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ORIGINAL PAPER

Expression of a *Bacillus thuringiensis* toxin (*cry1Ab*) gene in cabbage (*Brassica oleracea* L. var. *capitata* L.) chloroplasts confers high insecticidal efficacy against *Plutella xylostella*

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Abstract Chloroplast genetic engineering is an environmentally friendly approach, where the foreign integrated gene is often expressed at a higher level than nuclear transformation. The *cry1Ab* gene was successfully transferred into the cabbage chloroplast genome in this study. The *aadA* and *cry1Ab* genes were inserted into the pASCC201 vector and driven by the *prrn* promoter. The cabbage-specific plastid vectors were transferred into the chloroplasts of cabbage *via* particle gun mediated transformation. Regenerated plantlets were selected by their resistance to spectinomycin and streptomycin. According to antibiotic selection, the regeneration percentage of the two cabbage cultivars was 4–5%. The results of PCR, Southern, Northern hybridization and western analyses indicated that the *aadA* and *cry1Ab* genes were not only successfully integrated into the chloroplast genome, but functionally expressed at the mRNA and protein level. Expression of Cry1Ab protein was detected in the range of 4.8–11.1% of total soluble protein in transgenic mature leaves of the two species. Insecticidal effects on *Plutella xylostella* were also demonstrated in *cry1Ab* transformed cabbage. The objectives of this study were to establish a gene transformation system for *Brassica* chloroplasts, and to study the possibility for insect-resistance in dicot vegetables using chloroplast gene transformation.

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Introduction

Plant genetic engineering via chloroplast transformation has recently become an established technology for the improvement of crop plants. When commercial genes are integrated into plant plastids via homologous recombination, there are no position effects and gene silencing does not occur (Svab et al. 1990). All angiosperms and land plants have prokaryotic circular genomes in their plastids which range in size from 120 to 270 kb (Sugiura et al. 1998). The expression of foreign genes in plastid genomes not only dramatically enhances the level of gene expression (there are up to 10^5 copies of the foreign gene in chloroplasts per plant cell) (Bendich 1987), but proteins from plastid transgenes may be expressed at very high levels. Consequently, this technique is currently broadly utilized, and has proven for successful stable delivery of DNA to plastids of tobacco (Svab et al. 1990), potato (Chakrabarti et al. 2006; Sidorov et al. 1999), tomato (Ruf et al. 2001; Wurbs et al. 2007), *Arabidopsis* (Sikdar et al. 1998), *Lesquerella* (Skarjinskaia et al. 2003), oilseed (Hou et al.

2003), soybean (Dufourmantel et al. 2004), cotton (Kumar et al. 2004b), cauliflower (Nugent et al. 2006), rice (Lee et al. 2006a, b) and cabbage (Liu et al. 2007).

Genetic modification of plastids has been used to produce vaccines and therapeutic proteins, and confer agronomically important traits to key commercial crops (Bock 2007). Recently, many reports have confirmed that plastid transformation is an environmentally friendly approach (Cao et al. 2005; He et al. 2006). Foreign genes expressions via plastid transformation now bestow useful agronomic traits, for example insect resistance by multigene engineering of the *cry* operon cassette (De Cosa et al. 2001), high-level expression of Cry9Aa2 for resistance to potato tuber moth (Chakrabarti et al. 2006), drought resistance (Lee et al. 2003), herbicide resistance (Bock 2007; Dufourmantel et al. 2007), improve nutritional quality (Wurbs et al. 2007), environmental stress resistance (Kumar et al. 2004a) and especially for therapeutic protein production (Tregoning et al. 2005). The higher levels of foreign proteins required for use as vaccine antigens or biopharmaceuticals can be more easily achieved in transgenic plants with chloroplast transformation than with nuclear transformation (Dhingra and Daniell 2004). The first vaccine engineered via chloroplast transformation was reported to be the cholera toxin B subunit gene (Daniell et al. 2001). Since then, several studies have reported that plastid transformation can provide efficacious vaccines and various human therapeutic proteins. For example, anthrax protective antigen (Koya et al. 2005), viral peptide antigen for virulent canine parvovirus (Molina et al. 2004), and other vaccines efficacious against bacterial, fungal, viral and protozoan pathogens (Kamarajugadda and Daniell 2006) were all developed in previous studies. Furthermore, a significant advantage for producing vaccines and various human therapeutic proteins from plants has been demonstrated using multigene transformation, by means of chloroplast-derived operons or polycistrons (Quesada-Vargas et al. 2005). The highest levels of human serum albumin (*hsa*) and cholera toxin b-subunit (*ctb*) proteins were reported in the transgenic tobacco (Quesada-Vargas et al. 2005). Since the first report of tobacco chloroplast transformation was described (Svab et al. 1990), more than four transgenes have been simultaneously stably integrated and expressed using tobacco chloroplast genome transformation vectors (Daniell et al. 2005; Daniell 2007).

Genetically engineered cultivars have rapidly become commercially important crop plants. In 2005, the global area of approved biotech crops was 90 million hectares (ha), equivalent to 222 million acres. Compared with 81 million ha in 2004 the increase was 9 million ha, equivalent to an annual growth rate of 11% (James 2006). The ninety million ha were cultivated with genetically modified (GM) soybean (60%), maize (24%), cotton (11%) and canola (5%) (James 2006). In 2005, herbicide tolerant crops (including soybean, maize, canola and cotton) occupied 63.7 million ha, representing 71% of global transgenic area. The second most dominant GM trait was insect resistance: Transgenic crops harboring *Bacillus thuringiensis* (*Bt*) protein occupied 16.2 million ha (18%) of global biotech crop area. Because the use of Bt-genetic varieties can lead to substantial reductions in insecticide use in many commercial crops, the area worldwide that cultivates transgenic plants expressing insecticidal Cry proteins derived from *Bacillus thuringiensis* (*Bt*) is increasing (Romeis et al. 2006).

Bacillus thuringiensis is a spore-forming gram-positive bacterium, which produces the intracellular insecticidal proteins when in sporulation, encoded by *cry* genes. Cry genes, one of the most popular genes for insect-resistance, encode the *Bacillus thuringiensis* (*Bt*) crystal protein (Cry protein or δ-endotoxin), which is produced as phase-bright inclusions (Bulla et al. 1980). The crystal proteins have proven effective and are widely used in controlling insect larvae infestation, including those by *Diptera*, *Lepidoptera*, *Mallophaga*, *Homoptera* and *Coleoptera* (Schnepf et al. 1998). For more than 40 years, *B. thuringiensis toxins* have been developed in industry into successful biological agents to control a wide variety of insect pests (Romeis et al. 2006). These insecticidal proteins have been grouped CryI, CryII, CryIII, and CryIV, according to the degree of amino acid homology and crystal shape. The incorporation of *cry* genes into major crops has included rapeseed (Schuler et al. 2004), collards (Cao et al. 2005), rice (High et al. 2004), potato (Banerjee et al. 2006), cotton (Perlak et al. 1993), maize (He et al. 2006) and tomato (Kumar and Kumar 2004).

Diamondback moth (*Plutella xylostella*) is the most important insect pest of brassica crops worldwide. Diamondback moth (DBM) species occur wherever brassica crops are grown and has developed widespread resistance to a range of biological agents including microbial Bt products (Schuler et al. 2004). Several major host plants of DBM, namely cabbage, broccoli, and rapeseed, have been transformed to produce insect resistant Bt cultivars (Zhao et al. 2003). Recently, one reported problem has been the evolution of Bt resistant pests (Morina et al. 2004). Despite this, *cry*-transgenic plants carrying a synthetic or fully modified Bt gene, or expressing both groups of *cry* genes, caused rapid mortality of resistant larvae, showing excellent control of *P. xylostella* (Bhattacharya et al. 2002).

The possible flow of toxicity to non-target insects via transgenic pollen is one important environmental concern (Daniell 2002). Also, the most debated environmental worry posed by transgenic crops is the idea of transgenes escapes and their persists in the environment can be detrimental if they confer increased fitness or if they change the

phenotype of the plant, causing it to function differently in the ecosystem (Schmidt and Linder 1993).

In this paper, we are the first to report the use of the stable cabbage-plastid transformation system for *cry1Ab* gene expression by using a species-specific vector. The transgenic cabbage expressed the toxin, and effective control of *P. xylostella* larvae resulted. Our results suggest that the introduction of the *cry1Ab* gene into the cabbage chloroplast genome leads to high levels of protein expression. The establishment of a plastid transformation system in cabbage, which has several advantages over the conventionally used nuclear transformation system, offers new possibilities for genetic improvement and biological control in brassica crops.

Materials and methods

Plant cultivars

Two varieties of commercial cabbage (*Brassica oleracea* L. var. *capitata* L.) were used in this study. Seeds of 'K–Y cross' and 'Summer Summit' were surface-sterilized (70% ethanol, 1.5 min) and germinated in basic MS medium (Murashige and Skoog 1962) supplemented with 2% sucrose and 0.7% Bacto agar (DIFCO) for 7 days. Healthy and vigorous seedlings were selected and grown in a growth chamber. The chamber was set to 25/20°C, 80% relative humidity (RH) and developed all day with light photoperiod (Liu et al. 2007). The seedlings were sub-cultured for another 14 days if necessary. The mature leaves were better for plastid transformation from cabbage plants that had been grown aseptically on agar-solidified 1/2 MS basic medium, after 4 weeks supplemented with 3% sucrose and 0.7% Bacto agar. Fully expanded leaves of 1–2 month old plants were developed and harvested for plastid transformation.

Construction of transformation vectors

The sequence of the Arabidopsis chloroplast genome (NCBI accession No. AP000423) was used to design the primers, as it is a fellow member of the *Cruciferae*. Total plant DNA was isolated for use in polymerase chain reaction (PCR). The PCR was performed using a Perkin Elmer 2400 in a volume of 50 µl, containing 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 0.1 mM of each primer, 200 µM of each deoxynucleotide triphosphate, 50 ng DNA and 0.5 U ExTaq™ polymerase (TaKaRa). The 16S and 23S ribosomal RNA fragments from the chloroplast genome of 'K–Y cross' were amplified by PCR. The following oligonucleotide primers were used: 5′-GGTAATTCTCTTTCTCGAGGG GACGGGGA AGGG-3′ (primer1), which was complementary to the *trn*V gene of IR in the chloroplast genome, and 5′-CAGCCCATGGCACAACGACGCAATTATCA GGGG-3′ (primer2), specific to *trn*I. The amplified product was 2.1 kb in length. Primer3 (5′-CCAAGGTCAACATTA GCATGGCGTACTCCTCC-3′) was complementary to the *trn*I gene of IR and primer4 (5′-CAGCCCATGGCACAAC GACGCAATTATCAGGGG-3′) was specific to the 23S ribosomal RNA gene. The amplified product was 2.2 kb long. The PCR conditions were as follows: the 1st cycle, 94°C for 5 min; the 2nd–30th cycles: 94°C for 1 min, 64°C for 40 s then 72°C for 2 min, and a final extension for 10 min at 72°C. We constructed the chloroplast expression cassette pASCC201 carrying the *aadA* gene flanked by the cabbage 16S and 23S rRNA sequences as recombination regions (Liu et al. 2007).

We obtained the *cry1Ab* gene encoding the crystal protein in plasmid pKcBn (a kind gift of L. J. Chen, National Chung-Hsing University, Taiwan). For subcloning of the full-length *cry1Ab* gene, PCR amplification was used to generate appropriate cloning sites (*Sma*I at the 5′- and 3′-ends). The *cry1Ab* gene was amplified by PCR with the primers Bt-1 (5′-CCCGGGTGGTCAGTCCCTTCCATG GATAAC-3′) and Bt-2 (5′-CGACGGCCCGGGAATTC GATCTCACTCAAC-3′). Subsequently, the recovered PCR product was ligated into pGEM-T Easy Vector (Promega) for pGEM-*cry1Ab*, and then DNA sequencing was performed to check the accuracy of the *cry1Ab* fragment. For cloning into the plasmid pBpGUSp plamid, pGEM-*cry1Ab* was digested with the restriction enzyme *Sma*I. Subsequently, the *cry1Ab* fragment was cloned into plasmid pBpBT for control by the 5′-regulatory sequences of the tobacco plastid ribosomal RNA operon promoter (*rrn*). This modified 16S rRNA promoter is a strong and constitutive-expression promoter in plants (Svab and Maliga 1993). Subsequently, the *psbA* 3′ region (transcription terminator) was cloned downstream of the *cry1Ab* coding sequence as an *Sma*I/*Kpn*I fragment from plasmid pBluepsbA to generate the *cry1Ab* 3′ region and to introduce *Pst*I sites at the 3′-end. Finally, the *prrn*:*uidA*:*psbA* fragment from plasmid pBpBTp was inserted into the *Pst*I site of the species-specific expression cassette pASCC201 to produce the chloroplast transformation vector pASCCBT (Fig. 1b). The plastid recombination vector pASCC201 incorporated the *aadA* gene for selection on spectinomycin, and the homologous flanking regions (*trnV–rrn16S* and *trnI–trnA–rrn23S*). The plastid transformation vector contained the *aadA* and *cry1Ab* genes between the *trnV–rrn16S* and *trnI–trnA–rrn23S* regions, designed to insert into the chloroplast genome by homologous recombination after particle bombardment. Transgenic plants were selected by antibiotic resistance and bioassay.

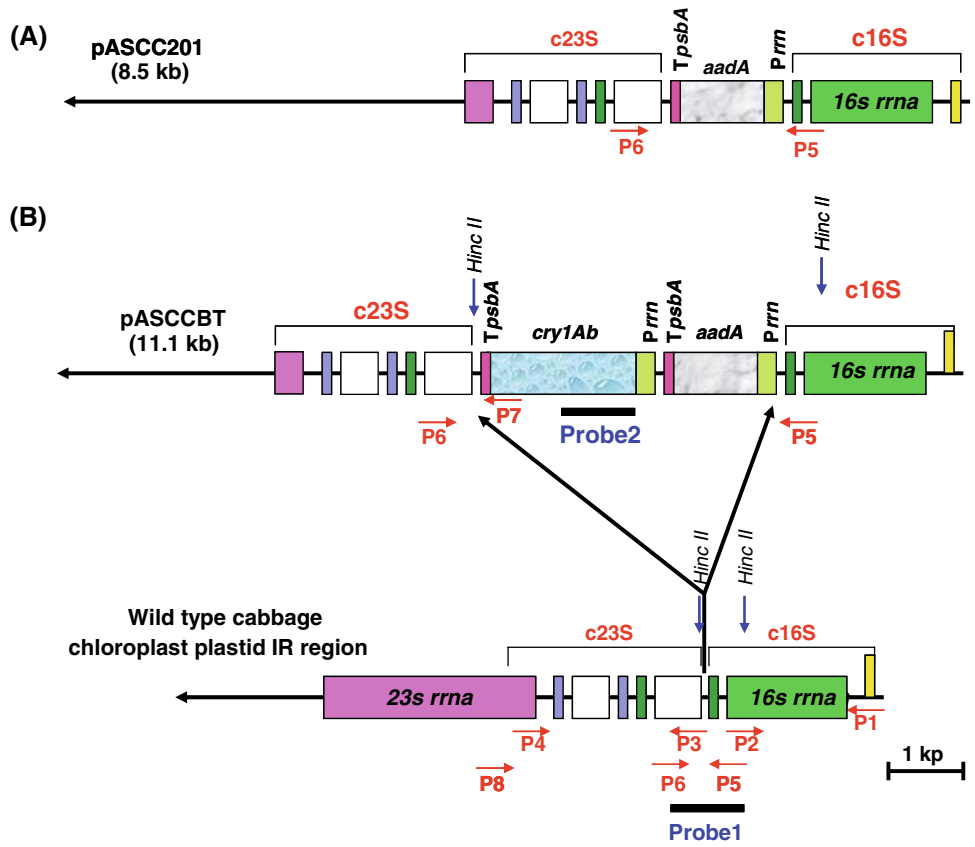

Fig. 1 Schematic representation of the *aadA* and *cry1Ab* gene cassette in the different constructs: **a** The species-specific cabbage plastid vector targets insertions in the *c16S* and *c23S* intergenic region. The chimeric *aadA* gene in the pASCC201 vector is expressed from the *Prrn* promoter and has *TpsbA* at the 3′-end. The plastid genes denoted by the *colored boxes* from *right* to *left* are *c16S* (*trnV-GAC-rrn16S-trnI-GAU*)-promoter *prrn-aadA*-terminator *psbA-c23S* (spacer region-*trnI-GAU-trnA-UGC*-spacer region-*trnA-UGC-rrn23S*). **b** Plasmid pASCCBT construct. The *colored boxes* from *right* to *left* denote: *c16S-prrn-aadA-psbA-prrn-cry1Ab-psbA-c23S*. The transgenes are targeted to the intergenic region between the plastid *trnI* and *trnA* by homologous recombination within the flanking regions. The hybridization position of the 1.2 kb *cry1Ab*-specific probe is shown on the T-DNA of construct B. The red arrows labelled P1-6 show the annealing positions of the PCR primers used for amplification of the c16s and c23s region fragments

Cabbage chloroplast transformation procedure

The transformation protocol was performed using the PDS-1000/He Particle Delivery System (Bio-Rad) according to previously published procedures (a rupture disc pressure of 1,100 psi, and a target distance of 6 cm were used for bombardment) (Sanford 1990). Firstly, 2 mg of 1 µm gold particles (Bio-Rad) were washed once with 70% ethanol and twice with sterile water. Washed gold particles were then resuspended by sonication in 220 µl of sterile water. In addition, 12.5 µl of a 1 µg/µl solution of plasmid DNA (pASCCBT), 250 µl of 2.5 M $CaCl_2$, and 50 µl of 0.1 M spermidine were added sequentially to the particle suspension. Plant samples, as described in the section *Plant cultivars* above, were placed in the circle within a 2-cm radius and were shot under a pressure of 28–29 in Hg. Transformation procedures were as follows: explants were plated onto L1 medium (MS supplements, 3% sucrose, 0.04% MES, 0.05 mg/l $AgNO_3$ and 0.7% Bacto-agar pH 5.7), and cultured in dark conditions. One week later, transformed samples were cut into 9 mm^2 pieces and were transplanted to new L1 medium containing 50 mg/l spectinomycin for callus-induction and primary antibiotic selection. Transformed explants were sub-cultured every 2 weeks and changed to L2 medium (MS supplements, 3% sucrose, 0.04% MES, 0.05 mg/l Picloram, 0.5 mg/l BA, 0.05 mg/l $AgNO_3$, and 0.7% Bacto-agar pH 5.7) plus 100 mg/l spectinomycin and streptomycin until new shoots appeared. Survival shoots were transferred to L3 medium (MS supplements, 3% sucrose, 0.04% MES, 0.1 mg/l Picloram, 0.2 mg/l BA, 0.05 mg/l $AgNO_3$, 200 mg/l spectinomycin, and 0.7% Bacto-agar pH 5.7) for rooting. After screening, the rooted shoots were further transferred to L4 medium (MS supplements, 3% sucrose, 0.04% MES, and 0.6% Bacto agar pH 5.7) for full plantlet formation. Finally, the surviving transformants were transplanted and hardened off in L5 medium (1/2 MS basic medium, 3% sucrose, and 0.6% Bacto-agar pH 5.7). Hardened plants with sufficient

shoots, leaves and roots were finally transferred to potted soil and grown in growth chambers.

Polymerase chain reaction (PCR) and Southern blot analysis

PCR amplification and Southern blot analysis were used to confirm the integration of the *cry1Ab* gene into the chloroplast genome. Plastid DNA was extracted from chloroplasts of transplastomic plants and wild-type cabbage leaves based on the CTAB (cetyltrimethylammonium bromide) method (0.02% CTAB, 1.4 M NaCl, 0.1 M Tris–HCl, 20 mM EDTA) (Doyle and Doyle 1987). One pair of specific primers was used to detect the *region of aadA-psbA-prrn promoter-cry1Ab*: primer 5 (forward) 5′-CGC GAGGGTGAGCTAACTCCAAAAACCCGTCC-3′ and primer 6 (reverse) 5′-CAAACCTGCTCCCATTTCGAGG CGG-3′ (Fig. 1b). Primer 7 (5′-GGCCT ATCAATCAGG GTAACTTCT-3′) binds at the *cry1Ab* 3′-end and primer 8 (5′-CTCCAC CGCTTCGCCTAGCAGCAC-3′) binds at the *23* s *rrna* 5′ region. PCR was also carried out using primer 8 in the external (flanking) regions and primer 7 in the partial *cry1Ab* gene. The PCR was performed in a volume of 25 µl, containing 10 mM Tris–HCl pH 9.0, 50 mM KCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 0.1 mM of each primer, 200 µM of each deoxynucleotide triphosphate (dNTP), 50 ng DNA and 1U Ex Taq-polymerase (TaKaRa). The expected fragment length for PCR with primers 5 and 6 was 0.7 kb and 4.5 kb (Fig. 1b) for wild-type plastid and transgenic plastid, respectively.

For Southern analysis, the DNA of chloroplasts was isolated from transgenic plant leaves based on the protocol of Liu et al. (2007). Plastid DNA was treated with 0.5 mg/ml DNase-free RNase A (Sigma) for 10 min at 37°C. Fifty micrograms of plastid DNA from each sample was digested with *Hinc* II overnight, and was subjected to electrophoresis in a 1.5% SeaKem LE agarose gel (FMC). The fractionated DNA was transferred to a Zeta-Probe GT hybridization nylon membrane (Bio-Rad) according to the manufacturer's instructions. A 0.4 kb *Hinc* II fragment of plastid containing the partial *rrn16S/trnI* coding region of the control plant was used as a probe for Southern hybridization (Fig. 1 probe 1). The probe was labeled with [α-^{32}P]-dCTP (Amersham) using the Random Primer Labeling Kit (Stratagene) and hybridized for 8–12 h at 65°C with agitation in a hybridization oven. After hybridization and washing, the blotted membranes were ready for autoradiography.

Northern blot analysis

Total RNA was prepared using Ultraspec RNA reagent (Biotecx), according to the manufacturer's instructions. Northern blot analysis was carried out utilizing the Random Primer Labelling Kit (Stratagene) to produce the isotope-labelled DNA. Twenty micrograms of total RNA was electrophoresed on a 2.0% denaturing agarose gel (1× MOPS buffer, 50% formamide, 6% formaldehyde, 2.0% agarose), and the RNA was transferred onto a Zeta-Probe GT hybridization membrane (Bio-Rad) by the VacuGene XL vacuum blotting system (Amersham Biosciences). The transferred membrane was then cross-linked with GS Gene Linker™ UV irradiation (Bio-Rad). The integrity of the RNA and equality of RNA loading were verified by ethidium bromide staining. A ^{32}P-labelled *cry1Ab* fragment was used as a probe in the hybridization reaction at 65°C for 16 h. After hybridization and washing, the membrane was dried and exposed to BioMax MS film (Kodak).

Protein extraction and Immunoblotting analysis

Mature leaves (2.0 g) of control and transgenic plants were ground into powder in liquid N_2, and homogenized with 2 ml extraction buffer then diluted with another 2 ml extraction buffe (100 mM potassium phosphate, pH 7.5; 2 mM EDTA; 1% PVP-40). The sample was centrifuged at 15,000×*g* for 20 min at 4°C. Soluble protein was extracted as described by Anderson et al. (1995). Quantity of foliar protein was determined according to the method of Bradford (1976) with BSA as a standard. The protein marker was rainbow molecular weight markers (10,000-250,000 kDa) (Amersham Biosciences). The 40 µg total soluble proteins (TSP) of transgenic plants were separated by 12% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred onto PVDF Immobilon-P membranes (Millipore). The membranes were blocked in PBST (155 mM NaCl, 1.1 mM KH_2PO_4, 3.0 mM K_2HPO_4–$7H_2O$, pH 7.4, 0.25% Tween 20) plus 5% skimmed milk for 1 h at room temperature and then incubated with primary antibody in PBST (containing 5% skimmed milk) overnight at 4°C. The primary antibody used for western blot analyses was monoclonal mouse anti-Cry1Ab antibody (1:1000; RDI, USA). The membranes were then washed three times with PBST, followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:4000; Abcam, UK) in PBST (containing 2% skimmed milk). Bound antibody was detected using the Enhanced Chemiluminescence System (Amersham). Chemiluminescent signals were quantified with the Fujifi lm LAS 3000 system (Fujifilm). All experiments were performed at least three times in duplicate.

Insect bioassay

This study used the susceptible third instar neonatal larvae of *Plutella xylostella*. Entomocidal toxicity of the Cry1Ab polypeptide expressed in the tissue of the plastid-trans-

formed cabbage was assayed through a no-choice detached leaf feeding bioassay, and we also directly applied the insects onto the intact plants. About 800–1,000 mg of fresh leaves was placed onto moist 3 M filter paper in plastic petri dishes (150 × 14.5 mm) and infested with 20 neonate insect larvae. The dishes were sealed with Parafilm to prevent desiccation and were kept in the insect rearing room at 25°C, coupled with a 16 h photoperiod and 75% relative humidity. Feeding was allowed for 3 days with one change of fresh leaves on day 2, and data on survival and mortality were collected. Each treatment was repeated twice with three replicates each. Identical infestation was also carried out for control plants. Mortality rates were presented as the proportion of dead larvae to applied larvae (%). Plant damage was estimated after 3 days of infestation using a leaf area meter (LI3100, Li-Cor Inc., Lincoln, NE, USA) and a 0–5 rating scale was performed to distinguish different situations of plant damage:

0–5 Rating scale (Whorl stage)

Scale 1. No visible leaf feeding or a small amount of shot-hole injury on a few leaves. Total damage-rating area <10%.
Scale 2. Small amount of shot-hole injury on a few leaves or common on several leaves. Total damage-rating area <30%.
Scale 3. Several leaves with shot-hole and large injury lesions. Total damage-rating area <50%.
Scale 4. Long lesions common on about two-third of the injured leaves. Total damage-rating area <70%.
Scale 5. Most leaves with large injury lesions. Total damage-rating area >70%.

Results

Transformation and regeneration of transgenic plants

A biolistic PDS-1000/He (Bio-Rad) gene gun system was used for this research. Deep green leaves were selected and cultured on L1 medium with 24 h dark photoperiod for 2 days before bombardment. Each shot carried 500 µg gold particles and 2 µg DNA of the pASCCBT plasmid. Explants were bombarded at a helium pressure of 1,100 psi. Gap distance between the target tissue and the rupture disc was 6 cm. Following particle bombardment, the tissue was incubated on new L1 medium in the dark at 25°C for approximately 48 h post-bombardment (Fig. 2a).

After 2 days, the leaves were dissected into small sections and were cultured on L2 regeneration medium containing 50 mg/l spectinomycin for primary antibiotic selection (Fig. 2b). Shoots were regenerated from transplastomic callus induced on selected medium (Fig. 2c), and non-transformed callus died under spectinomycin selection (Fig. 2d). The antibiotic resistance of green shoots and calli appeared over 4–6 weeks. The tissue was then transferred onto new L2 medium containing 100 mg/l spectinomycin dihydrochloride for recipient leaf section bleaching. Plants were regenerated from transplastomic callus tissue in 8 weeks post-bombardment (Fig. 2e). Subsequently, the regenerated shoots from resistant leaf sections were transferred onto rooting medium (L3 medium) for root development and secondary antibiotic selection (Fig. 2f). Transplastomic cabbage tissue was more resistant to high concentrations of antibiotic in L3 medium (200 mg/l spectinomycin and streptomycin), whereas the wild-type or non-transformed cabbages were killed under antibiotic selection (Fig. 2f, plant in middle culture bottle). The transplastomic shoots were further transferred to hardening medium (L4 medium) for full plantlet formation and matured in the greenhouse (Fig. 2 g). No visible differences were observed between the transformed plants and wild-type plants at maturity. Following this biolistic process, we successfully obtained transplastomic plants after about 5 months.

Integration of the cry1Ab gene and transgenic plant analysis

After the particle bombardment of 200 cabbage explants, 16 resistant transplastomic plants (8 for 'K–Y cross' and 8 for 'Summer Summit' variety) were obtained (Table 1). In order to demonstrate that the *cry1Ab* gene had integrated into the chloroplast genome through two homologous recombination events, the spectinomycin-resistant shoots were initially screened by PCR and Southern blot analysis. The plasmid pASCCBT (Fig. 1b) was used in this study, which contains the *aadA* and *cry1Ab* genes under control of chloroplast promoter *rrn,* and the *psbA* transcription termination region. The *aadA* cassette is used to recruit genes of interest into the IR region of the cabbage chloroplast genome at the intergenic spacer between *trnV-rrn16 s* and *trnI-trnA-rrn23 s* (Fig. 1). Genome integration occurs through two homologous recombination events between the chloroplast border sequences of the pASCCBT vector, and the corresponding homologous sequences of the chloroplast genome. PCR was performed to confirm integration of the foreign genes into the chloroplast genome of antibiotic resistant plants. PCR was carried out using specific primers in the external (flanking) regions, designed to detect the pASCCBT cassette. Forward primer 5, designated *rrn16S*, anneals to a region upstream of the chloroplast border used for recombination (refer to Fig. 1). Reverse primer 6, designated *rrn23S,* anneals to the chloroplast genome downstream border. Chloroplast DNA was isolated from five lines of 'K–Y cross' and four lines of 'Summer Summit'.

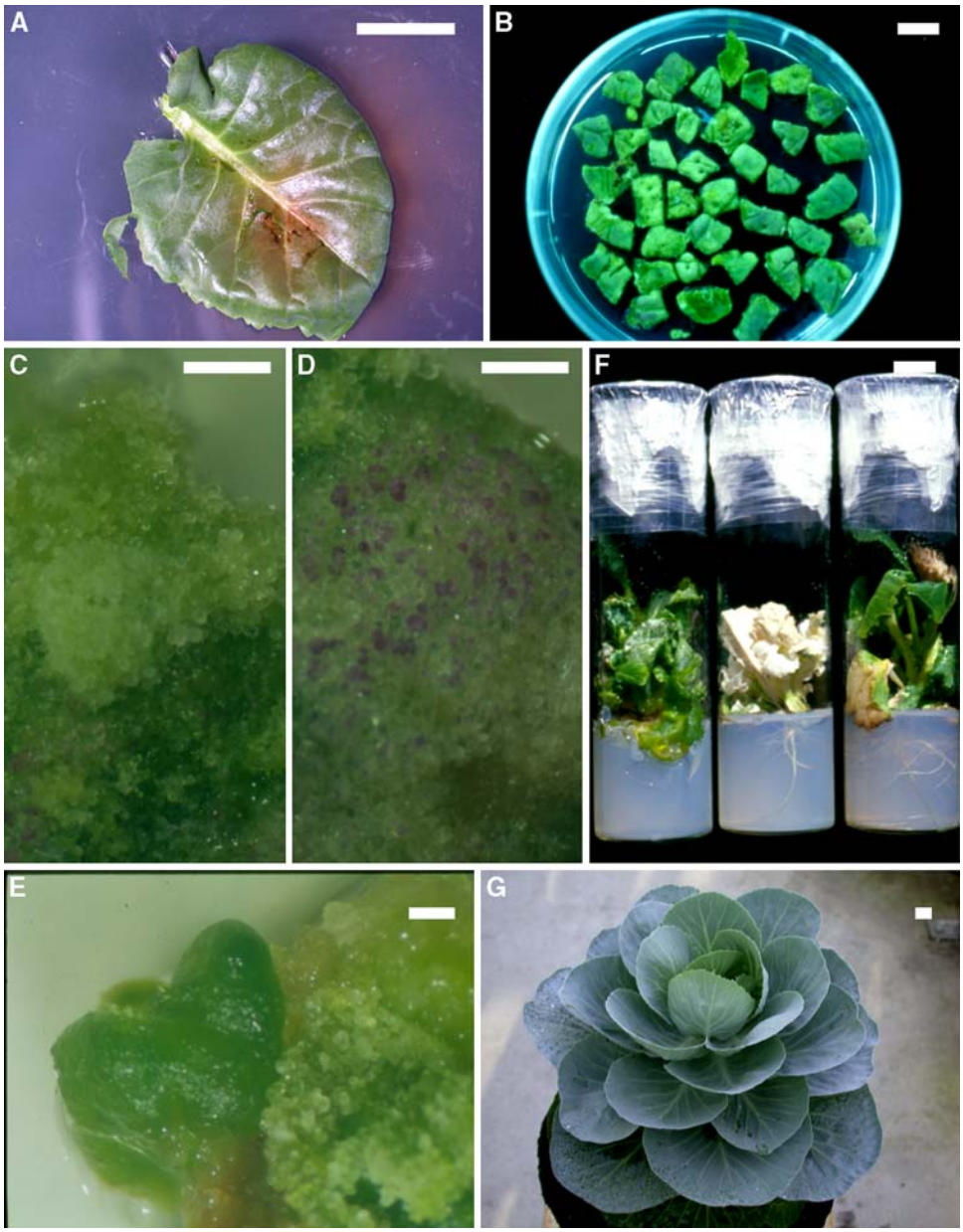

Fig. 2 Regeneration of chloroplast-transformed *Brassica oleracea* L. (K–Y cross). **a** Cabbage chloroplast transformation was achieved by biolistic bombardment of mature sterile leaves. The area of the leaf is approximately 4 cm^2. **b** After bombardment, leaf samples were cut into small pieces (9 mm^2) and transformed tissue was selected on 50 mg/l spectinomycin. **c** Transplastomic callus induced on selective medium. **d** The death of non-transformed callus on selective medium. **e** Plant regeneration from transplastomic callus tissue 8 weeks post bombardment. **f** Secondary selection of chloroplast transformed plants on 200 mg/l spectinomycin. **g** Transgenic plants were transferred to pots and grown in the greenhouse. The *bar* is 1 cm for all figures except C, D and E where the *bar* is 1 mm

Using primers 5 and 6, a 4.9-kb product was amplified only from the pASCCBT-transformed tissues, due to the insertion of the *aadA* and *cry1Ab* genes into the chloroplast region between *trnV-rrn16 s* and *trnI-trnA-rrn23 s* (Fig. 3a). Nine putative transgenic (spectinomycin and streptomycin resistant) plants were confirmed by PCR analysis for *aadA-cry1Ab* transgenes. Eight homoplasmic lines were acquired; these have only one 4.9 kb amplicon after PCR analysis (Fig. 3a lanes 3–10). A 0.9 kb product was obtained from untransformed tissue (Fig. 3a, lane 12). One transgenic line was heteroplasmic, where PCR amplified both wild-type (0.9 kb) and transgenic (4.9 kb) products (Fig. 3a, lane 2).

A second PCR analysis was performed to confirm integration of the foreign genes into the chloroplast genome. PCR was carried out using specific primers in the external (flanking) regions (forward primer 7 and reverse primer 8), designed to detect the foreign gene (*cry1Ab*) only when inserted in the chloroplast genome. The PCR results showed that all 8 of the transgenic lines carry the *cry1Ab* gene from the inverted repeat (IR) vector pASCCBT (Fig. 3b, lanes 2–9). After confirmation by PCR analysis, a Southern blot was performed. Total leaf genomic DNA was extracted from nine lines of 'K–Y cross' and 'Summer Summit' transplastomic plants. Digestion of pASCCBT with *Hin*c II restriction endonuclease yields a 4.3 kb frag-

Table 1 Regeneration and genetic analysis of chloroplast-transformed cabbage ('K–Y cross' and 'Summer Summit') after *cry1Ab* (pASCCBT) gene transformation *via* particle bombardment

Cabbage cultivars	Bombarded leaf samples	Antibiotic resistant calli	Southern positive	PCR positive	Northern positive
K–Y cross	100	8	8	5	4
Summer Summit	100	8	8	5	5

ment containing both the targeting *rrn16S*/*trnI* sequences and *prrn*:*aadA*:*psbA*:*prrn*:*cry1Ab*:*psbA*. A partial *rrn16S*/*trnI* fragment was used as the hybridization probe (Fig. 1b). The wild-type had a 0.7 kb fragment hybridizing with this probe. Southern blot results showed that some transgenic lines carried only the 4.3 kb fragment and therefore were homoplasmic (Fig. 3c, lines A3, A6, A7 B4, B6 and B7). Other transgenic plants carried both wild-type and transgenic fragments, and so were heteroplasmic (Fig. 3c, lines A1 and B3). Stable homoplasmic plants were selected for further characterisation by northern blot and insect bioassay.

Transcription of the *rrn* promoter-controlled *cry1Ab* gene in chloroplast- transformed plants was confirmed by northern blot analysis. The northern was performed utilizing the *cry1Ab* gene as a probe to determine *cry1Ab* gene transcription in each transplastomic line. Total RNA of leaf tissues of plants with positive patterns of PCR and Southern blot assay were prepared. Each sample) was electrophoresed on a 2.0% formaldehyde/agarose gel and then transferred onto a nylon membrane for hybridization. The *cry1Ab* gene was used as the hybridization probe. As shown in Fig. 4, the probe hybridized to a 1.9 kb transcript,

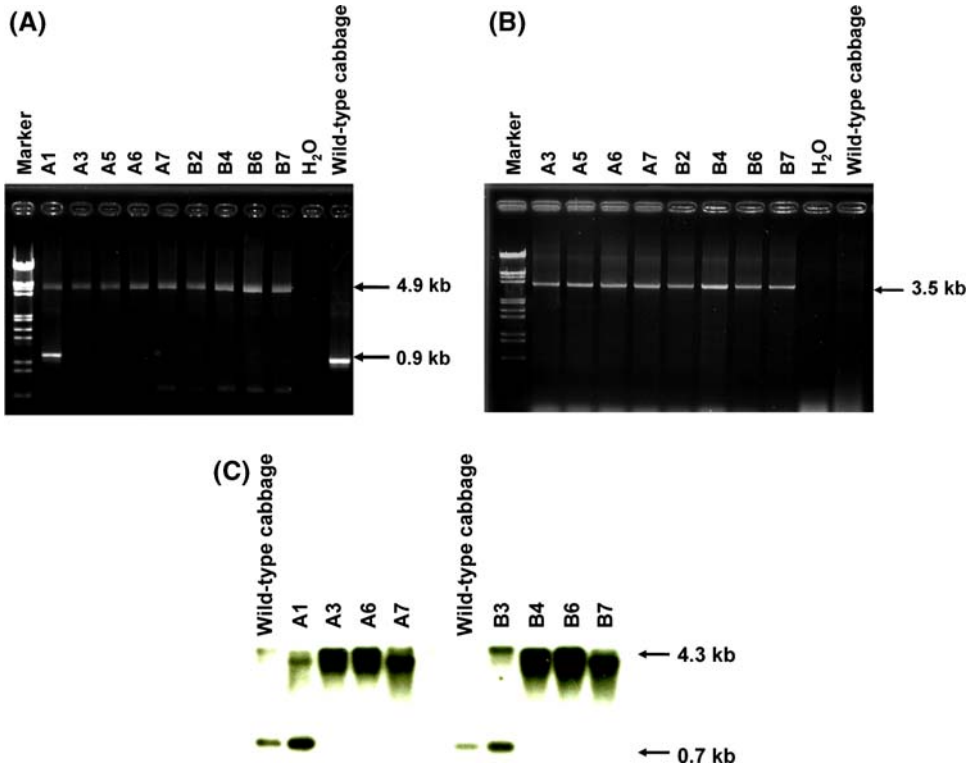

Fig. 3 PCR and Southern-blot analysis of *cry1Ab* gene (pASCCBT) transformed cabbage. **a** PCR analysis of the *cry1Ab* gene in transformed cabbages ('K–Y cross' and 'Summer Summit') using primer 5 and primer 6. The 4.9 kb fragment indicates amplification of the recombined *aadA* and *cry1Ab* genes whereas the 0.9 kb fragment indicates the wild-type plastid region. **b** PCR analysis in transformed cabbages using primer 7 and primer 8. The 3.5 kb fragment indicates amplification of the *23 s rrna* and *cry1Ab* genes, not seen in the wild-type plastid. All the PCR products were amplified using plastid DNA as a template, and analyzed on a 1.3% agarose gel by electrophoresis. *Lanes A1*, *A3*, *A5*, *A6* and *A7* were transplastomic 'K–Y cross' cabbage; *lanes 12* (A) and *11* (B) were control cabbage (wild-type 'K–Y cross' and 'Summer Summit', respectively); *lanes B2*, *B4*, *B6* and *B7* were transplastomic 'Summer Summit' cabbage; *lane 1* DNA Ladder (*λ*/*Hind*III + *Eco*RI, MBI Fermentas); *lanes 11*(A) and 10(B): no template control, H_2O. **c** Analysis of the *cry1Ab* gene in transformed cabbages by Southern-blot hybridization. Southern blot of plastid DNA from chloroplasts of wild-type and eight transgenic homozygous plants. Fifty micrograms of plant DNA from each sample was digested with *Hin*c II, the fragments were separated by electrophoresis on a 1.5% agarose gel, and then transferred onto a nylon membrane. The ^{32}P-labelled partial *rrn16S*/*trnI* fragment (probe1) was used as a probe in the hybridization (see Fig. 1)

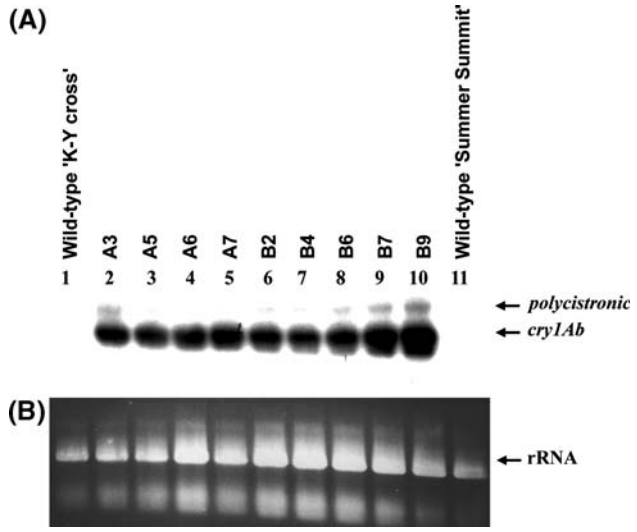

Fig. 4 Northern blot analysis of *cry1Ab* mRNA in the transformants. Total RNA from chloroplasts of wild-type (WT) cabbage and nine transgenic homozygous plants and 20 μg of total RNA from each sample ('K–Y cross' or 'Summer Summit' transformed cabbage) was electrophoresed on a 2.0% formaldehyde/agarose gel, and then transferred onto a nylon membrane. **a** A ^{32}P-labelled *cry1Ab* fragment (probe2) was used as probe in the hybridization reaction. **b** The ethidium bromide-stained gel is shown in the bottom panel as a quantitative control. *Lanes 2–5* transplastomic 'K–Y cross' cabbage from A3, A5, A6 and A7 plants; *lanes 6–10* transplastomic 'Summer Summit' cabbage from B2, B4, B6, B7and B9 plants; *lane 1* wild-type 'K–Y cross' cabbage; *lane 11* wild-type 'Summer Summit' cabbage

corresponding to the expected size of the *cry1Ab* mRNA. In the bottom panel (Fig. 4b), the rRNA, shown by staining the gel with ethidium bromide, was included as a quantitative control. Analysis of *cry1Ab* gene expression by northern analysis in the nine spectinomycin and streptomycin-tolerant plants and their clones revealed that all expressed both *aadA* mRNA (data not shown) and *cry1Ab* mRNA (Fig. 4a). Transgenic 'K–Y cross' cabbages gave strong hybridization signals (Fig. 4a, lanes 2–5), as did transgenic 'Summer Summit' cabbages (Fig. 4a, lanes 6–10). Non-transformed plants showed no *cry1Ab* hybridization signal, and no significant difference in rRNA levels was observed in the ethidium bromide-stained gel (Fig. 4b, lanes 1, 11).

In summary, these data demonstrate that the chloroplast transformation cassette pASCCBT was successfully integrated into the cabbage plastid genome by homologous rearrangement. Initial spectinomycin plus streptomycin screening, PCR, Southern hybridization and northern hybridization have together proved the presence and expression of the transgenes in the chloroplast genomes of transplastomic plants.

The accumulation of Cry1Ab protein was examined in mature leaves of different transgenic cabbage plants ('K–Y cross' and 'Summer Summit'). We examined Cry1Ab protein accumulation by immunoblotting analysis. Total lprotein extracts from leaves of each transformant and wild-type plants were separated by SDS-PAGE. Immunoblotting analysis showed that the novel Cry1Ab protein was present in mature leaves of transgenic plants (Fig. 5). A novel band seen only in transgenic plants confirmed the presence of Cry1Ab insecticidal protein by using a specific monoclonal antibody against the *Bacillus thuringiensis* toxin. Moreover, the recombinant protein could be detected at around the expected molecular weight of 78 kDa in the leaves of transgenic lines. The highest level of Cry1Ab accumulation was estimated to be between 4.8 and 11.1% of total soluble protein (TSP) using this approach (Fig. 5b).

All of the confirmed transgenic plants were subjected to feeding third instar neonatal larvae of *Plutella xylostella*. Insect mortality was recorded to assess the effect of the Cry1Ab protein on the larvae. The extent of leaf damage was also measured after 3 days of infestation. Eight transgenic cabbage lines were assessed along with a wild-type plant for comparison. Bioassays on detached transgenic leaves showed significant variation from the control wild-type plants, with leaf injury caused by feeding larvae rated on a five-point scale (Fig. 6). Variable degrees of leaf injury were observed, which might be due to differences in the expression levels of the *cry1Ab* gene. Three days after infestation by the neonatal larvae, control leaves were badly damaged (Fig. 6, WT1 and WT2). Contrary to this, the leaves of plastid- transgenic plants showed less leaf damage (Fig. 6). Some transgenic plants (e.g., Fig. 6, A1, A6, B2,

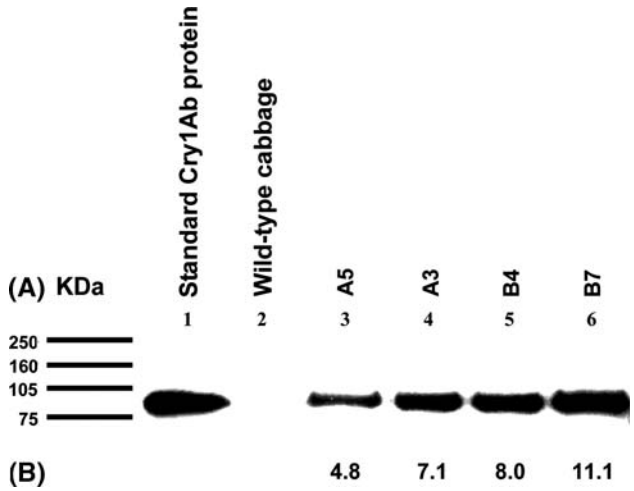

Fig. 5 Cry1Ab expression in transformants, evaluated by western blot analysis using monoclonal mouse anti-Cry1Ab antibody. **a** Total soluble protein (TSP) was extracted from mature leaves of wild-type (*WT*) and four transgenic homozygous cabbage plants. Forty micrograms of TSP from each cabbage was loaded into each lane. *Lane 1* four micrograms of standard Cry1Ab protein was loaded and used as reference; *lane 2* control cabbage, 'K–Y cross' wild-type; *lane 3*, *4* transplastomic 'K–Y cross' cabbage; *lane 5*, *6*: transplastomic 'Summer Summit' cabbage. **b** Relative Cry1Ab quantification as a percentage of total soluble protein in transgenic chloroplasts

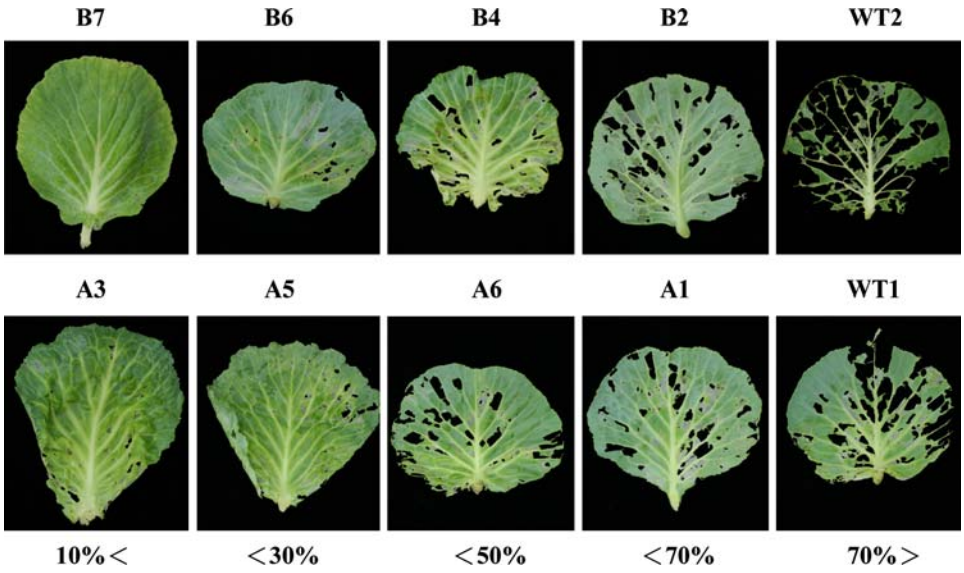

Fig. 6 The appearance of control (*right*) and *cry1Ab* (pASCCBT) transgenic cabbage leaves after feeding by 20 *Plutella xylostella* larvae. Leaves B2, B4, B6 and B7 are transgenic 'Summer Summit' cabbage (*top row*), leaves A1, A3, A5 and A6 are transgenic 'K–Y cross' (*bottom row*). *WT1* wild-type 'K–Y cross' cabbage. *WT2* wild-type 'Summer Summit' cabbage. Photographs were taken on the third day of the assay. The leaf injury rating scale is shown in the bottom panel

and B4) showed a higher injury rate after larval feeding than others (e.g., Fig. 6, A3, A5, B6, and B7), however, all transgenic plants showed some resistance towards *P. xylostella* larvae compared to wild-type plants. These results clearly indicate the expression of the *Bt* gene and its effectiveness in controlling *P. xylostella* larvae. Mortality of the *P. xylostella* larvae was assessed after 24 h of feeding on the leaves (Table 2). When feeding on control cabbage (Table 2 WT1 and WT2), no larval mortality was recorded after 24 h. When feeding on the transgenic lines a range of larval mortality rates was observed. In the case of A1, B2 or B9 transgenic plants, approximately 70% larval mortality was recorded 24 h after commencement of feeding. The highest mortality rate found was amongst those larvae feeding on transgenic plant lines A3, A5, A6, B4, B6 and B7 (Fig. 6), where in some instances the death rate was 100%. The insect death rate negatively correlated with the amount of leaf damage seen (i.e., less damage was seen when 100% larvae died). Thus, this study shows high-efficiency insect resistance in plant lines transformed using the species-specific plastid vector pASCCBT.

Table 2 The death rate of *Plutella xylostella* after 24 h of feeding on the leaves of *cry1Ab* transformed cabbage

Cultivar	Insect death (%)[a]
K–Y cross	
WT1	0
A1	67
A3	100
A5	93
A6	91
Summer Summit	
WT2	0
B2	67
B4	80
B6	100
B7	100
B9	67

WT wild-type cabbage

[a] Each leaf was infested with twenty *Plutella xylostella* larvae and the insect death rate was counted after 24 h. The data shown are the means of two individual leaves from same plant. A1, A3, A5 and A6 are transgenic 'K–Y cross' cabbage. B2, B4, B6, B7 and B9 are transgenic 'Summer Summit'

Discussion

Genetic engineering of the chloroplast genome is nowadays an important technique for developing useful transgenic plants. Chloroplast-transformation is currently a routine technique for tobacco, and has been successful developed in several crops (Ruf et al. 2007). The transformation efficiencies of non-solanaceous species were lower than in the tobacco system when using tobacco vectors, and the transformed plants were sterile (Sidorov et al. 1999). Recently, fertile plastid- transformed plants were obtained by using species-specific vectors in carrot (Kumar et al. 2004a), cotton (Kumar et al. 2004b), soybean (Dufourmantel et al. 2004), and cauliflower (Nugent et al. 2006). We devised a stable transformation system suitable to operate the action of transgenes in plastids of cabbage (*Brassica oleracea*). We constructed and established a selection system and performance protocol for insect-resistance. Present efforts are aimed at acquiring transgenic cabbage lines that display more efficient resistance against *Plutella xylostella* using stringent protocols of antibiotic selection. Each transplastomic line regenerated from fully expanded leaves was recovered using a two-step regeneration protocol (with L1

and L2 medium). The L1 medium is a callus-induction medium containing high concentration of spectinomycin for primary selection. The L2 medium is a shoot-induction medium and comprises both spectinomycin and streptomycin for secondary selection. The *aadA* gene (originally the bacterial aminoglycoside 3′-adenylyltransferase gene), the first chloroplast-specific antibiotic resistance marker, confers resistance to a number of antibiotics including spectinomycin and streptomycin (Goldschmidt-Clermont 1991). Consequently, transformation with the *aadA* gene is currently the most broadly utilized strategy for screening transplastomic plants, and has been used to prove that DNA could be delivered into plastids in a wide variety of plants (Sikdar et al. 1998). In this study, we successfully obtained 16 surviving explants after antibiotic selection. There is a requisite for a callus phase in the recovery of plastid-transformed cabbage, but it is different from that of tobacco and oilseed which only recruit a single step protocol (Svab and Maliga 1993; Skarjinskaia et al. 2003). This study also differs from that reported for tomato and tobacco (Ruf et al. 2001; Nguyen et al. 2005); following particle bombardment, our tissue was incubated in the dark at 25°C for 48 h on L1 medium, then the explants were dissected into 9 mm^2 sections. It may be important to cut the leaves into small explants for callus induction and shoot regeneration, since our plastid transformation efficiency was much higher than for larger leaf explants as reported for *Solanaceae* (Nguyen et al. 2005).

Successful plastid transgenic and homoplastic plants were all confirmed by PCR and southern analyses. Therefore, a practical protocol for *Brassica* stable plastid transformation by particle bombardment was constructed and established in this study. By the use of specific flanking PCR primers, the characteristics of ten homoplasmic lines were demonstrated by the presence of a 4.9 kb transgene-specific amplicon, and the absence of the 0.9 kb wild-type band. A PCR product of 4.9 kb (Fig. 3a) or 3.5 kb (Fig. 3b) was obtained only in pASCCBT-transformed tissues due to the insertion of the *aadA-cry1Ab* cassette in the chloroplast genome between *trnV-rrn16 s* and *trnI-trnA-rrn23 s*. Another six regenerated lines that showed both the 4.9 and 0.9 kb PCR products were deemed to be heteroplasmic or streptomycin insensitive mutants.

Some researchers have inferred that the frequency of potato, tomato or arabidopsis plastid transformants generated using tobacco homologous vectors is lower than that for tobacco (Ruf et al. 2001; Sikdar et al. 1998). Sidorov et al. (1999) reported 1 transgenic potato was obtained per 15 attempts transformed from the *rbcL-accD* region of the LSC (large single-copy) and 1 per 35 attempts for a vector targeting the *rps12-rrn16* intergenic region of the IR (invert repeat). Most reports have indicated that the species-specific vectors have contributed to efficient plastid transformation by particle bombardment (Kumar et al. 2004a, b). However, a recent report has showna low plastid transformation frequency was obtained in cauliflower using PEG-mediated uptake from the site of integration of the *B. napus*-specific plastid (Nugent et al. 2006). Our study is the first report of plastid transformation in a vegetable brassica using a transformation vector containing *Brassica oleracea* plastid DNA and particle bombardment. The plastid transformation frequencies reported here, between 4 and 5%, are higher than those reported in other crops (Sidorov et al. 1999; Nguyen et al. 2005). However, the transformation frequency reported here is lower than that for plasmid pASCC201 (12% for ‘K–Y cross’ and 16% for ‘Summer Summit’) (Liu and Tseng, 2005). In this study, we selected fully expanded leaves of 1–2-month-old plants for chloroplast transformation. The old foliage has more mature chloroplasts, but it is hard to obtain transplastomic plants from mature foliage after bombardment. Moreover, the universal flanking cabbage sequences *(trnV–rrn16S* and *rnI–trnA–rrn23S)* in pASCCBT used in this study are 99% identical to the arabidopsis chloroplast region and 97% identical to the same region of the tobacco chloroplast. Unfortunately, plastid transformation using plastid DNA sequences of less than 100% homology has been demonstrated in several other cases to result in much lower transformation frequencies (Zubko et al. 2004). Especially when using tobacco vectors to target the intergenic regions, the technique was widely unsuitable in other crop species (Daniell et al. 2005). This is the major reason for the lower transformation frequencies and the limitations of plastid genetic engineering in other crops (Ruf et al. 2001). In this report, practical vectors for *Brassica* species were created and used for the demonstration of plastid transformation. By using the sites-specific cassette from plasmid pASCC201, plastid transformation has been shown to be more efficient in cabbage.

The level of mRNA from the *cry1Ab* gene in each transformant was detected by northern blot analysis, and the transcript was expected to be approximately 1.9 kb in length. The level of *cry1Ab* mRNA found in two homoplasmic ‘Summer Summit’ lines was 87% higher (Fig. 4, lines B7 and B9) than that of other heteroplasmic transgenic lines of ‘Summer Summit’ (not shown), and no signal was observed in the same tissues of wild-type plants. No significant difference in mRNA level was detected in any of the ‘K–Y cross’ lines. Moreover, we have also noticed *cry1Ab* transcripts showed slightly different sizes in northern blot analysis (Fig. 4a). The plastid translational machinery has the capacity to co-express multiple genes (DeCosa et al. 2001). Because of the presence of three 16S rRNA promoters (two tobacco *rrn* promoters and one cabbage *rrn* operon), different transcripts may be detected in transgenic plants. It has been demonstrated that expressions of multiple genes from polycistronic mRNAs were visible using the

strong *rrn* operon promoter (Daniell et al. 2005). Significantly, we also detected another processed mRNA (Fig. 4 arrow indicates) in some transplastomic lines, which may be due to readthrough (Tregoning et al. 2005). In this study, polycistronic mRNAs of *cry1Ab-aadA* were detectable in mature leaves, similarly to the study reported by Chakrabarti et al. (2006), who examined RNA expression patterns from young leaves. We will continue to examine this phenomenon in the future. A strong hybridization signal indicated that the *cry1Ab* gene has integrated into the IR_A (inverted repeat) or IR_B regions in B7 and B9 homozygous plants. It is possible that 2 copies integrated in the lines with the higher expression levels. The highest expression level of a recombinant protein expressed in plastids is reported to be 46.1% of total soluble protein (TSP) for the cry2Aa2 operon protein of *Bacillus thuringensis* in old bleached tobacco leaves (De Cosa et al. 2001). Our results show that Cry1Ab accumulation in cabbage is less than 15% TSP, the best plastid expression yielding 11.1% TSP as Cry1Ab protein crystals, which were very stable proteins (data not shown). We report here that the overexpression of the Cry1Ab protoxin was at a level similar to that of Cry9Aa2 (Chakrabarti et al. 2006), but more than that of Cry2Aa2 (Kota et al. 1999), and GUS (Liu et al. 2007). The low transgene expression level in cabbage is likely to be because Cry1Ab was assessed in mature tissue, since transcription levels are highly dependent on tissue type, plant age and culture conditions (Herz et al. 2005). In the present study, stable Cry1Ab protein was observed in mature leaves of fully grown plants. Some data has shown that the highest protein yields are obtained in the young leaves of fast growing lines. Mature leaves from homoplasmic lines sometimes contain very low levels or even no desired protein (Birch-Machin et al. 2004). This demonstrates the success of our technique in comparison to others; furthermore, pest control can be achieved in mature plants.

Insect bioassays indicated that different Cry1Ab protoxin levels resulted in differential insect control and mortality. Wild-type leaves were highly damaged and no insect death occurred within 24 h of infestation with *P. xylostella* larvae. After feeding for 24 h, 3 transgenic cabbage lines (1 'K–Y cross' and 2 'Summer Summit') resulted in 100% insect mortality, and 100% larval death had occurred after feeding for 3 days on another 6 lines (Table 2). In this study, the expression of toxic protein may be at a low level, but the control and mortality of neonate insects was highly efficacious. The homoplasmic plant lines that showed the highest level of foreign gene expression were also the most efficient at killing *P. xylostella* larvae (lines A3, B6 and B7), with no visible leaf damage after the insect bioassay. Recently, we mimicked insect stress in the natural environment by releasing 100 *P. xylostella* third instar larvae onto individual transgenic and wild-type cabbage plants. The primary field trial has demonstrated that transgenic plants displayed significantly higher resistance to insect-stress, and 100% insect mortality after 7 days (data not shown). Transmission electron micrographs (TEMs) of transgenic plants showed bipyramidal-crystal formation in chloroplasts of mature transgenic leaf tissue (data not shown). These results clearly demonstrate the expression of the plastid-transferred *cry1Ab* gene, and its effectiveness in exterminating *P. xylostella* larvae. Moreover, the growth morphology of the transgenic plants was identical to the wild-type plants. This study reports the successful introduction of *cry1Ab* into the chloroplast genome. We developed a *Brassica* species-specific vector that can transform the chloroplast genome of any *Brassica* species, and may have opportunity to conquer the major limitations of chloroplast transformation in important crops. Recently, more than eighty transgenes have been expressed via the chloroplast genome, the most recent is the expression of insulin in lettuce (Ruhlman et al. 2007). It is becoming more popular to use plastid-based systems for human therapeutic protein production. In the future, because the cabbage is edible, this species-specific vector could have potential as a delivery system to express vaccines or biopharmaceuticals in an edible format.

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