stages of fruit development (A.P. Gleave & Z. Luo, unpublished).

5.3 Functional genomics in *Actinidia* and heterologous hosts

Prior to the initiation of generating the *Actinidia* EST resource in 2000, reports of functional genomics through expression of *Actinidia* genes in either a heterologous or an *Actinidia* host were somewhat limited (Guo et al., 1999; Lay et al., 1996; Paul et al., 1995; Schroder et al., 1998; Z.C. Xu et al., 1998). The EST resource has facilitated a significant increase in *Actinidia* functional genomics, through expression of genes in various microbial and plant hosts.

Actinidia genes encoding: a pectin methylesterase inhibitor, with applications in fruit juice production (Hao et al., 2008); Bet v 1 and profilin-homologous allergens (Bublin et al., 2010; Oberhuber et al., 2008); an *L*-galactose-1-phosphate phosphatase and *l*-galactose guanyltransferase, (Laing et al., 2004; 2007) and *L*-galactose dehyrogenase (Shang et al., 2009), involved in vitamin C production; a lycopene beta-cyclase, involved in carotenoid production (Ampomah-Dwamena et al., 2009); three xyloglucan endotransglucosylase/ hydrolases involved in cell wall structure (Atkinson et al., 2009); two terpene synthases, involved in the production of floral sesquiterpenes (Nieuwenhuizen et al., 2009); and three glycosyltransferases of the anthocyanin pathway (Montefiori et al., 2011), have all been successfully expressed in *Escherichia coli,* with the recombinant proteins being used to study protein/enzyme function. The yeast species, *Pichia pastoris* or *Saccharomyces cerevisiae*, have also been used to express recombinant *Actinidia* proteins, a pectin methylesterase inhibitor (Mei et al., 2007), and three alcohol acyltransferases, involved in the production of volatile esters (Gunther et al., 2011) and actinidin, which was found to have a negative effect on *S. cervesiae* growth (Yuwono, 2004).

In planta functional genomics of *Actinidia* genes has been used to study genes involved in a variety of processes. Paul et al. (1995) expressed *A. deliciosa* preproactinidin in transgenic *Nicotiana tabacum*, showing that the protein was correctly processed and detrimental to plant growth when it accumulated to high levels. Yin et al. (2010) showed that expression of the *A. deliciosa ETHYLENE INSENSITIVE3*-like *EIL2* and *EIL3* transcription factor cDNAs in *Arabidopsis thaliana* stimulated ethylene production, and up-regulation of host ACC synthase and ACC oxidase gene family members, as well as a number of xyloglucan endotransglycoylase (*XET*) genes. Yin et al. (2010) also used the *N. benthamiana* transient expression system, described by Hellens et al. (2005), to demonstrate transactivation of *A. deliciosa* ripening-related *ACO1* and *XET5* promoters by *EIL2* and *EIL3*, confirming their role in the signal transduction pathway connecting ethylene signalling and ripening processes.

To understand the role of *Actinidia* lipoxygenase (*LOX*) genes, which in other plants are involved in a range of processes, including senescence and fruit ripening, B. Zhang et al. (2006) used transient expression of *A. deliciosa LOX1 and LOX2* genes in *N. benthamiana*. qPCR had shown that *LOX1* increased in expression in ethylene-treated fruit, in contrast to *LOX2 expression,* which was repressed by ethylene. The transient expression studies revealed that *LOX1* significantly accelerated chlorophyll degradation and chlorophyll fluorescence, whereas *LOX2* had no apparent effect on senescence.

Varkonyi-Gasic et al. (2011) expressed cDNAs of nine *Actinidia* MADS-box genes in *A. thaliana,* to determine their role in floral meristem and floral organ fate. Resulting transgenic plants showed a variety of phenotypes. *FUL-like* expression promoted floral transition in both long day (LD) and short day (SD) conditions, with a terminal flower phenotype evident in plants showing high levels of transgene expression. Expression of *FUL* promoted flowering, but less efficiently than *FUL-like*, and the floral phenotype was as wild-type. *SEP4* expression also promoted floral transition, with many plants showing small and curled leaves, and a reversion to vegetative growth and aerial rosettes during SD conditions. *SEP3* expression had a mild effect on floral transition under LD conditions and *PI* and *AP3-1* expression showed no effect. *A. thaliana* expressing the kiwifruit *AG* flowered earlier than the wild-type under SD conditions, and showed reduced height, curled leaves and a loss of inflorescence indeterminancy. Coupled with information on the patterns of expression of

these genes in *Actinidia* vegetative tissue and both normal and aberrant floral organs, these studies gave considerable insights into the role of these MADS-box transcription factors in the specification of *Actinidia* floral organs, phase change and flowering time.

Vitamin C is an essential metabolite for plants and animals, and the inability of some animals, including humans, to synthesize vitamin C means that they are dependent upon a dietary source. The *L*-galactose pathway is a significant route for vitamin C production in plants, although the enzyme responsible for the conversion of GDP-*L*-galactose to *L*-galactose-1-phosphate remained elusive until Laing et al. (2007) identified homologous genes from *Arabidopsis* and *A. chinensis* encoding a GDP-*L*-galactose guanyltransferase (GGT) capable of carrying out this function. Transient expression of the *A. chinensis GGT* gene in *N. benthamiana* leaves showed a 3-fold increase in vitamin C levels, and coupled with the biochemical studies, confirmed GGT's role in the *L*-galactose pathway. Further studies of *Actinidia* vitamin C production via the *L*-galactose pathway also made use of *in planta* functional genomics. As qPCR results had indicated GGT and GDP-mannose-3',5'-epimerase (GME) were key enzymes involved in the high vitamin C content of *A. eriantha*, Bulley et al. (2009) expressed the *A. eriantha GGT* in *Arabidopsis*, identifying plants with over four times the amount of vitamin C in leaves. In *N. benthamiana* leaves, transient *GGT* expression increased vitamin C levels 4.2 fold, a 20% increase resulted from transient *GME* expression, and simultaneous expression of *GGT* and *GME* gave an average increase of 8.6-fold in vitamin C.

Biochemical and gene expression studies on the production β-linalool, an acyclic monoterpene alcohol, were supplemented by transient expression of putative (*S*)-linalool synthase cDNAs from *A. eriantha* and *A. polygama*, to further understand their role in floral aroma (X.Y. Chen et al., 2010). The production of large amounts of linalool in *N. benthamiana* leaves transiently expressing these cDNAs confirmed their function as linalool synthases. A biochemical study of *A. deliciosa* 'Hayward' and its male pollinator 'Chieftain' identified the sesquiterpene (*E,E*)-α-farnesene as the major terpene floral volatile, with germacrene D, (*E*)-β-ocimene, (*Z,E*)-α-farnesene, also present (Nieuwenhuizen et al., 2009). Transient expression in *N. benthamiana* leaves of two *A. deliciosa* cDNAs encoding putative terpene synthases (*AFS*1 and *GDS1*), followed by dynamic headspace sampling and GC-MS analyses, showed that expression of *AFS1* resulted in the production of large quantities of (*E,E*)-α-farnesene and smaller quantities of (*Z,E*)-α-farnesene and (E)-β-ocimene. *GDS1* expression resulted in production of germacrene D.

Glycosyltransferases are responsible for much of the diversity of anthocyanins, a subgroup of the flavonoids that give much of the red, purple and blue pigmentation to plants. Montefiori et al. (2011) characterized two glycosyltransferases (*F3GT1* and *F3GGT1*) from a red-fleshed *A. chinensis*. Recombinant *F3GT1* produced in *E. coli* catalyzed the addition of galactose to the 3-*OH* position in cyanidin, whilst recombinant *F3GGT1* catalyzed the addition of UDP-xylose to cyanadin-3-galactosidase. Confirmation of the roles of these genes in the red pigmentation of fruit flesh was demonstrated firstly through establishing that transient expression of *Arabidopsis PAP1* and *TT8* transcription factors in *A. eriantha* fruit resulted in red pigmentation, localized mainly around the fruit core, and with the major accumulated anthocyanin being cyanidin 3-*O*-xylo-galactoside. Concomitant transient expression of the two *Arabidopsis* genes with an *F3GT1* RNAi construct resulted in little or

no visible red colour in *A. eriantha* fruit, indicating the *F3GT1* gene's critical role in anthocyanin biosynthesis. Concomitant transient expression of *PAP1* and *TT8* with an *F3GGT1* RNAi construct greatly reduced the amount of the major anthocyanin, cyanidin 3-*O*-xylo-galactoside.

Despite the availability of *Actinidia* transformation systems, to date there has been little published research on functional genomics of *Actinidia* genes through over-expression or silencing of genes in *Actinidia.* Such research is ongoing at Plant & Food Research and elsewhere, and the lack of published information is probably because many of these studies are related to fruit characteristics, and the time from initiating transformation to fruiting is at least three years. Of the few reports that have been published, one using gene silencing of ACC oxidase (Atkinson et al., 2011) has been discussed earlier in this chapter. Other studies have involved the over-expression of an *Actinidia Lfy* transcription factor cDNA, in an attempt to enhance early fruit set (Guo et al., 1999) and the silencing of ACC synthetase or ACC oxidase genes in *A. deliciosa* and *A. chinensis* (Li et al., 2003). In both these studies, only the production of transgenic plants was reported, with no analysis of their phenotype. Ledger et al. (2010), however, describe the use of transgenic *A. chinensis* plants to examine the role of *carotenoid cleavage dioxgenase* (*CCD*) genes in branching and vine architecture. The involvement of *CCD* genes, or their orthologs, in branching has been shown through the characterization of branching mutants in a range of annual plant species. Ledger et al. (2010) showed that expression of *A. chinensis CCD7* and *CCD8* cDNAs was able to complement their corresponding *Arabidopsis* branching mutants *max3* and *max4*. In *A. chinensis* plants transformed with a *CCD8* gene silencing construct, a number of plants showed greatly reduced *CCD8* expression levels at eight and 13 months of growth in the glasshouse. The *CCD8*-silenced plants showed significantly more primary and higher order branches, and a higher incidence of short branches, compared with control plants, but no difference in internode length on the main stem. Another finding was that leaves on some *CCD8*-silenced plants were slower to senesce and had a greater chlorophyll content than leaves of control plants. This study confirmed the role of *CCD8* in branching, and identified that *CCD8* plays a role in senescence in a deciduous woody perennial plant.

The studies described above give some valuable insights into the enzymatic or structural function of proteins encoded by these *Actinidia* genes and the roles they may play in the plant's phenotypic characteristics. However, in assigning a definitive function to a gene, it is essential also to understand the temporal and spatial regulation of its expression. In many of the studies described above, microarray and/or qPCR analysis were used to determine the transcriptional level of these genes in various tissues and in some cases at different developmental phases of the plant. Another approach to gain insights into the regulation of a gene's expression has been the analysis of promoter-reporter gene fusions in transgenic systems. Lin et al. (1993) fused an upstream region of an actinidin coding region to the β-glucuronidase (GUS) coding region and observed GUS expression during the later stages of transgenic petunia seed pod development, resembling the induction of actinidin in *Actinidia* fruit tissues. Similar promoter-GUS fusions were used to analyse an *A. chinensis* polygalacturonase promoter, and at the breaker stage of transgenic tomato fruit development, GUS expression was observed throughout the inner and outer pericarp, the columella and seeds, and became restricted to the inner pericarp and seeds at the later stages of ripening (Z.Y. Wang et al., 2000).

General Process	Metabolic pathway, process or gene classification		No. of over-expression	No. of silencing constructs
Flavour & Aroma	Terpenoid Biosynthesis		9	3
	Ester Biosynthesis		12	1
	Cytochrome P450		22	3
Sugars & Acids	Sugar metabolism		2	
	Aromatic amino acid		4	
Fruit Ripening	Cell wall structure		5	1
	Ethylene biosynthesis and		2	1
Colour	Chlorophyll degradation		3	1
	Carotenoid biosynthesis		2	2
	Anthocyanin biosynthesis		1	
	Phenylpropenoid biosynthesis		1	3
Vitamin C	Ascorbate biosynthesis		24	6
Protein Degradation	Ubiquitination		2	1
	Actinidin		3	2
Allergenicity	Allergens		2	1
Plant Development	Branching		1	1
	Phase change		2	1
	Hormone response		5	4
	Cell signalling		1	
Defence	Antimicrobial peptides		2	
DNA and Replication	Cell cycle			1
	Nucleotide synthesis & DNA			2
	Chromatin remodelling		1	5
Transport	Transporters		3	1
Unknown	Sex-linked			3
Transcription	Transcriptional machinery		2	
	miRNA		4	
	Transcription factors	*Myb* & *Myb*-	35	3
		bZIP	19	2
		MADS-box	15	7
		bHLH	13	1
		C2-C2 *Dof*	9	
		C2-C2 *CO*-	8	
		NAC Domain	5	
		AP2-EREB	2	1
		WRKY	1	1
		BZR	1	1
		WD40	1	
Total			224	59

Table 2. Plant & Food Research's *Actinidia in planta* cDNA over-expression and gene silencing construct collection. Over-expression constructs of full length cDNAs cloned into pART27-derived vectors (Gleave, 1992) and gene silencing constructs of hairpin cDNA structures cloned into pTKO2 (Snowden et al., 2005). cDNAs are under the control of the CaMV35S promoter.

Transcription factors play a central role in regulating gene expression, through activation or repression of target promoters, and are able to regulate complex developmental processes or entire metabolic pathways co-ordinately. The dual-luciferase reporter vector system, developed to identify transcription factor activation of promoters (Hellens et al., 2005), has been applied to studying transactivation of promoters of *Actinidia* ripening-related *ACO1* and *XET5* genes, by *EIL2* and *EIL3* (Yin et al., 2010). Exploiting this promoter activation tool is reliant on having a cloned *Actinidia* transcription factors; Plant & Food Research has developed such a resource, which includes *Actinidia* cDNA over-expression and RNAi gene silencing constructs, including 109 *Actinidia* transcription factors for use in many of the *in planta* functional genomics approaches described above (Table 2).

5.4 Molecular markers and mapping

Molecular markers have been used to carry out genetic characterization of the *Actinidia* genus. They allow germplasm enhancement through systematic crossing of plants, selected on the basis of their intra- and inter-specific phylogenic relationships and patterns of allelic diversity, and the selection of parent plants with desirable alleles for use in breeding programmes. Markers are also used to determine hybridity, pedigree, and for quality control during crossing. The development and application of molecular markers closely linked to desirable traits has the potential to assist kiwifruit breeding greatly through the early selection of those progeny, with a high probability of carrying the genetic information for the desired trait. Various genetic markers have been developed in *Actinidia* using restriction fragment length polymorphisms (RFLPs) (Crowhurst et al., 1990), amplified fragment length polymorphisms (AFLPs) (Novo et al., 2010; Testolin et al., 2001; X.G. Xiao et al., 1999), random amplified polymorphic DNAs (RAPDs) (Gill et al., 1998; H.W. Huang et al., 2002a; Shirkot et al., 2002), SSRs (Fraser et al., 2004; W.G. Huang et al., 1998; Korkovelos et al., 2008) or SNPs (Zhou et al., 2011). Much of the early molecular marker development was primarily to investigate the molecular phylogeny of *Actinidia* species, which in general was consistent with the traditional morphology-based classification. Studies were aimed also at sex determination, and molecular markers confirmed that the dioecious nature in *Actinidia* was a consequence of sex-determining genes localized on a pair of chromosomes that function like an XX/XY system (Gill et al., 1998; Harvey et al., 1997; Testolin et al., 1995). Although the genetic basis for sex determination in kiwifruit remains unknown, RAPD markers linked to this trait led to the development of sequence-characterized amplified regions (SCARs) (Gill et al., 1998). These are now deployed routinely in marker-assisted kiwifruit breeding, to eliminate male plants from crosses at the seedling stage, to select males when breeding for pollinizers, or to ensure a desirable male-to-female ratio of progeny are planted when characterizing families.

A framework *Actinidia* linkage map was first constructed using SSRs and the pseudo-test cross mapping strategy, often used for mapping out-crossing species, followed by the integration of AFLP markers (Testolin et al., 2001). Markers were screened over 94 individuals from a population generated from an inter-specific cross of a diploid *A. chinensis* female and a diploid *A. callosa* male. Linkage maps were produced for each parent, with the female framework map having 160 loci, 38 linkage groups and covering 46% of the estimated genome length, and the male framework map having 116 loci, 30 linkage groups, and covering 34% of the estimated genome length. The maps were produced with LOD

scores ≥2 (as an indication of coinheritance of loci). Continued progress in *Actinidia* mapping led to a significant advancement in *Actinidia* genetics, with the generation of a gene-rich linkage map of *A. chinensis,* constructed using 644 SSRs, and defining the 29 chromosomes of the haploid genome (Fraser et al., 2009). Again, SSRs were the marker of choice, owing to their abundance, distribution in coding and non-coding regions, reproducibility, Mendelian mode of inheritance and co-dominant nature. The inherent variability of SSRs, because of the high mutation rate, makes SSRs highly informative genetic markers. The linkage maps were produced using a mapping population of 272 individuals, created through an intra-specific cross of diploid *A. chinensis* parents, selected from two very distinct geographical locations in China, and exhibiting a diversity of fruiting characteristics. Resulting comprehensive genetic linkage maps of the male and female parents were produced and an integrated map of the cross was generated, using co-dominant SSR markers. The female and male linkage maps were composed of 464 and 365 markers, respectively, with markers estimated to be within 10 cM of each other in over 96% of the female genome and 94% of the male genome. The robustness of the maps was reflected by the LOD scores of 4–10. Using sex-linked SCAR markers, linkage group 17 was identified as the putative X and Y chromosomes. The sex-determining locus appeared to be sub-telomeric, occupying only a small portion of the chromosome, with little evidence of recombination in this region. These genetic linkage maps provide a valuable resource for the supply of markers for the breeding of novel cultivars, as tools for comparative and quantitative trait mapping. They will contribute to further investigations on the evolution and function of genetic control mechanisms in kiwifruit. They are an essential part of assembling the genome sequence of *Actinidia*.

5.5 Genome sequencing

Although the *Actinidia* EST database is a useful resource, it at best represents only 50–60% of the genes within the *Actinidia* genome, and contains no information on elements such as promoters, terminators and introns that play important roles in controlling gene expression. In addition, EST libraries, by the nature of their construction, under-represent genes that are expressed at relatively low levels and yet could play a critical role in a particular trait. Understanding key traits requires detailed information of not only the transcribed regions of a genome but also the intergenic and intron sequences, information that can be gained from the whole genome sequence (WGS) and its subsequent annotation. The advent of second-generation sequencing (2^{nd}GS) and advances in data handling and assembly software have now made it feasible to determine the WGS of a plant species at a fraction of the cost of the Sanger technology used to generate the WGS of *Arabidopsis,* for example (The Arabidopsis Genome Initiative, 2000). Plant & Food Research has recently initiated a research effort to determine the WGS of a diploid *A. chinensis* genotype, the haploid genome of which is predicted to be 650 Mbp. With no di-haploid or homozygous *Actinidia* genotypes available, the heterozygosity of the diploid *A. chinensis* may create some problems in WGS assembly. To minimize this, a genotype that has undergone two generations of sib-crossing and has an inbreeding coefficient of 0.375 has been selected. Genome sequencing is being carried out using an Illumina sequencing platform, using a variety of libraries and resulting sequencing data assembled into scaffolds. WGS assembly is being complemented by BAC-end sequencing, using an *A. chinensis* BAC library (Hilario et al., 2007), and use of the genetic-linkage map discussed earlier.

6. Future perspectives and challenges

Completion of the *A. chinensis* WGS will be the first within the Ericales, a large and diverse order that includes persimmon, blueberry, cranberry and tea, and the benefits to be gained in having the *Actinidia* WGS are enormous. Genome annotation is a key to the utility of any WGS, and the advances in transcriptome sequencing will greatly aid the defining and delineating of genes. Building on the availability of the genome sequence, characterization of the interrelationships between the *Actinidia* genome, transcriptome, proteome and metabolome, and functional genomics of alleles, will greatly aid in the understanding of biological processes, phenotypes and traits of kiwifruit. The annotated *A. chinensis* WGS will also provide a reference genome for the sequencing of genomes of other *Actinidia* species, to examine inter-species variability, and to identify SNPs. The knowledge gained from these efforts will open up greater opportunities for molecular breeding in kiwifruit, allowing the use of molecular markers for selective and accelerated introgression of desirable traits from the diverse *Actinidia* germplasm, to create new and novel cultivars.

As detailed in this chapter, much of the molecular research in *Actinidia* has targeted traits such as fruit flavour, aroma, ripening and colour, which could be exploited in the development of new cultivars with novel fruit characteristics. There has been very little molecular research in *Actinidia* targeted towards pathogens and disease. However, the recent devastating effect on commercial kiwifruit orchards in parts of Italy, due to kiwifruit canker, caused by the bacterium *Pseudomonas syringae* pv. *actinidiae* (Psa), may well change the emphasis of the immediate future of kiwifruit research. Although Psa was identified in Italy in 1992 (Scortichini, 1994), the bacterium caused little problem, until severe disease outbreaks in both *A. deliciosa* and *A. chinensis* cultivars in 2009. The presence of Psa has now been reported in most of the major kiwifruit growing regions of the world, although there appear to be a number of haplotypes, differing in their virulence. Minimizing the impact of Psa on the global kiwifruit industry will require a coordinated effort by pathologists, physiologists, breeders and growers. Many of the molecular tools, the knowledge and the *Actinidia* resources described in this chapter will aid in the understanding of the plant–pathogen interactions, the plant's response to infection, the identification and mapping of *Actinidia* genes offering Psa resistance, and ultimately the development of kiwifruit cultivars resistant to Psa, through breeding or genetic manipulation.

7. References

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