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# Production of transgenic kiwifruit plants harboring the *SbtCry1Ac* gene

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**ABSTRACT.** The kiwifruit (*Actinidia chinensis* Planch.) is an economically and nutritionally important fruit crop that has a remarkably high vitamin C content and is popular throughout the world. However, kiwifruit plants are vulnerable to attack from pests, and effective pest control is urgently required. Transgenic kiwifruit plants containing the synthetic chimeric gene *SbtCry1Ac* that encodes the insecticidal protein btCrylAc were obtained through an *Agrobacterium*-mediated transformation of kiwifruit leaf discs. The kanamycin resistance of the transgenic plants was then analyzed. Results from polymerase chain reactions and genomic DNA Southern blot analyses indicated that *SbtCrylAc* had been integrated into the genomes of these plants. The results of insect bioassays revealed that the average *Oraesia excavate* inhibition rate of plants tested at 10 days post-infestation was 75.2%. To our knowledge, this is the first study that has developed insect-resistant transgenic kiwifruit plants.

**Key words:** *Actinidia chinensis*; *SbtcrylAc*; Insect-resistant; Transgenic plant

 

## INTRODUCTION

The kiwifruit (*Actinidia chinensis* Planch.) is native to China, and is one of the most important commercial fruits in the world (Huang and Ferguson, 2001; Li et al., 2007). It contains sugars (mainly glucose and fructose) and is rich in vitamin C (most of which remains even after months in storage), and citric acid is the main organic acid. It has long been called "the king of fruits" because of its remarkably high vitamin C content and balanced nutritional composition of minerals, dietary fiber, and other beneficial metabolites (Huang et al., 2013). Extensive studies on the metabolic accumulation of vitamin C, carotenoids, and flavonoids have been conducted in kiwifruit (Ampomah-Dwamena et al., 2009; Ledger et al., 2010; Montefiori et al., 2011; Pilkington et al., 2012; Zuo et al., 2012; Park et al., 2013). Kiwifruits are now grown in Italy, Chile, France, and the United States, among other countries (Huang and Ferguson, 2001). China, Italy, New Zealand, and Chile account for around 80% of the world's commercial kiwifruit production. Currently, the international kiwifruit industry consists of more than 120,000 ha of orchards and has an annual production that exceeds 1.4 million metric tons of fresh fruit (Ferguson, 2007; Mabberley, 2008; Testolin and Ferguson, 2009; Huang et al., 2013). However, pests are a common problem in kiwifruit orchards. Kiwifruit vines are attacked by the fruit-piercing moth (*Oraesia excavate*), the salt marsh moth (*Estigmene acrea*), and the brown-headed leaf roller (*Ctenopseustis obliquana)*, amongst others. Pest may occur in abundance in some seasons and damage the fruit under the sepals, or create holes where two or more fruits touch each other. Therefore, pest control is very important in kiwifruit production.

There are several methods to control pests. For example, many species overwinter on weeds or plant debris in or near gardens, so removing weeds and organic mulches, which can provide homes for insects, is an efficient method. However, this is time-consuming work, particularly in large orchards. Pesticides may also be used as part of a pest management program, but even if a pesticide is botanical in origin it may still be toxic; indeed, a few botanical insecticides are more toxic than some commonly available synthetic insecticides. Microbial biopesticides are an alternative to some chemical pesticides, but once wet and compacted they lose their effectiveness, which is true of all pesticides. Breeding novel insect-resistant cultivars is an effective method of dealing with insect pests, but a large proportion of valuable kiwifruit germplasm may have already been lost through the popularity of certain cultivars in commercial planting, and continuous artificial selection (Ferguson, 2007). Therefore, there are few truly insect-resistant parents among the existing kiwifruit cultivars, and it will take a long time to introduce insect-resistant genes from the wild kiwifruit species into a certain cultivar. Alternatively, introducing a foreign gene into a kiwifruit cultivar genome could be a better approach, and transgenic studies on the kiwifruit have been conducted (Uematsu et al., 1991; Varkonyi-Gasic et al., 2013).

In this study, a plant expression vector (pBSbtCry1Ac) containing the synthetic chimeric gene *SbtCry1Ac* that encodes the insecticidal protein btCrylAc was constructed. Transgenic kiwifruit plants expressing this gene were obtained through an *Agrobacterium*-mediated transformation of kiwifruit leaf discs.

## MATERIAL AND METHODS

### Vector construction

*Escherichia coli* DH5α, *Agrobacterium tumefaciens* LBA4404 (containing the helper

plasmid pAL4404), pSbtCry1Ac, pD12, and pBin513 (Zhang et al., 2007) were preserved or constructed in our laboratory. The chimeric *SbtCry1Ac* gene was isolated from pSbtCry1Ac as a *Bam*HI and *Xho*I fragment and inserted into the *Bam*HI and *Sal*I sites of the binary vector pBin513 to form the plant expression vector pBSbtCry1Ac (Figure 1).

**Figure 1.** Gene structure of the plant expression vectors.

## Transformation method and kanamycin resistance analysis

An *in vitro* culture of kiwifruit plants and the conditions for regenerating plants from leaf discs were established as described by Uematsu et al. (1991). Calli and adventitious shoots were introduced using a Murashige and Skoog (MS) medium with 2.0 mg/L zeatin and 0.1 mg/L a-naphthaleneacetic acid; a proliferation rate of 3.7 was obtained using MS medium with 1.0 mg/L thidiazuron, and a rooting rate of 83.9% was obtained using 1/2 MS medium with 1.0 mg/L indolebutyric acid.

The expression vector was transformed into *A. tumefaciens* as described by Zhang et al. (2007). The transformation of kiwifruit Hongyang leaf tissues was conducted by an *Agrobacterium*-mediated method, as described by Uematsu et al. (1991). Kanamycin resistance in the transgenic plants was checked following the method of Zhang et al. (2007).

## DNA extraction

DNA was extracted from the shoots by the cetyltrimethylammonium bromide method (Zhang et al., 2007). DNA quantity was estimated by the 260/280 nm absorbance ratio by NanoDrop instrument (Thermo Scientific, Wilmington, DE, USA). DNA samples were diluted in sterile deionized water and stored at -20°C.

## Polymerase chain reaction (PCR) analysis

The gene-specific primers used for the *btCry1Ac* amplification were $P_1^{(+)}$ (5'ATC TAT GCA GAG TCT TTC AGA3') and $P_2^{(-)}$ (5'GAG GTT ATC CAA GGA GGT3'). A fragment that was 1370 bp in length was amplified using these two primers.

## Southern blot

A *Bam*HI-*Xho*I fragment of the *SbtCry1Ac* gene was [$^{32}$P]-labeled and used as a probe in Southern blot analysis. DNA transfer and hybridization was performed according to Sambrook et al. (1989). The DNA was digested with *Bam*HI and *Xho*I, electrophoresed on 0.8% agarose, and transferred to nylon membranes. Prehybridization blots were hybridized with the *SbtCry1Ac* probe overnight at 65°C. They were then washed three times with sodium phosphate buffer (pH 7.2) and 1% SDS at 65°C for 5, 30, and 15 min, and visualized by autoradiography.

 

## Insect bioassay

An insect bioassay of the transgenic kiwifruit plants was conducted, as described by Zhang et al. (2007). Thirty same-age *O. excavate* larvae were placed on each of seven non-transgenic plants (controls) and seven transgenic plants (which cultured from a PCR and Southern blot positive plant #21, and these seven plants could be treated as the same strain), and three replicates were included. Student *t*-tests were used to assess the significance of differences between treatments.

## RESULTS

Approximately 250 independently transformed plants were obtained from the co-cultured transformation of leaf tissues using *A. tumefaciens* LBA4404 containing pBSbtCry1Ac. More than 80% of the regenerated plants could produce the expected gene-specific PCR product. A comparison of neomycin phosphotransferase detection by kanamycin infiltration and transgene detection by PCR amplification using specific primers was then conducted. The kanamycin infiltration test and transgene amplification by PCR exhibited an extremely high level of agreement when the transgenic plants were screened. Only one plant was positive in the kanamycin infiltration test, which was negative in the PCR analysis. The results indicated that the infiltration of the kanamycin into the kiwifruit leaves was effective in identifying the transgenic from the non-transformed plants.

The results demonstrate that using healthy, actively growing leaves as experimental materials can improve gene transfer frequencies in kiwifruit (Table 1). The results also indicate that the pre-culture of leaf explants can significantly inhibit gene transfer, particularly at the cut edge of the explants (Table 1), and at least one transgenic plant can be regenerated from each leaf inoculated.

**Table 1.** Comparison between regeneration frequencies from transformation experiments with different materials.

| Experimental material | Number of calli used for transformation | Number of positive transgenic plants obtained (% regeneration frequency) |
|---|---|---|
| Growing leaves | 30 | 83 (276.7%) |
| Pre-culture growing leaves | 30 | 67 (223.3%) |
| Grown leaves | 30 | 54 (180.0%) |
| Pre-culture grown leaves | 30 | 48 (160.0%) |

Some PCR-positive plants were selected for Southern blot hybridization. Figure 2 shows the results of the PCR and of the transgenic plant DNA hybridized with [$^{32}$P]-dCTP-labeled *btCry1Ac* probes. The PCR and Southern blot results suggest that the insect-resistant gene had been inserted into the genomes of these plants as a single copy.

The average mortality of *O. excavate* on plants tested at 10 days post-infestation was up to 75.2%, although it was only 38% on one plant. No significant difference in mortality was observed between the replicates. The leaf damage rate and the average weight of the surviving larvae on the transgenic plants were about 42.1 and 41.85% lower, respectively, than that in the non-transformed plants (Figure 3), and no significant difference was observed between the replicates.

 

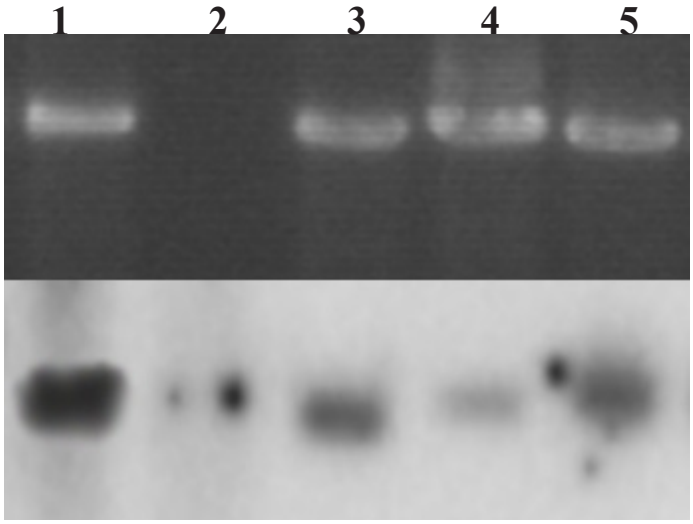


**Figure 2.** Polymerase chain reaction and Southern blot hybridization patterns of pBSbtCry1Ac-transformed kiwifruit plants. *Lane 1* = positive controls, fragments from plasmids containing the *btCry1Ac* gene; *lane 2* = negative controls, fragments from non-transformed kiwifruit plants; *lanes 3-5* = fragments from pBSbtCry1Ac transgenic plants #9, #21, and #35. Transgenic kiwifruit genomic DNA was digested with *Bam*HI and *Xho*I, with only one cutting site in the plasmid for each enzyme. The digested samples were run on 0.8% agarose gel, transferred to nylon membranes, and probed with a [$^{32}$P]-labeled *SbtCry1Ac* gene fragment.

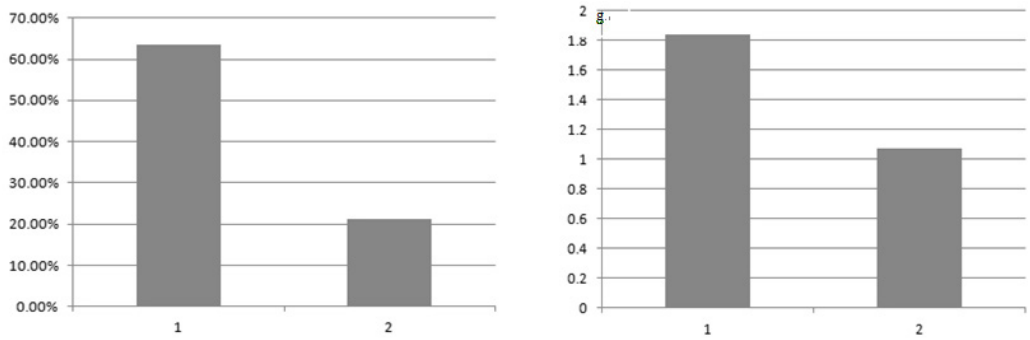


**Figure 3.** Insect bioassay of transgenic and non-transgenic kiwifruit plants. **A.** Leaf damage rate. Column 1 represents the average leaf damage rate in seven non-transgenic plants (63.4%), and column 2 represents the average leaf damage rate in seven transgenic plants (21.3%) from an *in vitro* culture from #21. **B.** Average weight of surviving larvae. Column 1 represents the average weight of surviving larvae on seven non-transgenic plants (1.84 g), and column 2 represents the average weight of surviving larvae on seven transgenic plants (1.07 g) from an *in vitro* culture from #21.

## DISCUSSION

Transgenic technology is the fastest growing technology in agriculture. Plant transgenic technology refers to a set of techniques used for transferring desirable gene(s) across taxonomic boundaries into a certain plant. The donors of the genes can be other plants, animals, or microbes, or even artificial, synthetic, or chimeric DNA. In this study, a chimeric *Bt*

gene, which produces a protein known as Bt toxin, was used as the donor gene. Bt toxin occurs naturally, is only toxic to certain herbivorous insects, and is completely safe to humans (Yang et al., 2013). Current plant transgenic technology includes all of the techniques that are required for the subsequent stable integration of introduced DNA into the plant genome, and its expression. Transgenic technology provides the possibility of not only adding desirable characteristics from other varieties of the species but also of adding characteristics from other, unrelated species. Transgenes are introduced into plant cells, tissues, or organs by a variety of methods, allowing the production of new plant varieties, which are usually normal in appearance and differ from the parent only with respect to the function of the inserted foreign gene.

Because the use of plant transformation methods to introduce resistance genes into plant genomes may have an important impact on many crop yields (Zhang et al., 2007), attempts have been made to produce insect-resistant transgenic plants through the introduction and expression of foreign genes that encode insecticidal or insect-inhibiting proteins, such as Bt endotoxins, protease inhibitors, and lectins (Guo et al., 2004; Jube and Borthakur, 2007). Transgenic plants that express Bt endotoxins or lectins (e.g., *Amaranthus caudatus* agglutinin) have been used successfully against lepidopterans, coleopterans, and aphids (Guo et al., 2004; Jube and Borthakur, 2007). In this study, transgenic kiwifruit plants that contained *btCrylAc*-expressed protein exhibited a high insect resistance rate. Consistent results were obtained when intermediary leaves were infiltrated with a 100-mg/mL kanamycin solution, using a syringe and a needle. The volume of the infiltrated solution varied according to leaf size, but this did not seem to interfere with the results. Conspicuous chlorotic spots were seen on the non-transgenic plants, whereas no spots were seen on the transformed plants. The reliability of the kanamycin resistance test in this study indicates that it could be used for the first screening of transgenic kiwifruit plants. Furthermore, the test is simple, rapid, non-destructive, relatively cheap, and can be conducted at any developmental stage of the plant.

In conclusion, in this study we developed transgenic kiwifruit plants that contained *btCrylAc*. Kanamycin resistance tests, PCR detection, and genomic DNA Southern blot analysis illustrated that the selection marker and the *SbtCrylAc* gene had been integrated into the genomes of these plants, and the results of insect bioassays revealed that transgenic kiwifruit plants can be partially protected against *O. excavate* by the expression of the insecticidal *btCrylAc* in their tissues. The introduction of this novel resistance gene into the germplasm of kiwifruit makes the trait available for conventional kiwifruit breeding programs, in order to resist *O. excavate* attacks.

## Conflicts of interest

The authors declare no conflict of interest.

## ACKNOWLEDGMENTS

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## REFERENCES

Ampomah-Dwamena C, McGhie T, Wibisono R, Montefiori M, et al. (2009). The kiwifruit lycopene beta-cyclase plays a significant role in carotenoid accumulation in fruit. *J. Exp. Bot.* 60: 3765-3779.

Ferguson AR (2007). Genetic resources of kiwifruit: domestication and breeding. *Hort. Rev.* 33: 1-121.

Guo HN, Jia YT, Zhou YG, Zhang ZS, et al. (2004). Effects of transgenic tobacco plants expressing *ACA* gene from *Amaranthus caudatus* on the population development of *Myzus persicae*. *Acta Bot. Sin.* 46: 1100-1105.

Huang H and Ferguson AR (2001). Kiwifruit in China. *New Zeal J. Crop Hort.* 29: 1-14.

Huang S, Ding J, Deng D, Tang W, et al. (2013). Draft genome of the kiwifruit *Actinidia chinensis*. *Nat. Commun.* 4: 2640. Doi: 10.1038/ncomms3640.

Jube S and Borthakur D (2007). Expression of bacterial genes in transgenic tobacco: methods, applications and future prospects. *Electron. J. Biotechnol*. 10: 452-467.

Ledger SE, Janssen BJ, Karunairetnam S, Wang T, et al. (2010). Modified CAROTENOID CLEAVAGE DIOXYGENASE8 expression correlates with altered branching in kiwifruit (*Actinidia chinensis*). *New Phytol.* 188: 803-813.

Li JQ, Li XW and Soejarto DD (2007). Actinidiaceae. In: Flora of China. Vol. 12: 334-362 (Wu ZY, Raven PH and Hong DY, eds.). Science Press, Beijing and Missouri Botanical Garden Press, St. Louis.

Mabberley DJ (2008). Mabberley's plant-book, 3rd edition (2009 reprint with corrections). Cambridge University Press, Cambridge.

Montefiori M, Espley RV, Stevenson D, Cooney J, et al. (2011). Identification and characterisation of F3GT1 and F3GGT1, two glycosyltransferases responsible for anthocyanin biosynthesis in red-fleshed kiwifruit (*Actinidia chinensis*). *Plant J.* 65: 106-118.

Park YS, Im MH, Ham KS, Kang SG, et al. (2013). Nutritional and pharmaceutical properties of bioactive compounds in organic and conventional growing kiwifruit. *Plant Foods Hum. Nutr.* 68: 57-64.

Pilkington SM, Montefiori M, Jameson PE and Allan AC (2012). The control of chlorophyll levels in maturing kiwifruit. *Planta* 236: 1615-1628.

Sambrook J, Fritish EF and Maniatis T (1989). Molecular cloning a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 474-491.

Testolin R and Ferguson AR (2009). Kiwifruit (*Actinidia* spp.) production and marketing in Italy. *New Zeal. J. Crop Hort.* 37: 1-32.

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

Varkonyi-Gasic E, Moss SM, Voogd C, Wang T, et al. (2013). Homologs of FT, CEN and FD respond to developmental and environmental signals affecting growth and flowering in the perennial vine kiwifruit. *New Phytol.* 198: 732-746.

Yang Y, He J, Dong B, Xu H, et al. (2013). Effects of two *Bt* rice lines T2A-1 and T1C-19 on the ecological fitness and detoxification enzymes of *Nilaparvata lugens* (Hemiptera: Delphacidae) from different populations. *J. Econ. Entomol.* 106: 1887-1893.

Zhang HY, Liu XZ, Wei L, Zhou LY, et al. (2007). Transgenic tobacco plants containing *Bt* and *GNA* genes. *Biol. Plant* 51: 746-748.

Zuo LL, Wang ZY, Fan ZL, Tian SQ, et al. (2012). Evaluation of antioxidant and antiproliferative properties of three Actinidia (*Actinidia kolomikta, Actinidia arguta, Actinidia chinensis*) extracts *in vitro*. *Int. J. Mol. Sci.* 13: 5506-5518.

 