Transgenic Plant Journal ©2007 Global Science Books

High Throughput Transformation of *Actinidia:* A Platform for Kiwifruit Functional Genomics and Molecular Breeding

Tianchi Wang* • **Kui Lin-Wang**

The Horticulture and Food Research Institute of New Zealand Ltd., Private Bag 92169, Auckland, New Zealand

Corresponding author: * **tianchi.wang@hortresearch.co.nz**

ABSTRACT

Plant transformation is an important tool for charactering the function of genes that will assist in the breeding of novel cultivars. Establishing a transformation platform, which can produce transformants in a highly efficient, reliable and reproducible way, is a prerequisite to using this tool for both genetic breeding programmes and functional genomic analysis. *Agrobacterium*-mediated transformation is still the most effective mechanism for transforming genes into the fruit species *Actinidia* (kiwifruit). Great progress has been made in *Actinidia* transformation since the first transgenic kiwifruit plants were reported 17 years ago. This progress includes expression from the fruit-related actinidin promoter, development of high transformation efficiency, the choice of *Agrobacterium* strain, various genes of interest, different species and field trials of transgenic plants. To date, transformation systems have been developed for three *Actinidia* species for varying purposes. The emerging scientific revolution, sparked by genomics-based technologies, has produced enormous amounts of gene sequence information. An *Actinidia* database of over 130,000 expressed sequence tags (ESTs) has been developed to exploit the genetic potential of kiwifruit. The platform of high throughput transformation of *Actinidia* has been set up for characterizing kiwifruit genes from the EST database in an efficient way. A range of factors, which are associated with *Agrobacterium*-mediated transformation systems, will be discussed. An overview will be provided on the use of transgenic technology and the new emerging concepts of transformation techniques.

Keywords: *Agrobacterium*-mediated transformation, biotechnology, cisgenics, transgenic plant
Abbreviations: **AS**, acetosyringone; **CAT**, chloramphenicol acetyl transferase; **EST**, Expressed sequence tag; **GUS**, β-glucuronidase; ***nptII***, neomycin phosphotransferase II; **PEG**, polyethylene glycol

CONTENTS

INTRODUCTION

Actinidia is a genus of deciduous vines with more than 60 species naturally distributed mainly in China and South Eastern Asia, mostly found in the temperate, mountainous forests (Liao and Wang 1984; Ferguson 1990). The plants are normally dioecious (male and female flower are separate plants), but occasional *Actinidia* plants have perfect flowers (Ferguson 1984). Kiwifruit (*Actinidia* spp.) was introduced into New Zealand from its original areas in China, and subsequently developed as a successful fruit crop in world trade in the 20th century. The commonly cultivated species are *A. deliciosa* (green kiwifruit), *A. chinensis* (yellow kiwifruit) and *A. arguta* (baby kiwifruit). Globally it is a small fruit industry compared with apple, banana and citrus, with it representing around 0.25% of total world pro-

Received: 9 March, 2007. Accepted: 4 May, 2007.

Invited Review

duction of major fruit crops (Belrose Inc. 2006). Because kiwifruit meets the requirements seen as of primary importance in new fruit development, such as health and pleasure, there is good potential for increased consumer demand and, hence, increased commercial production. The total area of known kiwifruit plantings throughout the world was estimated at more than 100,000 hectares in 2005. The total world kiwifruit production was above 1.5 million tonnes, about 48% above the level achieved in 1998 (Belrose Inc. 2006).

Breeding programmes for *Actinidia* have been set up, in particular in New Zealand, Italy and China, with the objectives of these breeding programmes directed primarily at producing *A. deliciosa* and *A. chinensis* varieties with large fruit size, good flavour, novel flesh colour, variations in harvest period, improved yield and growth habit, and hermaphroditism (Ferguson *et al*. 1996). Longer term breeding objectives include the development of novel high value fruit with increased consumer health and convenience attributes (Seal 2003). In addition to the conventional breeding methods, various modern molecular biology methodologies and tools have been employed to understand processes that control fruit characters, and to assist in reaching breeding goals.

Applications of biotechnology in *Actinidia*

The genus *Actinidia* has an unusually wide range of species diversity and a rich genetic resource. This provides tremendous potential for continuing cultivar improvement and enhancing the sustainability of the world kiwifruit industry. However, kiwifruit commerce in the world is currently based on very few selected cultivars, which were bred by selection from natural phenotypic variations of two species, *A. deliciosa* and *A. chinensis*. Natural genetic resources are and will be the fundamental basis for future genetic improvement. Nevertheless, there are some features in *Actinidia* which make them less amenable to conventional breeding programmes and application of biotechnology than other crops. These features include: 1) vigorous climbing vines; 2) relative long juvenile periods of 3-5 years; 3) all species are functionally dioecious; 4) a reticulate polyploid structure with diploids, tetraploids and octoploids; and 5) inter and intra-specific compatibility (Fairchild 1927; Ferguson *et al*. 1997; Atkinson and MacRae 2007).

Efforts have been made to overcome the problems mentioned above by employing a range of modern molecular tools and techniques. Considerable progress has been made to achieving the breeding goals. Research has focused on molecular markers for sex (Michelmore *et al*. 1991; Harvey *et al*. 1997; Gill *et al*. 1998); RAPD and microsatellite markers for cultivar identification and somatic mutation (Cipriani *et al*. 1996, Fraser *et al*. 2001; Palombi and Damiano 2001; Zhen *et al*. 2004); Southern and Northern analysis (Kobayashi *et al*. 1996; Schröder *et al*. 1998; Chen *et al*. 1999), cDNA library production (Ledger and Gardner 1994; Langenkämper *et al*. 1998); constructing a genetic map of *Actinidia* species (Testolin *et al*. 2001; Fraser *et al*. 2004) as well as protein and enzymatic analysis (MacRae *et al*. 1992; Marquis and Bucheli 1994; Sugiyama *et al*. 1996; Laing *et al*. 2004). Tissue culture techniques e.g. multiplication, embryo rescue, somatic embryogenesis, haploid recovery, triploid recovery from endosperm culture, protoplast culture and cryopreservation applied to kiwifruit have been reviewed (Kumar and Sharma 2002; Oliveira and Fraser 2005). Encapsulated buds from *in vitro* shoots of 'Hayward' kiwifruit (*A. deliciosa*) were used for non-embryogenic synthetic seed production (Gardi *et al*. 1999; Adriani *et al*. 2000). Somatic hybridization has been achieved in *Actinidia* (Xiao and Han 1997; Xiao *et al*. 2004). Protoplasts isolated from cotyledon-derived callus of *A. chinensis* ($2N = 2x = 58$) were fused with cotyledon protoplasts of *A. deliciosa* ($2N = 6x = 174$) or with mesophyll protoplasts of *A. kolomikta* ($2N = 2x = 58$) using PEG (polyethylene glycol) method. Plantlets of interspecific somatic hybrid of *A. chinensis* and *A. deliciosa* ($2N = 8x = 232$) and interspecific somatic hybrid of *A. chinensis* and *A. kolomikta* ($2N = 4x = 116$) were obtained, respectively (Xiao and Han 1997; Xiao *et al*. 2004). Recent advances in protoplast culture and somatic hybridization of *Actinidia* are summarised in **Table 1**.

Great progress has been made in *Actinidia* transformation since the first transgenic plant was reported 17 years ago (Matsuta *et al*. 1990; Rugini *et al*. 1991; Uematsu *et al*. 1991; Janssen and Gardner 1993; Fraser *et al*. 1995; Yamakawa and Chen 1996; Rugini *et al*. 1997; Wang *et al*. 2006a, 2006b). The emerging scientific revolution in genomic-based technologies has produced enormous amounts of gene sequence information. By January 2007, there were 482 en-

Table 1 Protoplast culture and somatic hybridization in *Actinidia*.

Species	Tissue types used	Purposes	Results	References
A. arguta	Leaf mesophyll, calli	Protoplast culture	Regenerated cell walls	Derambure and Hirsch 1995
A. arguta	Leaf mesophyll	Protoplast culture	Microcalli	Xiao and Hirsch 1996
A. arguta	Leaf calli	Protoplast culture	Cell colonies	Zhu *et al*. 2001
A. chinensis	Petiole segments	protoplast culture	Plantlets	Pais *et al*. 1987
A. chinensis	Leaf calli	Protoplast culture	Plantlets	Tsai 1988
A. chinensis	Cotyledon callus	Protoplast culture	Plantlets	Xiao *et al*. 1992
A. deliciosa	Suspension culture cells	Protoplast culture	Plantlets	Mii and Ohashi 1988
A. deliciosa	Leaf, petiole and stem fragment calli	Protoplast culture	Plantlets	Cai *et al*. 1991
A. deliciosa	Leaf mesophyll, calli	Protoplast culture	Regenerated cell walls	Derambure and Hirsch 1995
A. deliciosa	Leaf mesophyll	Protoplast culture	Microcalli	Xiao and Hirsch 1996
A. deliciosa	leaves	Protoplast culture and direct DNA transfer	Plantlets	Raquel and Oliveira 1996
A. deliciosa	Leaf mesophyll	Protoplast culture	Cell colonies	Ferraiolo 1997
A. deliciosa	Stem segment calli	Protoplast culture	Plantlets	Hu *et al*. 1998
A. deliciosa	Suspension culture cells	Protoplast culture	Plantlets	Gan *et al*. 2003
A. eriantha	Leaves	Protoplast culture	Plantlets	Zhang *et al*. 1998
A. kolomikta	Leaf mesophyll	Protoplast isolation	Protoplasts	Derambure and Hirsch 1995
A. kolomikta	Leaf mesophyll	Protoplast culture	Microcalli	Xiao and Hirsch 1996
A. polygama	Leaf mesophyll	Protoplast isolation	Protoplasts	Derambure and Hirsch 1995
A. polygama	Leaf mesophyll	Protoplast culture	Microcalli	Xiao and Hirsch 1996
A. arguta x *A. deliciosa*	Shoot tips and young leaves	Somatic hybridization	Fused cells	Lindsay *et al*. 1995
A. kolomikta x *A. polygama*	Leaf mesophyll	Somatic hybridization	Fused cell division	Xiao and Hirsch 1997
A. chinensis x *A. deliciosa*	Cotyledon callus Cotyledon callus	Somatic hybridization	Plantlets	Xiao and Han 1997
A. chinensis x *A. kolomikta*	Cotyledon callus Leaf mesophyll	Somatic hybridization	Plantlets	Xiao and Han 1997; Xiao *et al*. 2004

tries deposited in the GenBank database for all *Actinidia* species (http//:www.ncbi.nlm.nih.gov/). A proprietary *Actinidia* database of 130,000 expressed sequence tags (ESTs) has been developed at HortResearch, New Zealand, to exploit the genetic potential of kiwifruit (Wang *et al*. 2006a; Atkinson and MacRae 2007). With the advent of post-genomics era where gene functions are being systematically uncovered by functional genomics methodologies in model plants such as *Arabidopsis* and tobacco, exploiting genes of *Actinidia* as a whole for its cultivar improvement has become very attractive. Establishing a transformation platform, which can produce transformants in a highly efficient, reliable and reproducible way, is a prerequisite to using this tool to complement the traditional breeding and functional genomics studies. In this review, we describe work carried out on transformation in *Actinidia* with emphasis on *Agrobacterium*-mediated transformation and we also discuss some emerging concepts of transformation techniques and their perspectives.

TRANSFORMATION SYSTEMS

Agrobacterium-mediated transformation

Agrobacterium-mediated transformation is an important tool for delivering novel genes into established cultivars and for use in plant functional genomics studies. Matsuta *et al*. (1990) first reported experiments on producing *A. deliciosa* transgenic plants by *Agrobacterium*-mediated transformation. Since then researchers have applied this tool to transfer genes in different species for various purposes (**Table 2**).

In general, most work on increasing plant transformation efficiency focuses on (1) DNA delivery vehicles, (2) delivery methods and conditions and (3) recovering the transformed cells to regenerate into plantlets. More recently, several plant genes involved in *Agrobacterium*-mediated transformation have been identified, suggesting alternative approaches to increasing transformation might come from manipulation of the plant itself (Gelvin 2003). Here some key factors that affect *Actinidia* transformation systems are discussed.

Agrobacterium strains

Agrobacterium strains LBA4404, A281, C58, EHA101 and EHA105 are the common strains used in *Actinidia* species transformation (**Table 2**). *Agrobacterium* strains exhibit differences in their capacity to transfer T-DNA to various plant species (Godwin *et al*. 1991). When strain A281 (a virulent L,L-succinamopine strain (Sciaky *et al*. 1978)) and C58 (a virulent stain carrying the nopaline Ti plasmid pTiC58 (Depicker *et al*. 1980)), both carrying the plasmid pKIWI105 (Janssen and Gardner 1990) were used for *A. chinensis* transformation, there was no marked difference in efficiency of transformation between these two strains (Fraser *et al*. 1995). However, results from Janssen and Gardner (1993) showed differences in ability to transform *A. deliciosa* between different strains of *Agrobacterium*. In general, strain A281 produced slightly higher gene transfer rates than C58 and EHA101 (Janssen and Gardner 1993). They also pointed out that because of variability in source material strain comparisons had to be repeated several times. In fact, strain A281 harbours a tumour-inducing plasmid pTiBo52 (Hood *et al*. 1986) and it also presents an extra copy of the transcription activator involved in the regulation of the *vir* genes. More recently a comparison using four *Agrobacterium* strains A281, GV3101, EHA105 and LBA4404 to compare their transformation efficiency in *A. chinensis* has been undertaken (Wang *et al*. 2006b). All strains harboured the pART27-10 binary vector, which contains *nptII* and *uidA* genes. For strain A281, 27% of leaf strips produced a callus, compared with 22.2% for EHA105, 18.1% for LBA4404 and 13.9% for GV3101. The results suggest that using strain A281, or its non-oncogenic derivative EHA105, generates the highest rates of transformants in *A. chinensis*. These two strains, which have the Ti-plasmid pTiBo542 in the C58 chromosomal background (Hood *et al*. 1986; Hood *et al*. 1993), have also been shown to be superior in facilitating gene transfer in other plant species e.g. apple (de Bondt *et al*. 1994) and blueberry (Cao *et al*. 1998). However, high callus formation derived from A281 does not mean high transformation efficiency of transgenic plant production. We found that transformants derived from A281 responded differently to exogenous phytohormones from transformants derived from other three strains during shoot regeneration and rooting. The transformants from A281 had less than 20% shoot and root development. By contrast, over 70% of calli derived from strains EHA105, GV3101 and LBA4404 regenerated shoots and roots. Overproliferation of callus derived from strain A281 was even more severe in *A. eriantha* and no regenerated shoots were obtained from these calli (Wang *et al*. 2006a). PCR (Polymerase Chain Reaction) analysis revealed that high callus formation and poor bud and root initiation of the transformants from A281 was related to the oncogenes of strain A281 (such as *iaaH* and *ipt*, which are involved in the biosynthesis of auxin and cytokinin), presumably co-integrated into plant genome (unpublished data). Because of the endogenous hormone production, strain A281 is no longer applied in *Actinidia* transformation for our functional genomics studies. Instead, EHA105 is the strain of choice of *Agrobacterium* for the *Actinidia* transformation in our laboratory.

Table 2 *Agrobacterium*-mediated transformation in *Actinidia*.

Species	Explants	*Agrobaterium* strains	Plasmid vectors	Gene and Source	References
A. deliciosa	Leaves, petioles, stems	LBA4404	pBI121		Matsuta *et al*. 1990
A. deliciosa	Hypocotyls, stem segments	EHA101	pLAN411, pLAN421	GUS, *nptII*	Uematsu *et al*. 1991
A. deliciosa	Leaf discs	LBA4404	pRi 1855	*rol* A, B, C genes of *A. rhizogenes* GUS, *nptII*	Rugini *et al*. 1991; Rugini *et al*. 1997; Balestra *et al*. 2001
A. deliciosa	Leaves, petioles	*A. rhizogenes*	ArM 123	Mannopine	Yamakawa and Chen 1996
A. deliciosa	Leaf strips	A281, C58, LBA4404, EHA105	pKIWI105, pKIWI110, pKIWI109, pLAN421	GUS, *nptII*	Janssen and Gardner 1993
A. deliciosa	Leaves, petioles, stems	LBA4404	pBI121	Rice homeobox gene, *OSH1*	Kusaba *et al*. 1999
A. deliciosa	Leaves, petioles, stems	LBA4404	pROKla-EG	Soybean β-1-3-endoglucanase	Nakamura *et al*. 1999
A. deliciosa	Leaf discs, petiole segments	LBA4404	pBE121	*Vitis* stilbene gene	Kobayashi *et al*. 2000
A. chinensis	Leaves, petioles, stem segments	A281, C58	pKIWI105	GUS, *nptII*	Fraser *et al*. 1995
A. chinensis	Leaf discs, petiole segments	LBA4404	pBE121	Human epidermal growth factor	Kobayashi *et al*. 1996
A. chinensis	Leaf strips	A281, EHA105, GV3101, LBA4404	pART27-10	GUS, *nptII*	Wang *et al*. 2006b
A. eriantha	Leaf strips	A281, EHA105	pART27-10	GUS, *nptII*	Wang *et al*. 2006a

In addition, Janssen and Gardner (1993) investigated the effect of binary vectors on stable transformation frequency. They tested five different binary vectors transferred to strain A281 and found the highest frequencies were obtained with vectors based on pGA643. It was thought that the variation might be due to differences in their kamamycin-resistance gene, the particular borders in each vector, or the size of their T-DNA.

Transformation conditions

The processing of the T-DNA from the Ti plasmid and its subsequent export from the bacterium to the plant cell results in large part from the activity of virulence (*vir*) genes carried by the Ti plasmid (Stachel *et al*. 1986). Phenolics, sugars, temperature and pH can affect the virulence of *Agrobacterium* and presumably its capacity to transform plant cells (Alt-Moerbe *et al*. 1988). However, the degree to which these factors influence the transformation efficiency varies with species and reports. Acetosyringone (AS), which is one of 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 applied to increase transformation efficiency in various crops (Guivarc'h *et al*. 1993; James *et al*. 1993; Wu *et al*. 2003). Janssen and Gardner (1993) found the addition of 20 μM AS to the *Agrobacterium* growth medium and to the co-cultivation plates increased GUS expression approximately 2-fold in *A. deliciosa* leaf pieces. Wang *et al*. (2006a, 2006b) added 100 μM of AS in bacterial culture for inoculation to improve transformation efficiency in *A. chinensis* and *A. eriantha* transformation. The effects of AS pre-treatment and co-cultivation temperature, and the correlations of these two factors with four tested *Agrobacterium* strains were also evaluated in *A. deliciosa* transformation. The results from this preliminary experiment demonstrated some positive role of AS pre-treatment two weeks prior to infection. Also, the *Agrobacterium* strains could respond differently to various concentrations of AS pre-treatment. Strain EHA 105 had the highest transformation frequency at 10 μM AS, whereas LBA 4404 had the highest transformation rate at 100 μM AS. However, because the experiment involved too many variable factors, the independent effect of AS pre-treatment remains unclear. The effect of co-cultivation temperature on transformation was assessed in different *Agrobacterium* strains. Strain EHA105 had a similar transformation rate at all co-cultivation temperatures, whereas transformation frequencies for LBA4404 and A281 increased at 18 and 21°C compared with co-cultivation at 24°C (unpublished data).

Co-cultivation duration of usually 2 days, but sometimes up to one week, has been applied to *A. deliciosa* transformation (Uematsu *et al*. 1991). 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 transformation frequencies in kiwifruit (Janssen and Gardner 1993). They found that the inclusion of filters substantially improved gene transfer, but for reasons this was not clear.

Plant regeneration

Establishing a reliable tissue culture system is a pre-requisite for transformation processes in kiwifruit. Once that is achieved, a choice of explants and the right hormone combinations are the two main components for success. Tissue culture of kiwifruit (*A. chinensis* and *A. deliciosa*) was found to be relatively easy for both callus induction and adventitious bud initiation when appropriate explants were used. Young leaves, petioles and stem segments have been used by most researchers for kiwifruit transformation (**Table 2**). As with most other crops, the younger the explants of kiwifruit used, the easier regeneration will be. However, some narcosis of explants occurs after co-cultivation with *Agrobacterium* if the explants are too young. For the trans-formation of *A. arguta*, a considerable narcosis is observed when young leaf strips were used (unpublished data). To maintain the explants in active and amenable condition for infection, it is essential to subculture *in vitro* shoots at intervals of 3-4 weeks (Fraser *et al*. 1995; Wang *et al*. 2006a). MS basal (Murashige and Skoog 1962) medium has been used successfully by many researchers for callus induction as well as regeneration in *Actinidia* species (Kumar and Sharma 2002). However, applications of auxins and cytokinins and combinations thereof are complicated by variations in explant conditions. A comparison of combinations of growth regulators for regeneration was carried out in *A. chinensis* by Fraser *et al*. (1995). They found that TDZ (thidiazuron) and kinetin, at a range of concentrations between 0.1 and 10 mg/l, were clearly inferior to other cytokinins. Differences between NAA (1-naphthalene acetic acid) and IAA (indole-3-acetic acid) were insignificant but NAA is stable in media under light. The most satisfactory combination of growth regulator additives was found to be zeatin alone, or in combination with BAP (6-benzyl-aminopurine), combined with 0.1 mg/l of NAA. Zeatin was clearly superior to BAP when used alone, but the combination of the two cytokinins gave the best overall result in terms of numbers of normal-looking shoots. Wang *et al*. (2006a) had similar results in *A. eriantha* when they compared different combinations of zeatin and BAP at different concentrations. The highest shoot regeneration rates (61% and 55% for two different varieties of *A. eriantha*) were obtained using medium containing a combination of 2 mg/l zeatin and 3 mg/l BAP. Shoot regeneration in *A. eriantha* showed a synergistic response to the mixture of zeatin and BAP. Uematsu *et al*. (1991) reported that the regeneration frequency varied with the basal medium used, and B5 basal medium (Gamborg *et al*. 1968) containing zeatin was most suitable for obtaining transformed shoots. The high level of callus formation and shoot regeneration of kiwifruit in tissue culture makes it possible to obtain transformed shoots at a high frequency. However, in a transformation system minimal callus development and maximum shoot development are desirable, as the chance of random somaclonal variation should be kept at a minimum during the process.

For establishing a good transformation system, it is critical to experiment with the conditions for plant regeneration, including genotype, explant type and medium. In discussions about transformation efficiency, which is one of key components for measuring a transformation system, the importance of plant regeneration is often a minor consideration in many plant transformation studies, including for those agronomic crops species categorized as recalcitrant. Since *Agrobacterium* is the major tool for plant transformation, its molecular mechanism of Ti-plasmid transfer, integration, and expression has been intensively studied for the last three decades (Tzfira and Citovsky 2002). *Agrobacterium* T-DNA integration normally occurs at random sites in the plant genome (Gelvin 2000; Chilton and Que 2003). Great efforts have been made to increase transformation efficiency by employing recent advances in the molecular mechanism of foreign genes as delivery vehicles. However, for plant genetic engineering, ultimately it is not the number of transformed cells but the number of transformed plants that are important. The journey from transformed cells to transformed plants is sometimes difficult and full of challenges. Making these transformed cells develop into a plantlet requires a variety of tissue culture techniques. For woody crops, the methods of plant regeneration play a key role in increasing transformation efficiency.

Species

Transformation systems have been developed for three predominant kiwifruit species - *A. chinensis*, *A. deliciosa* and *A. eriantha* (**Table 2**). All *Actinidia* genotypes so far 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, such as apple (James *et al*. 1989) and orange (Kaneyoshi *et al*. 1994), relatively high rates of transformation and regeneration were achieved in *A. deliciosa* (Uematsu *et al*. 1991). Up to 27.8% transformation efficiency was obtained from *A. chinensis* (Wang *et al*. 2006b). Among the three *Actinidia* species, *A. eriantha* displayed relatively low rates of regeneration and transformation (Wang *et al*. 2006a).

Stable expression and inheritance

Gene transfer systems have been developed for a range of plant species and they provide a powerful tool for crop improvement. Practical application of this tool in breeding programmes typically requires introduced genes that can express stably in the transformants and be inherited in progeny plants. When generating transgenic plants, the most conclusive evidence demonstrating integration of foreign DNA into the host genome is transmission of the transgenic phenotype to progeny. Wang *et al*. (2006a) evaluated GUS expression in calli, leaves, buds, stems, roots, petals and fruit tissues in *A. eriantha* transgenic plants, including their progeny. They demonstrated that the 35S-CaMV driven *uidA* gene was constitutively expressed in tissues of transgenic plants and inherited into their progeny. Fung *et al*. (1998) also examined the expression of three-year-old transgenes in field-grown *A. deliciosa*, and the inheritance and expression of transgenes in progeny from these plants. They found that a gene encoding resistance to kanamycin and a reporter gene expressing the β-glucuronidase (GUS) enzyme were both inherited and expressed in progeny of kiwifruit plants. For two plants examined in detail (Fung *et al*. 1998), molecular evidence suggested that there were multiple copies of both the kanamycin resistance (*nptII*) gene and the *uidA* gene. None of the actively expressed copies of *nptII* or *uidA* were linked. Expression of GUS was variable in some progeny plants, consistent with the idea that gene silencing phenomena were operating. Rugini *et al*. (1997) evaluated genetic stability and phenotypic changes of expressing the *rol*B and *rol*ABC genes in transgenic kiwifruit plants over a 6-year period. They found that all *rol*ABC and *rol*B microcuttings rooted in hormone-free-media, whereas non-transformed material showed 30-40% rooting under similar conditions and *rol*ABC transformants maintained the "hairy root" phenotype for over 6 years. Segregation of inserted genes was observed, such that there were 50% offspring with the "hairy root" phenotype when transgenic plants were crossed with wild types.

Direct gene transfer

A gene transfer system involves the use of several technologies that have the capacity to shuttle isolated DNA into a viable host cell. The efficiency of a transfer system is dependent upon the species to be transformed. Although *Agrobacterium* and particle bombardment-mediated gene transfer are the most common used systems, they are not the only techniques available. Every system has its advantages and limitations, making continuous development of new systems, and improvements upon established systems, essential. The PEG (polyethylene glycol)-mediated gene transfer was the technique commonly used in early 1980's for integration of foreign genes into protoplasts. Molecules ranging in size from small gene sequences or plasmids to large molecules such as chromosomes or micronuclei can be transferred into the protoplast in the presence of PEG. PEG-mediated DNA uptake in protoplasts to produce transgenic potato and sunflower has been described (Fehér *et al*. 1991; Binsfeld *et al*. 2000). It is still not completely clear how PEG induces protoplasts to take up DNA; some evidence shows a reversible permeabilization of the plasma membrane, which allows the passage of different molecules (Songstad *et al*. 1995).

Oliveira *et al*. (1991) reported their PEG-mediated gene transfer in kiwifruit. They used chloramphenicol acetyl transferase (CAT) gene as a reporter gene to assess the conditions for polyethylene glycol (PEG)-mediated transfection of kiwifruit protoplasts. Using this method, they optimised plasmid and PEG concentration, and also assessed the effect of heat shock on transfection. The best CAT activity was obtained using 30% of polyethylene glycol 4000 and by submitting protoplasts to heat shock (45°C, 5 min) prior to transfection. However, no transgenic plants were reported from their experiments.

The lack of interest in this system is primarily due to the existence of more efficient transformation systems, the low yields of transformants and the inability of many species to be regenerated from protoplasts into viable plants. However, as a genetic engineering approach for functional genomics, *Agrobacterium*-mediated transformation is facing concerns due to unwanted DNA sequences (T-DNA, antibiotic resistant markers and backbone), which integrate along with the genes of interest into the plant genome. One of the advantages of pursuing PEG-mediated gene transfer in *Actinidia* is that there are successful protocols for regeneration of whole plants from protoplasts of *A. chinensis* (Pais *et al*. 1987; Tsai 1988; Xiao *et al*. 1992), *A. deliciosa* (Mii and Ohashi 1988; Cai *et al*. 1991; Raquel and Oliveira 1996; Hu *et al*. 1998; Gan *et al*. 2003) and *A. eriantha* (Zhang *et al*. 1998). It is anticipated that PEG-mediated gene transfer may address some of the concerns of *Agrobacterium*-mediated transformation and provide an alternative system described above for woody crops like *Actinidia* with highly heterogeneous genetic backgrounds that are amenable to protoplast culture (**Table 1**).

TRANSGENIC PLANTS AND FUNCTIONAL GENOMICS

Agronomic traits manipulated and altered in transgenic plants

Although several transformation systems have been developed for different *Actinidia* species, most of the transgenic plants produced only involved reporter genes such as the *npt*II gene that confers kanamycin resistance, or the *uid*A gene for monitoring GUS expression (**Table 2**). Nevertheless, significant advances have been achieved by using genetic engineering tools to exploit the manipulation of agronomic traits in *Actinidia*. The target agronomic traits in these reports include rooting ability, dwarfing, plant structure, disease resistance and nutrition.

Self-rooted cuttings in woody species, like kiwifruit, are highly desirable for propagation especially when the rootstock is not required. Rugini *et al*. (1991) transferred *rol* A, B, C genes of *A. rhizogenes* into *A. deliciosa* and generated transgenic plants that showed the typical "hairy root" phenotype described in herbaceous species. Explants from these plants showed an increased ability to produce roots. These transgenic plants and their progeny maintained the "hairy roots" phenotype over six years (Rugini *et al*. 1997). Interestingly, the transgenic plants with *rol*ABC genes had increased susceptibility to *Pseudomonas syringae* pv. s*yringae* and *P. viridiflava*. The susceptibility was probably correlated to the high nitrogen content in the leaves of the *rol*ABC plants (Balestra *et al*. 2001). Dwarfism and early flowering of fruit trees are useful characters for orchard management. *OSH1* is a rice homeobox gene, homologous to KN-1 (Matsuoka *et al*. 1993). When *OSH1* cDNA under 35S promoter was introduced into kiwifruit plants, the morphology of the transgenic plants fell into three categories: normal, mild, and severe phenotypes. Severe phenotype transgenic kiwifruit were much dwarfed with tiny leaves and lacked apical dominance (Kusaba *et al*. 1999).

β-1,3-endoglucanase catalyzes the hydrolysis of β-1,3-glucan, which is a major component of the cell wall of most fungi. Enzyme activity is usually low in a healthy plant but it increases during fungal infection. It has been proposed

that induction of enzyme activity is related to the plant defence reaction. Nakamura *et al*. (1999) transformed a soybean β-1,3-endoglucanase cDNA driven by 35S promoter into kiwifruit and these transgenic plants showed about a six-fold increase in enzyme activity over that in the control plants. When leaves of transgenic plants were inoculated with *Botrytis cinerea*, which causes grey mould disease, the disease lesion areas were smaller than on control plants. Kobayashi *et al*. (2000) introduced a stilbene synthase gene (which is related to resveratrol synthase and was isolated from a grapevine, *Vitis* spp.) into kiwifruit (*A. deliciosa*) plants by *Agrobacterium*-mediated gene transfer. Resveratrol is a phytoalexin produced in several plants in response to fungal infection or UV irradiation. A clear relationship between resveratrol and disease resistance of the leaf has been demonstrated with transgenic tobacco (Hain *et al*. 1993). However, the transformants that Kobayashi *et al*. (2000) obtained did not show resistance against *Botrytis cinerea*, although they produced higher amounts (182 µg/g in the leaves) of resveratrol-glucoside. Resveratrol has an inhibitory effect on platelet aggregation and cancer chemopreventive activity at very low concentrations (Bertelli *et al*. 1995; Jang *et al*. 1997). It is assumed that if there is a corresponding elevation of resveratrol in the fruit to the same concentration as observed in old leaves (20 µg/g) of the transgenic plant, approximately 2 mg resveratrol should be produced in an average sized fruit (about 100 g). It has been proposed that this amount of resveratrol could have a positive biological effect on human health (Kobayashi *et al*. 2000). Kobayashi and colleagues (1996) also transferred a chemically synthesized gene encoding the human epidermal growth factor (hEGG) into *A. chinensis* and the introduced hEGF gene(s) were expressed in the young leaves of the regenerated plants. This was the first demonstration of the production of human bioactive peptides, non-fused with other proteins, in fruit trees and demonstrates the possibility of producing some useful components in fruits in using transformation techniques.

Kiwifruit fruit-specific promoters

Actinidin genes encoding a cysteine protease that is highly expressed in fruit of *A. deliciosa* have been studied extensively for their character and expression (Baker 1980; Praekelt *et al*. 1988; Keeling *et al*. 1990; Lin *et al*. 1993; Sugiyama *et al*. 1996). In ripe kiwifruit, actinidin is the most abundant soluble fruit protein. The actinidin mRNA is rare in leaves and roots and increases in abundance during later stages of fruit development, suggesting that a tissue-specific regulation of transcription is important in controlling the levels of this protease (Praekelt *et al*. 1988). These features make the actinidin promoter a potential tool for studying gene expression in late fruit development. Six different isozymes have been isolated from the fruit, with slightly different enzyme properties (Sugiyama *et al*. 1996). An actinidin promoter was isolated from the actinidin genes that are the most highly expressed in fruit and the gene fragment was cloned and sequenced (Keeling *et al*. 1990). Lin *et al*. (1993) constructed fusions between actinidin promoter fragments and the GUS reporter gene and introduced the chimaeric gene into petunia plants. They demonstrated that a 1.36 kb actinidin promoter is able to direct induction of the GUS reporter gene in seed pods of transgenic *Petunia* plants. We recently transformed the GUS reporter gene driven by the actinidin promoter into *A. chinensis* and *A. eriantha*. These transgenic plants have produced fruit in the containment greenhouse and the characterization of the GUS expression in the fruit is in process. Despite the high levels of the protein found in fruit and its extensive characterization, the function of actinidin is still not known (Atkinson and MacRae 2007).

Polygalacturonase (PG) is one of the enzymes involved in the raft of cell wall modifications that occur during fruit ripening and have an impact on the texture and shelf life of fruit. Three fragments of a type A PG promoter were isolated from *A. deliciosa* (Atkinson and Gardner 1993) and cloned as translational fusions to the chimeric β-glucuronidase (*uidA*) reporter gene (Wang *et al*. 2000). These three promoter constructs were transformed into tomato and the expression of the PG promoter characterised in tomato fruit. The results showed that type A PG promoter fragments of 467, 860 and 1296 bp all directed fruit ripening-specific GUS gene expression in transgenic tomato. No histochemical staining was detected in leaves, roots, flowers, abscission zone or developing fruit prior to the breaker stage.

Large gene databases explored

The emerging scientific revolution sparked by genomics-based technologies has produced massive amounts of gene sequence information. By contrast, only 683 entries of gene sequences for all *Actinidia* species were deposited in the GenBank database by February 2007 (http//:www.ncbi.nlm.nih.gov). Of these sequences, 154 are from *A. deliciosa*, 145 from *A. chinensis*, 39 from *A. arguta*, 30 from *A. eriantha*, 27 from *A. polygama* and the remaining 288 are from the rest of the *Actinidia* species (**Fig. 1**). HortResearch in New Zealand has developed a proprietary database of > 130,000 expressed sequence tags (ESTs) to exploit the genetic potential of kiwifruit (Wang *et al*. 2006a; Atkinson and MacRae 2007). Among these ESTs, 59,163 are from *A. deliciosa*, 47,568 from *A. chinensis*, 13,030 from *A. eriantha*, 7,370 from A. arguta and the rest are from other species. All the ESTs were derived from 35 libraries and the majority of them are from the tissues of fruit and shoot buds (Atkinson and MacRae 2007).

Despite the very impressive progress made in *Actinidia* gene sequencing, the understanding of these genes with their specific functions for commercial use remains to be elucidated.

High throughput transformation for functional genomics

Establishing a transformation system, which can produce transformants in a highly efficient, reliable and reproducible way, is a prerequisite to using this tool in functional genomic analysis. In particularly, if the transformation system is designed to be a platform for investigating plant functional genomic studies, the transformation efficiency and timing required for transformant production are particularly critical because of the cost and the large numbers of genes involved. We have established an optimised *Agrobacterium*-mediated transformation system for kiwifruit. The system allows us to produce transgenic plants efficiently, to test numerous ESTs in kiwifruit functional genomic studies. Thirty-two constructs for genes involved in flavour and aroma biosynthesis transcriptional control, chromatin remodelling, ascorbic acid biosynthesis and cell wall modification have been transformed into *A. chinensis*, *A. deliciosa* and *A. eriantha* using strains of *Agrobacterium* (Atkinson and MacRae 2007). The number of genes tested is increasing continually.

Currently, all transgenic plants have to be potted, grown and maintained in a containment greenhouse. As current commercial cultivars are mainly from *A. chinensis* and *A. deliciosa*, most research interests are focused on

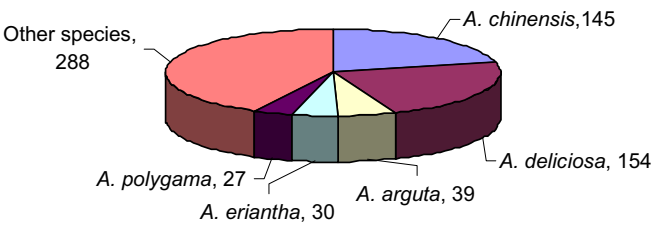

Fig. 1 Number of gene sequences from *Actinidia* species deposited in GenBank.

these two species (**Fig. 1**). However, the vine characters and relatively long juvenility of these two species make functional genomics analysis, in particular of those genes related to fruit characters, difficult and time consuming. In this regard, several characters of *A. eriantha*, rather than *A. chinensis* and *A. deliciosa*, make this species of particular interest for functional genomics studies in *Actinidia*. The flowers of *A. eriantha* form all over the vine including on the lower axillary branches, and the vines are relatively small and non-vigorous in nature. We have established a transformation system for *A. eriantha* and their transgenic plants have flowered and fruited within 2 years (starting from *Agrobacterium* infection) in a containment greenhouse (Wang *et al*. 2006a). We believe that *A. eriantha* is an ideal model species for functional genomics studies in *Actinidia* because of its early flowering, small sized vine, reduced winter chilling requirement and economical maintenance in the containment greenhouse.

FUTURE PERSPECTIVES

Genetic transformation is a useful tool for analysing the function of plant genes. There are many innovative methods and strategies proposed by researchers for carrying out plant transformation for various purposes. We should take great care firstly to clarify the objectives of using genetic transformation tools when we discuss what methods should be used and how the transgenic plants would be produced. With fully sequenced genomes in *Arabidopsis* and rice, the problem now for researchers who are using genetic transformation to analyse plant gene function is not lack of data, but how best to interpret the wealth of publicly-available gene sequence and expression information. To this end, an ideal vector and transformation efficiency are the most important components for establishing a transformation system. Rigorous regulations that consider environmental risk and public acceptance are surmountable factors that need to be considered even when the transformation experiments are aimed at identifying function in plant genes, rather than a goal of plant improvement.

Ultimately we face the challenge of applying genetic transformation to improve crop plants used in food, feed and industrial production. The testing and release of genetically modified organisms (GMOs), in particular GM plants, is regulated internationally. Because transgenic plants embody numerous components and processes, each of which may have intellectual and tangible property (IP/TP) right attached, a product clearance analysis leading to freedom to operate (FTO) becomes a complex issue (Kowalski *et al*. 2002). Nevertheless, gene transfer has been used to produce GM-varieties of corn, canola, soybean and cotton, which were grown on over 100 million hectares in 22 countries by over 10 million farmers in 2006 (http://www.isaaa.org). However, the majority of transgenic crops produced in most countries are not allowed to be released for commercial field production. Public concern about the introduction of foreign DNA into food crops is at the centre of many objections to transgenic plants. For example, the presence of marker genes encoding for antibiotic or herbicide resistances in genetically modified plants poses a number of problems (Scutt *et al*. 2002).

Recently, great effort has been made to respond to public concern. Here, we discuss some advanced and innovative approaches and techniques which may hold great promise for using genetic transformation to improve or accelerate woody plant breeding programmes.

Obtaining marker-free transgenic plants by PCR analysis

Although several methods to create marker-free transformed plants have been reported, for example co-transformation, transposable elements, site-specific recombination and intrachromosomal recombination (Komari *et al*. 1996; Gleave *et al*. 1999; Zubko *et al*. 2000; Zuo *et al*. 2001), most of these are time-consuming and inefficient (Ow 2001). Vetten *et al*. (2003) reported a method that permits the identification of transgenic plants without the use of selectable makers. Their strategy was very simple and straightforward and uses PCR to identifying the transformants. They were able to get 0.2% of the transformants from the harvested shoots infected with *Agrobacterium* strain LBA4404 in potato transformation, whereas with the strain AGL0 this percentage was on average 4.5%. Because this system does not require genetic segregation or site-specific DNA-deletion systems to remove marker genes, it may provide a reliable and simple tool for generating transgenic plants like woody crop kiwifruit, that need to be propagated clonally.

Using plant original visual marker genes to select transformants

Using activation tagging, Borevitz *et al*. (2000) isolated a bright-purple mutant, involving production of an anthocyanin pigment *1-Dominant* (*pap1-D*). In this, genes encoding enzymes involved in the biosynthesis of phenylpropanoid natural products exhibit massive and widespread activation throughout plant development. This finding provides a new approach to generating transgenic plants by expressing visually screened marker genes that are under the control of promoters from genes encoding enzymes. When this colour marker gene is fused with fruit development tissue-specific promoters like the actinidin promoter, we may be able to produce transgenic plants that have coloured fruit but unchanged vegetative tissue.

All-native DNA transformation

Genetic engineering in plants usually relies on the introduction of foreign DNA into plant genomes. Although genetically engineered traits provide valuable alternatives to those available through conventional breeding, public concern about the extent to which transgenic crops differ from their traditionally bred counterparts has limited the commercial application of this technology. Rommens *et al*. (2004) reported a plant-derived (P-) DNA fragment can be used to replace the universally employed *Agrobacterium* transfer (T) DNA. They identified plant border-like sequences from potato (*Solanum tuberosum*) that shared most homology with the left border of nopaline strains (21 of 25 bp) and the right border of octopine strains (22 of 25 bp) of *Agrobacterium*. Hundreds of marker-free and backbone-free potato plants were produced and they represent the first example of genetically engineered plants that only contain native DNA. The plant contains five genetic elements from either potato or a sexually compatible wild relative. These are transfer DNA from wild potato (PDNA), promoter of potato granule-bound starch synthase gene (PGB), a potato vacuolar invertase inhibitor gene (INH), a terminator of the potato ubiquitin-3 gene and a potato polyphenol oxidase gene (PPO). As these plants are formed simply by the rearrangements of genomic material from within the same sexual compatibility group, they are called 'intragenic' plants instead of transgenic plants. Such modifications could alter traits in a similar but more efficient and precise manner to that of plant breeding (Rommens 2004). Subsequently, the concept of the intragenic vector system, consisting of only plant-derived DNA fragments, was developed (Conner *et al*. 2007).

Following this breakthrough of all-native DNA transformation, Schouten *et al*. (2006) proposed a rationale for a new subcategory of genetically modified (GM) plants known as cisgenics. They defined cisgenics as the genetic modification of a recipient plant with a natural gene from a crossable and sexually compatible plant. They proposed that cisgenic plants should be free of any regulation, and food derived from them should not be labelled as genetically engineered (GE). Indeed, cisgenesis is a particularly efficient method for cross heterozygous plants that propagate vegeta-

tively, such as kiwifruit, potato, apple and banana. It can directly improve an existing variety without disturbing the genetic make-up of the plant. Cisgenesis offers the potential to overcome bottlenecks in traditional *Actinidia* breeding but also to make possible 'designed fruit' in a safe and environmentally risk-free manner. Because the *Actinidia* genus is usually polyploid and highly heterogeneous genetically, with relative long juvenility, it is almost impossible to express a trait of interest from a wild plant by crossing it with a cultivated high-quality genotype, because of linkage drag. Using backcrossing to reduce linkage drag is also difficult and sometimes impractical because of *Actinidia*'s dioecism. By contrast, cisgenesis isolates only the genes of interest from the donor plant, which are then inserted into the recipient in one step. As no other genes are involved, there is no linkage drag problem. In addition, as *Actinidia* naturally have inter- and intra-specific compatibility, cisgenesis may allow us to assemble premium traits from different species and germplasm into one cultivar.

ACKNOWLEDGEMENTS

This work was supported by the New Zealand Foundation for Research, Science and Technology (contract C06X0207). We would like to thank Andrew Allan, Andrew Granger and Roger Hellens for critically reading the manuscript and for their valuable suggestions. The authors are also grateful for helpful comments from Anne Gunson, Sue Muggleston and Beth Stark.

REFERENCES

Adriani M, Piccioni E, Standardi A (2000) Effect of different treatments on the conversion of 'Hayward' kiwifruit synthetic seeds to whole plants following encapsulation of in vitro-derived buds. *New Zealand Journal of Crop and Horticultural Science* **28**, 59-67

Alt-Moerbe J, Neddermann P, Lintig J, Weiler EW, Schröder J (1988) Temperature-sensitive step in Ti plasmid *vir*-region induction and correlation with cytokinin secretion by *Agrobacteria*. *Molecular and General Genetics* **213**, 1-8

Atkinson RG, Gardner RC (1993) A polygalacturonase gene from kiwifruit (*Actinidia deliciosa*). *Plant Physiology* **103**, 669-670

Atkinson RG, MacRae EA (2007) Kiwifruit. In: Pua EC, Davey MR (Eds) *Biotechnology in Agriculture and Forestry Vol. 60*, Transgenic Crops V, Springer-Verlag, Berlin, pp 329-346

Baker EN (1980) Structure of actinidin, after refinement at 1.7 Å resolution. *Journal of Molecular Biology* **141**, 441-484

Balestra GM, Rugini E, Varvaro L (2001) Increased susceptibility to *Pseudomonas syringae* pv. *syringae* and *Pseudomonas viridiflava* of kiwifruit plants having transgenic *rol*ABC genes and its inheritance in the T1 offspring. *Journal of Phytopathology* **149**, 189-194

Belrose Inc. (2006) Production of kiwifruit. *World Kiwifruit Review*, pp 13-24

Bertelli AAE, Giovannini L, Giannessi D, Migliori M, Bernini W, Fregoni M, Bertellli A (1995) Antiplatelet activity of synthetic and natural resveratrol in red wine. *International Journal of Tissue Reactions* **17**, 1-3

Binsfeld PC, Wingender R, Schnabl H (2000) Characterization and molecular analysis of transgenic plants obtained by microprotoplast fusion in sunflower. *Theoretical and Applied Genetics* **101**, 1250-1258

Borevitz JO, Xia Y, Blount J, Dixon RA, Lamb C (2000) Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. *Plant Cell* **12**, 2383-2394

Cai QG, Qian YQ, Ke SQ, He ZC (1991) Preliminary analysis of regenerated plants derived from protoplasts of *Actinidia deliciosa*. *Physiologia Plantarum* **82**, 12

Cao X, Liu Q, Rowland LJ, Hamerschlag FA (1998) GUS expression in blueberry (*Vaccinium* spp.) factors influencing *Agrobacterium*-mediated gene transfer efficiency. *Plant Cell Reports* **18**, 266-271

Chen KS, Li F, Zhang SL (1999) The expression pattern of xyloglucan enotransglycosylase gene in fruit ripening of *Actinidia chinensis*. *Acta Botanica Sinica* **41**, 1231-1234

Chilton MD, Que Q (2003) Targeted integration of T-DNA into the tobacco genome at double-stranded breaks: new insights on the mechanism of T-DNA integration. *Plant Physiology* **133**, 1-10

Cipriani G, Di Bella R, Testolin R (1996) Screening RAPD primers for molecular taxonomy and cultivar fingerprinting in the genus *Actinidia*. *Euphytica* **90**, 169-174

Conner AJ, Barrell PJ, Baldwin SJ, Lokerse AS, Cooper PA, Erasmuson AK, Nap JP, Jacobs JME (2007) Intragenic vectors for gene transfer without foreign DNA. *Euphytica* **154**, 341-353

de Bondt A, Eggermont K, Druart P, de Vil M, Goderis I, Vanderleyden J, Borekaert WF (1994) *Agrobacterium*-mediated transformation of apple (*Malus* x *domestica* Borkh): an assessment of factors affecting gene transfer efficiency during early transformation steps. *Plant Cell Reports* **13**, 587-593

Depicker A, de Wilde M, de Vos G, de Vos R, van Montagu M, Schell J (1980) Molecular cloning of overlapping segments of the nopaline Ti-plasmid pTiC58 as a means to restriction endonuclease mapping. *Plasmid* **3**, 193-211

Derambure A, Hirsch AM (1995) Obtaining protoplasts from *Actinidia deliciosa* (kiwifruit) and from frost resistant *Actinidia* species and cultivars. *Acta Botanica Gallica* **142**, 5-21

de Vetten N, Wolters AM, Raemakers K, van der Meer I, ter Stege R, Heeres E, Heeres P, Visser R (2003) A transformation method for obtaining marker-free plants of a cross-pollinating and vegetatively propagated crop. *Nature Biotechnology* **21**, 439-442

Fairchild D (1927) The fascination of making a plant hybrid; being a detailed account of the hybridization of *Actinidia arguta* and *Actinidia chinensis*. *Journal of Heredity* **18**, 49-62

Fehér A, Felföldi K, Preiszner J, Dudits D (1991) PEG-mediated transformation of leaf protoplasts of *Solanum tuberosum* L. cultivars. *Plant Cell, Tissue and Organ Culture* **27**, 105-114

Ferguson AR (1984) Kiwifruit: a botanical review. *Horticultural Review* **6**, 1-64

Ferguson AR (1990) The genus *Actinidia*. In: Warrington IJ, Weston GC (Eds) *Kiwifruit Science and Management*, Ray Richards Publisher, Auckland, New Zealand, pp 15-35

Ferguson AR, O'Brien IEW, Yan GJ (1997) Ploidy in *Actinidia. Acta Horticulturae* **444**, 67-71

Ferguson AR, Seal AG, McNeilage MA, Fraser LG, Harvey CF, Beatson RA (1996) Kiwifruit. In: Janick J, Moore JN (Eds) *Fruit Breeding* (Vol II): *Vine and Small Fruit Crops*, John Wiley and Sons, Inc., New York, pp 371-417

Ferraiolo G (1997) Isolation and culture of fruit tree protoplasts. *Italus Hortus* **4**, 31-41

Fraser LG, Harvey CF, Crowhurst RN, de Silva HN (2004) EST-derived microsatellites from *Actinidia* species and their potential for mapping. *Theoretical and Applied Genetics* **108**, 1010-1016

Fraser LG, Harvey CF, Gill GP (2001) Application of microsatellite-based DNA profiling in *Actinidia* species. *Acta Horticulturae* **546**, 401-405

Fraser LG, Kent J, Harvey CF (1995) Transformation studies of *Actinidia chinensis* Planch. *New Zealand Journal of Crop and Horticultural Science* **23**, 407-413

Fung RWM, Janssen BJ, Morris BA, Gardner RC (1998) Inheritance and expression of transgenes in kiwifruit. *New Zealand Journal of Crop and Horticultural Science* **26**, 169-179

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* **50**, 151-158

Gan L, Xiong X, Wang R, Power JB, Davey MR (2003) Plant regeneration from cell suspension protoplasts of *Actinidia deliciosa*. *Acta Horticulturae* **610**, 197-202

Gardi T, Piccioni E, Standardi A (1999) Effect of bead nutrient composition on regrowth of stored in vitro-derived encapsulated microcuttings of different woody species. *Journal of Microencapsulation* **16**, 13-25

Gelvin SB (2000) *Agrobacterium* and plant genes involved in T-DNA transfer and integration. *Annual Review of Plant Physiology and Plant Molecular Biology* **51**, 223-256

Gelvin SB (2003) Improving plant genetic engineering by manipulating the host. *Trends in Biotechnology* **21**, 95-98

Gill GP, Harvey CF, Gardner RC, Fraser LG (1998) Development of sex-linked PCR markers for gender identification in *Actinidia*. *Theoretical and Applied Genetics* **97**, 439-445

Gleave AP, Mitra DS, Mudge SR, Morris BAM (1999) Selectable marker-free transgenic plants without sexual crossing: transient expression of *cre* recombinase and use of a conditional lethal dominant gene. *Plant Molecular Biology* **40**, 223-235

Godwin I, Todd G, Ford-Lloyd B, Newbury HJ (1991) The effects of acetosyringone and pH on *Agrobacterium*-mediated transformation vary according to plant species. *Plant Cell Reports* **9**, 671-675

Guivarc'h A, Caissard JC, Brown S, Marie D, Dewitte W, Vanonckelen H, Chriqui D (1993) Localization of target-cells and improvement of *Agrobacterium*-mediated transformation efficiency by direct acetosyringone pretreatment of carrot root disks. *Protoplasma* **174**, 10-18

Hain R, Reif HJ, Krause E, Langebartels R, Kindl H, Vornam B, Wiese W, Schmelzer E, Schreier PH, Stöcker RH, Stenzel K (1993) Disease resistance results from foreign phytoalexin expression in a novel plant. *Nature* **361**, 153-156

Harvey CF, Gill GP, Fraser LG, McNeilage MA (1997) Sex determination in *Actinidia*. 1. Sex-linked markers and progeny sex ratio in diploid *A. chinensis*. *Sex Plant Reproduction* **10**, 149-154

Hood EE, Helmer GL, Fraley RT, Chilton M (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. *Journal of Bacteriology* **168**, 1291-1301

Hood EE, Gelvin SB, Mechers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgenic Research* **2**, 208-218

Hu JJ, Xiong XY, Zhang QM, Gan L (1998) Studies on technology of protoplast culture and plant regeneration of *Actinidia deliciosa*. *Journal of Hunan Agricultural University* **24**, 184-190

James DJ, Passey AJ, Barbara DJ, Bevan M (1989) Genetic transformation of apple (*Malus pumila* Mill.) using a disarmed Ti-binary vector. *Plant Cell Reports* **7**, 658-661

James DJ, Uratsu S, Cheng J, Negri P, Viss P, Dandekar AM (1993) Acetosyringone and osmoprotectants like betaine or proline synergistically enhance *Agrobacterium*-mediated transformation of apple. *Plant Cell Reports* **12**, 559-563

Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CWW, Fong HHS, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC, Pezzuto JM (1997) Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **275**, 218-220

Janssen BJ, Gardner RC (1990) Localized transient expression of GUS in leaf discs following cocultivation with *Agrobacterium*. *Plant Molecular Biology* **14**, 61-72

Janssen BJ, Gardner RC (1993) The use of transient GUS expression to develop an *Agrobacterium*-mediated gene transfer system. *Plant Cell Reports* **13**, 28-31

Kaneyoshi J, Kobayashi S, Nakamura Y, Shigemoto N, Doi Y (1994) A simple and efficient gene transfer system of trifoliate orange (*Poncirus trifoliate* Raf.). *Plant Cell Reports* **13**, 541-545

Keeling J, Maxwell P, Gardner RC (1990) Nucleotide sequence of the promoter region from Kiwifruit actinidin genes. *Plant Molecular Biology* **15**, 787-788

Kobayashi S, Ding CK, Nakamura Y, Nakjima I (2000) Kiwifruit (*Actinidia deliciosa*) transformed with a *Vitis* stilbene synthase gene produce piceid (resveratrol-glucoside). *Plant Cell Reports* **19**, 904-910

Kobayashi S, Nakamura Y, Kaneyoshi J, Higo H, Higo K (1996) Transformation of kiwifruit (*Actinidia chinensis*) and trifoliate orange (*Poncirus trifoliata*) with a synthetic gene encoding the human epidermal growth factor (hEGF). *Journal of the Japanese Society for Horticultural Science* **64**, 763-769

Komari T, Hiei Y, Saito Y, Murai N, Kumashiro T (1996) Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of tranformants free from selection markers. *Plant Journal* **10**, 165-174

Kowalski Sp, Ebora RV, Kryder RD, Potter RH (2002) Transgenic crops, biotechnology and ownership rights: what scientists need to know. *The Plant Journal* **31**, 407-421

Kumar S, Sharma DR (2002) *In vitro* propagation kiwifruit. *Journal of Horticultural Science and Biotechnology* **77**, 503-508

Kusaba S, Kano-Murakami Y, Matsuoka M, Matsuta N, Sakamoto T, Fukumoto M (1999) Expression of the rice homeobox gene, *OSH1*, causes morphological changes in transgenic kiwifruit. *Journal of Japanese Society for Horticultural Science* **68**, 482-486

Laing WA, Frearson N, Bulley S, MacRae E (2004) Kiwifruit L-galactose dehydrogenase: molecular, biochemical and physiological aspects of the enzyme. *Functional Plant Biology* **31**, 1015-1025

Langenkämper G, McHale R, Gardner RC, MacRae E (1998) Sucrose-phosphate synthase steady-state mRNA increases in ripening kiwifruit. *Plant Molecular Biology* **36**, 857-869

Ledger SE, Gardner RC (1994) Cloning and characterization of five cDNAs for genes differentially expressed during fruit development of kiwifruit (*Actinidia deliciosa* var. *deliciosa*). *Plant Molecular Biology* **25**, 877-886

Liao JS, Wang T (1984) A preliminary investigation on the distribution and ecological characteristics of Chinese *Actinidia* in Fujian. *Journal of Fujian Agricultural College* **13**, 291-298

Lin E, Burns DJW, Gardner RC (1993) Fruit developmental regulation of the kiwifruit actinidin promoter is conserved in transgenic petunia plants. *Plant Molecular Biology* **23**, 489-499

Lindsay GC, Hopping ME, Binding H, Burge GK (1995) Graft chimeras and somatic hybrids for new cultivars. *New Zealand Journal of Botany* **33**, 79-92

MacRae E, Quick WP, Benker C, Stitt M (1992) Carbohydrate metabolism during postharvest ripening in kiwifruit. *Planta* **188**, 314-323

Marquis H, Bucheli P (1994) Inhibition of tomato pectin methylesterae by partially purified kiwi pectin methylesterase inhibitor protein. *International Journal of Food Scientific Technology* **29**, 212-128

Matsuoka M, Inchikawa H, Saito A, Tada Y, Fujimura T, Kan-Murakami Y (1993) Expression of rice homeobox gene causes altered morphology of transgenic plants. *Plant Cell* **5**, 1039-1048

Matsuta N, Iketani H, Hayashi T (1990) Effect of acetosyringone on kiwifruit transformation. *Japanese Journal of Breeding* **40**, 184-185

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease resistance genes by bulk segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. *Proceedings of the National Academy of Sciences USA* **88**, 9828-9832

Mii M, Ohashi H (1988) Plantlet regeneration from protoplasts of kiwifruit, *Actinidia chinensis* Planch. *Acta Horticulturae* **230**, 167-170

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum* **15**, 473-497

Nakamura Y, Sawada H, Kobayashi S, Nakajima I, Yoshikawa M (1999) Expression of soybean β-1,3-endoglucanase cDNA and effect on disease tolerance in kiwifruit plants. *Plant Cell Reports* **18**, 527-532

Oliveira MM, Barroso J, Pais MSS (1991) Direct gene transfer into *Actinidia deliciosa* protoplasts: analysis of transient expression of the *CAT* gene using TLC autoradiographay and a GC-MS-based method. *Plant Molecular Biology* **17**, 235-242

Oliveira MM, Fraser L (2005) *Actinidia* spp. Kiwifruit. In: Litz RE (Ed) *Biotechnology of Fruit and Nut Crops*, CABI Publishing, Wallingford, pp 2-27

Ow DW (2001) The right chemistry for marker gene removal? *Nature Biotechnology* **19**, 115-116

Nakamura Y, Sawaada H, Kobayashi S (1999) Expression of soybean B β-1,3-endoglucanase cDNA and effect on disease tolerance in kiwifruit plants. *Plant Cell Reports* **18**, 527-532

Pais MSS, Oliveira MM, Barroso J (1987) Use of petiole segments of *Actinidia chinensis* (kiwi) for plant differentiation and production of friable calli for protoplast isolation. *Acta Horticulturae* **212**, 687-690

Palombi MA, Damiano C (2001) Use of different molecular markers, RAPDs and SSRs, to investigate clonal stability in micropropagated kiwifruit (*Actinidia deliciosa* A. Chev.). *Acta Horticulturae* **546**, 609-614

Praekelt Um, McKee RA, Smith H (1988) Molecular analysis of actinidin, the cysteine proteinase of *Actinidia chinensis*. *Plant Molecular Biology* **10**, 193-202

Raquel MH, Oliveira MM (1996) Kiwifruit leaf protoplasts competent for plant regeneration and direct DNA transfer. *Plant Science* **121**, 107-114

Rommens CM (2004) All-native DNA transformation: a new approach to plant genetic engineering. *Trends in Plant Science* **9**, 457-464

Rommens CM, Humara JM, Ye J, Yan H, Richael C, Zhang L, Perry R, Swords K (2004) Crop improvement through modification of the plant's own genome. *Plant Physiology* **135**, 421-431

Rugini E, Craricato G, Muganu M, Taratufolo C, Camilli M, Cammilli C (1997) Genetic stability and agronomic evaluation of six-year-old transgenic kiwi plants for *rol* ABC and *rol* B genes. *Acta Horticulturae* **447**, 609-610

Rugini E, Pellegrineschi A, Mencuccini M, Mariotti D (1991) Increase of rooting ability in the woody species kiwi (*Actinidia deliciosa* A. Chev.) by transformation with *Agrobacteriuim rhizogenes rol* genes. *Plant Cell Reports* **10**, 291-295

Schröder R, Atkinsoan RG, Langenkämper G, Redgwell RJ (1998) Biochemical and molecular characterisation of xyloglucan endotransglycosylase from ripe kiwifruit. *Planta* **204**, 242-251

Schouten HJ, Krens FA, Jacobsen E (2006) Cisgenic plants are similar to traditionally bred plants. *EMBO Reports* **7**, 750-753

Sciaky D, Montoya AL, Chilton MD (1978) Fingerprints of *Agrobacterium* Ti plasmids. *Plasmid* **1**, 238-253

Scutt CP, Zubko E, Meyer P (2002) Techniques for the removal of marker genes from transgenic plants. *Biochemie* **84**, 1119-1126

Seal AG (2003) The plant breeding challenges to making kiwifruit a worldwide mainstream fresh fruit. *Acta Horticulturae* **610**, 75-80

Songstad DD, Somers DA, Griesbach RJ (1995) Advances in alternative DNA delivery techniques. *Plant Cell, Tissue and Organ Culture* **40**, 1-15

Stachel SE, Messens E, van Montague M, Zambryski P (1985) Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. *Nature* **318**, 624-629

Stachel SE, Nester EW, Zambryski P (1986) *virA* and *virC* control the plant induced activation of the T-DNA transfer process of *A. tumefaciens*. *Cell* **46**, 325-333

Sugiyama S, Ohtsuki K, Sato K, Kawabata M (1996) Purification and characterization of six kiwifruit proteases isolated with two ion-exchange resins, Toyopearl-SuperQ and Bakerbond WP-PEI. *Bioscience, Biotechnology and Biochemistry* **60**, 1994-2000

Testolin R, Huang WG, Lain O, Messina R, Vecchione A, Cipriani G (2001) A kiwifruit (*Actinidia* spp.) linkage map based on microsatellites and integrated with AFLP markers. *Theoretical and Applied Genetics* **103**, 30-36

Tsai CK (1988) Plant regeneration from leaf callus protoplasts of *Actinidia chinensis* Planch. var '*chinensis*'. *Plant Science* **54**, 231-235

Tzfira T, Citovsky V (2002) Partners-in-infection: host proteins involoved in the transformation of plant cells by *Agrobaterium*. *Trends in Cell Biology* **12**, 121-129

Uematsu C, Murase M, Ichikawa H, Imamura J (1991) *Agrobacterium*-mediated transformation and regeneration of kiwi fruit. *Plant Cell Reports* **10**, 286-290

Wang ZY, MacRae EA, Wright MA, Bolitho KM, Ross GS, Atkinson RG (2000) Polygalacturonase gene expression in kiwifruit: relationship to fruit softening and ethylene production. *Plant Molecular Biology* **42**, 317-328

Wang T, Ran Y, Atkinson RG, Gleave AP, Cohen D (2006a) Transformation of *Actinidia eriantha*: A potential species for functional genomics studies in *Actinidia*. *Plant Cell Reports* **25**, 425-431

Wang T, Atkinson RG, Janssen BJ (2006b) The choice of *Agrobacterium* strain for transformation of kiwifruit. *Acta Horticulturae*, in press

Wu H, Sparks C, Amoah B, Jones HD (2003) Factors influencing successful *Agrobacterium*-mediated genetic transformation of wheat. *Plant Cell Reports* **21**, 659-668

Xiao ZA, Shen DX, Lin BN (1992) Plant regeneration from protoplasts of *Actinidia chinensis* Planch. *Acta Botanica Sinica* **24**, 763-742

Xiao XG, Hirsch AM (1996) Microcallus formation from leaf mesophyll protoplasts in the genus *Actinidia* Lindl. *Plant Cell Reports* **15**, 896-899

Xiao XG, Hirsch AM (1997) Isolation, culture and fusion of protoplasts in *Acti-*

nidia spp. *Acta Horticulturae* **444**, 119-124

Xiao ZA, Han BW (1997) Interspecific somatic hybrids in *Actinidia*. *Acta Botanica Sinica* **39**, 1110-1117

Xiao ZA, Wan LC, Han BW (2004) An interspecific somatic hybrid between *Actinidia chenensis* and *Actinidia kolomikta* and its chilling tolerance. *Plant Cell, Tissue and Organ Culture* **79**, 299-306

Yamakawa Y, Chen LH (1996) *Agrobacterium rhizogenes*-mediated transformation of kiwifruit (*Actinidia deliciosa*) by direct formation of adventitious buds. *Journal of Japanese Society for Horticultural Science* **64**, 741-747

Zhang YJ, Qian, YQ, Mu XJ, Cai QG (1998) Plant regeneration from *in vitro*-cultured seedling leaf protoplasts of *Actinidia eriantha* Benth. *Plant Cell Reports* **17**, 819-821

Zhen Y, Li Z, Huang H, Wang Y (2004) Molecular characterization of kiwifruit (*Actinidia*) cultivars and selections using SSR markers. *Journal of the American Society for Horticultural Science* **129**, 374-382

Zhu DY, Qin YH, Zhi YB, Yi ML, Chen ZK (2001) Protoplast culture and cell colony regeneration of *Actinidia arguta*. *Journal of Henan Agricultural University* **35**, 221-224

Zubko E, Scutt C, Meyer P (2000) Intrachromosomal recombination between attP regions as a tool to remove selectable marker genes from tobacco transgenes. *Nature Biotechnology* **18**, 442-445

Zuo J, Nui QW, Moller SG, Chua NH (2001) Chemical-regulated, site-specific DNA excision in transgenic plants. *Nature Biotechnology* **19**, 157-161