

# Chloroplast transformation of rapeseed (*Brassica napus*) by particle bombardment of cotyledons

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**Abstract** A protocol for chloroplast transformation of an elite rapeseed cultivar (*Brassica napus* L.) was developed based on optimized conditions for callus induction and regeneration from cotyledonary tissues. Comparison of six different media with three elite cultivars showed that B5 medium plus 3 mg/l AgNO<sub>3</sub> supplemented with 0.6 mg/l 2,4-dichlorophenoxyacetic acid and 0.2 mg/l 6-furfurylaminopurine was optimal for callus formation and maintenance without differentiation, while the medium suitable for regeneration was B5 medium supplemented with 1 mg/l 6-benzylaminopurine, 1 mg/l 6-furfurylaminopurine and 0.5 mg/l  $\alpha$ -naphthaleneacetic acid. A rapeseed-specific chloroplast transformation vector was constructed with the *trnI* and *trnA* sequences amplified from the rapeseed

chloroplast genome using two primers designed according to *Arabidopsis* homologs. The *aadA* gene was used as a selection marker regulated by the ribosome-binding site from the bacteriophage T7 gene 10L, the tobacco 16S rRNA promoter and the *psbA* terminator. After bombardment, cotyledonary segments were cultured for callus formation on media containing 10 mg/l spectinomycin and regeneration was carried out on medium with 20 mg/l spectinomycin. Heteroplasmic plastid transformants were isolated. An overall efficiency for the chloroplast transformation was one transplastomic plant per four bombarded plates. Southern blot analyses demonstrated proper integration of the target sequence into the rapeseed chloroplast genome via homologous recombination. The expression of the *aadA* gene was confirmed by Northern blot analysis. Analysis of T1 transplastomic plants revealed that the transgenes integrated into the chloroplast were inheritable with a ratio of about 8%. These results suggest that rapeseed may be a suitable crop for chloroplast transformation with cotyledons as explants under appropriate conditions.

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## Introduction

Rapeseed (*Brassica napus* L.) is one of the most important oil-producing crops worldwide. In China, rapeseed production has been increasing steadily since the 1980s, and the annual cultivation area has reached up to 6.9 million hectares (Fu et al. 2003). Moreover, in both winter and summer of Southern and Northern China, rapeseed has

been the main nectar source for insects for a long history since it has a long flowering period (Li et al. 2003). Thus, rapeseed plants play a very important role in China, where genetic transformation to breed new cultivars is important for rapeseed production. However, since China is the centre of evolutionary origin and biodiversity of many crucifer species (Lu et al. 2005), the foreign genes in cultivars bred by nuclear transformation technology could escape through pollen from transgenic rapeseed plants to other closely related species under such a complicated ecological environment. Cross-pollination can occur when pollen is transported by wind or insects although rapeseed is self-compatible and can produce a large quantity of seed without cross-pollination. Transgenes may be transferred from *B. napus* to vegetables and wild species of *B. rapa* or *B. juncea* by cross-pollination in fields where the crops and weedy species exist in close proximity (Lu et al. 2002). Studies have revealed that natural crosses occur among *B. napus*, *B. rapa* and *B. juncea*, indicating that hybridizations among these species do occur under field conditions (Bing et al. 1996).

Chloroplast transformation in plants has many advantages over nuclear transformation (Daniell and Dhingra 2002). First, chloroplast genes are inherited in a strictly maternal fashion in most angiosperm plant species including rapeseed. This minimizes the possibility of outcrossing transgenes to related weeds or species and reduces the potential toxicity of transgenic pollen to non-target insects (Daniell et al. 2002). This maternal inheritance eliminates or reduces the environmental concerns about foreign gene proliferation by pollen dispersal. Second, gene silencing and position effect have not been reported in chloroplast transformation due to their site-specific transgene integration into the spacer region of the chloroplast genome. As photosynthetic cells of higher plants contain a large number of chloroplasts cells can carry up to 100 identical copies of the plastome, resulting in high levels of protein expression with proper folding and disulfide bonds (Daniell et al. 2001; Fernandez-San Millan et al. 2003; Koya et al. 2005; Lee et al. 2003; Staub et al. 2000). In addition, gene silencing, position effects, and random integration in the genome have been reported with nuclear transgenic technology. Chloroplast transformation has been used to improve agronomic traits in different plant species such as resistance to insects (De Cosa et al. 2001; Hou et al. 2003; Liu et al. 2008), and tolerance to drought (Lee et al. 2003), salt (Kumar et al. 2004a) and herbicides (Kang et al. 2003; Lutz et al. 2001; Ye et al. 2001).

Although chloroplast transformation is very attractive, this technology is not as widely used as nuclear transformation. It has been mostly focused on a few plant species, especially in tobacco (Craig et al. 2008; Scotti et al. 2009; Soria-Guerra et al. 2009). This circumstance might be

related to several difficulties associated with development of chloroplast transformation for major crops, such as limitations of currently available tissue culture systems and efficient regeneration protocols. In rapeseed, technology for nuclear transformation is well established, in which cotyledon petioles have been widely used as explants. However, cotyledon petioles appeared to be not proper explants for rapeseed chloroplast transformation (Hou et al. 2003). In this study, we first established a new tissue culture system from cotyledons of elite rapeseed cultivars, which was subsequently used for chloroplast transformation by bombardment through spectinomycin selection conferred by *aadA* gene. Southern blot analysis of transplastomic rapeseed plants demonstrated a specific integration of the target cassette that was inherited in their progenies. Expression of the *aadA* gene in the transplastomic plants was further confirmed by Northern blot analysis. These results may serve as a basis for generation of chloroplast transgenic rapeseed plants to engineer agronomically important traits.

## Materials and methods

### Explant preparation

Three elite rapeseed (*Brassica napus* L.) cultivars, FY-1, FY-4, and FY-10 that have been cultivated in China were used in this study for optimization of culture conditions. The cultivar FY-4 was selected for chloroplast transformation due to its high efficiency of callus formation and regeneration. Seeds were surface-sterilized by 70% (v/v) ethanol for 40 s and then agitated for 35 min in 15% (v/v) hydrogen peroxide. After four rinses in sterile distilled water, the seeds were placed in hormone-free B5 medium (Gamborg et al. 1968) solidified with 0.7% agar and the seeds were cultured at 25°C under a 16-h photoperiod (50–70  $\mu\text{mol photons/m}^2/\text{s}$ ). Cotyledons were harvested from 4-day-old seedlings, which were cut into 0.3  $\times$  0.3 cm pieces for subsequent experiments.

### Culture medium

The B5 medium supplemented with 30 g/l sucrose, 7 g/l agar and 3 mg/l  $\text{AgNO}_3$  was used as a basal medium. To select an optimal medium for callus formation, six different media were compared that varied in plant growth regulators or their combinations. Compositions and combinations of phytohormones in the six media containing the B5 basal medium were made as follows: IA, 5 mg/l 6-benzylaminopurine (6-BA) and 5 mg/l  $\alpha$ -naphthaleneacetic acid (NAA) (Akasaka-Kennedy et al. 2005); IB, 0.04% MES omitting phytohormones (Liu et al. 2007; 2008); IC, 0.2

mg/l 2,4-dichlorophenoxyacetic acid (2,4-D); ID, 0.4 mg/l 2,4-D; IE, 0.6 mg/l 2,4-D and 0.2 mg/l 6-furfurylamino-purine (KT); IF, 0.8 mg/l 2,4-D and 0.3 mg/l KT. After culture for 4 weeks, the calli were transferred onto shoot regeneration media. The three shoot regeneration media all contained B5 medium but differed in phytohormones: RA, 3 mg/l 6-BA and 1 mg/l zeatin (ZT); RB, 0.5 mg/l 6-BA and 0.5 mg/l KT; RC, 1 mg/l 6-BA, 1 mg/l KT and 0.5 mg/l NAA.

#### Culture condition

All B5 media with carbon sources and agar were sterilized by autoclaving at 121°C for 20 min. Phytohormones and AgNO<sub>3</sub> were filter-sterilized by passing through a 0.22 µm syringe filter and added to the autoclaved medium in the laminar flow cabinet before pouring the medium into 9 cm sterile Petri dishes. In one Petri dish, 16 explants were placed and 48 explants were cultured per experiment. The explants were cultured under the conditions described above. Each experiment had three independent replicates. After culture for 4 weeks, calli formed from the explants were scored for comparative analysis and transferred onto shoot regeneration medium. After culture for another 4 weeks, shoots induced on explants were scored for analysis. Regenerated shoots were excised and transferred to 0.7% agar-solidified B5 medium without phytohormones for root initiation. All explants were cultured in plates containing 40 ml medium and incubated at 25°C under a 16-h photoperiod (50–70 µmol photons/m<sup>2</sup>/s). Regenerated plants were acclimatized, transferred to soil, and grown in a greenhouse.

#### Construction of a rapeseed chloroplast transformation vector

The rapeseed chloroplast transformation vector pCL308 carries a cassette containing the *aadA* selection marker gene plus a synthetic 1.8 kb human serum albumin gene (*HSA*) sequence (accession no. E03839) and targets the cassette into the *trnI-trnA* inverted repeat region of the rapeseed chloroplast genome. The sequence of the *Arabidopsis thaliana* chloroplast genome (accession no. NC000932) was used to design two sets of primers. The first set of primers trnIF1 (5'-ATAGAGCTCTGGAACCC TGAACAGACTG-3') and trnIR2 (5'-ATAGCGGCC GCATTTGAACCAGAGACCTC-3') were used for PCR amplification of the *trnI* gene sequence while the second set of primers trnAF1 (5'-ATAGTCGACGGGGATAT AGCTCAGTTGG-3') and trnAR2 (5'-TAAGGGCCCCGG TACTACTTCGCTATCG-3') were used for amplification of the *trnA* sequence. The chloroplast DNA isolated from rapeseed leaves (Kolodner and Tewari 1972) was used as

template for PCR amplification of *trnI* and *trnA* gene sequences. The PCR-amplified *trnI* and *trnA* genes were sequenced and subjected to vector construction. First, the *SacI-NotI trnI* fragment and *Sall-ApaI trnA* fragment were cloned into pBluescript SK(-) vector to form the intermediate plasmid pCLLR303. The expression cassette released as an *SpeI-EcoRV* fragment was cloned between the two chloroplast regions into the pCLLR303, to yield the pCL308 vector. The cassette contains one complete promoter of the tobacco 16S rRNA gene, fused with a ribosome-binding site (RBS) from the bacteriophage T7 gene 10 leader G10L (Ye et al. 2001), followed by the coding sequences of the *HSA* gene and the *aadA* gene, and the terminator of the tobacco plastid *psbA* gene that is located at the end of the entire cassette.

#### Transformation and regeneration of transplastomic rapeseed plants

Mature seeds were germinated on agar-solidified B5 medium supplemented with 3% sucrose and 0.7% agar for 4 days at 25°C under a 16-h photoperiod (50–70 µmol photons/m<sup>2</sup>/s). Intact cotyledons with no petiole were harvested and were then placed in the center of a 6 cm Petri dish. The concentric explants with a diameter of 2.5 cm were subjected to particle bombardment (Fig. 3a). The plasmid DNA was coated onto 1 µm gold particles as microcarriers. The particles were washed first with 70% ethanol and sterile water and then resuspended by sonication in sterile water. An aliquot of 15 µl of a 2 µg/µl solution of plasmid DNA, 150 µl of 2.5 M CaCl<sub>2</sub> and 60 µl of 0.1 M spermidine were added sequentially to the particle suspension for bombardment with the PDS 1000/He Biolistic Gun (Bio-Rad, USA) at 1,100 psi, and a target distance of 9 cm (Ye et al. 1990). Two days after bombardment, the cotyledons were cut into 0.3 × 0.3 cm segments that were transferred to callus induction medium supplemented with 10 mg/l spectinomycin. After selection for 2 months, green, resistant calli were sub-cultured onto the shoot regeneration medium containing 20 mg/l spectinomycin until green shoots appeared for rooting. The rooted shoots were transferred to B5 medium without phytohormones and spectinomycin for full plantlet formation and transplanted into pots filled with soil.

#### DNA extraction and PCR amplification

Total DNA was extracted from 100 mg leaf tissues of the transformed and wild type rapeseed plants. PCR was performed by using the Taq DNA polymerase (Takara Co., Japan) with 150 ng leaf DNA as template. For the PCR identification of transgenic plants, primers P1 (5'-ATGGCAGAAGCGGTGATCGC-3') and P2 (5'-CTAGACA

TTATTTGCCGACTAC-3') were used to amplify the complete sequence of *aadA* gene. Primers P3 (5'-TGTCCTACTACGTGAAGGCGAG-3') and P4 (5'-TCGGTCTTTTCCCCATTACTTAG-3') were located downstream of the *aadA* gene and upstream of the right homologous recombination region, respectively, and were used to detect the integration of the expression cassette into the rapeseed plastome. The PCR protocol consisted of an initial denaturation step at 95°C for 4 min, then 30 cycles (95°C, 50 s; 64°C 50 s; 72°C 50 s) followed by 10 min final extension at 72°C. PCR products were analyzed on a 1.2% agarose gels. PCR-positive plants were transferred to the greenhouse for plant growth and seed production.

#### Southern and Northern blotting

Southern blotting was carried out with leaf DNA (3 µg) isolated from PCR-positive and wild type plants. DNA was digested with restriction enzyme *EcoRI* and separated on a 1.0% agarose gel. Lambda DNA digested by *PstI* was run in parallel as a molecular marker and after electrophoresis the gel containing the DNA marker was cut off, stained with ethidium bromide and photographed with a ruler under UV illumination (Sambrook et al. 2000). DNA fragments were transferred onto nylon membranes and hybridized with  $\alpha$ -[<sup>32</sup>P]-dCTP-labeled DNA fragments (TaKaRa, Dalian, China) derived either from the *trnA* region (probe 1, Fig. 2) or the coding region of the *aadA* gene (probe 2, Fig. 2). Pre-hybridization and hybridization were performed as previously described (Li et al. 2008). Washed membranes were exposed to a phosphor screen and scanned in Fuji Film BAS-1800II Imaging Plate Scanner. After stripping of probe 1, the membranes were re-hybridized with probe 2.

For Northern blotting, total leaf RNA was extracted from Southern blotting-positive and wild type plants using Trizol Reagent following the manufacturer's instruction (Tiangen Biotech Co., LTD). Total RNA (5 µg) was denatured and electrophoresed on 1.2% agarose/formaldehyde gel. An RNA marker RL6000 (TaKaRa, Dalian, China) was run in parallel on the gel as a molecular marker and after electrophoresis the gel was treated as described above for Southern blot analysis (Sambrook et al. 2000). The membranes were hybridized with the *aadA* gene probe that was radioactively labeled as described above for the Southern blotting.

#### Transgene analysis in T1 generation

Seeds harvested from three T0 transplastomic rapeseed plants (referred as lines 1, 2 and 3 of T1 generation) and wild type control plants were surfaced-sterilized as described above, placed on B5 medium containing 20 mg/l

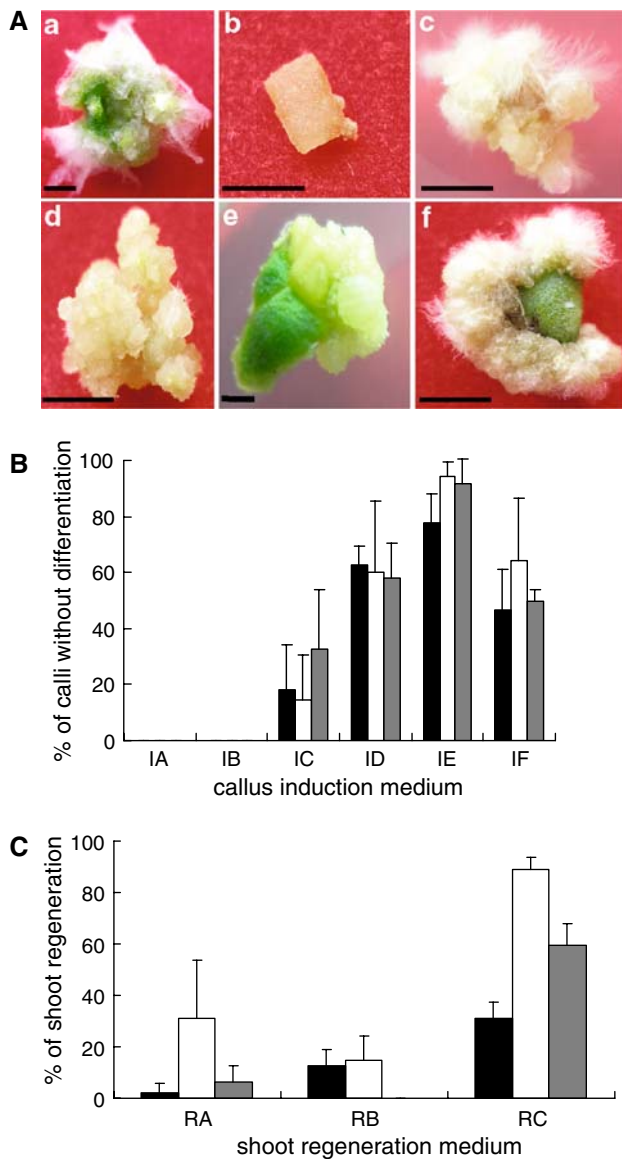
spectinomycin and cultured at 25°C under a 16-h photoperiod (50–70 µmol photons/m<sup>2</sup>/s). After culture for 14 days, the seedlings were transferred into soil and grown in greenhouse. The leaves from three transplastomic lines (three seedlings of the line 1, and two seedlings from the lines 2 and 3, respectively) were used for PCR analysis with the two pairs of primers as described above for PCR identification of T0 plants.

## Results

### Optimization of the culture system for efficient regeneration of rapeseed

To select a medium suitable for chloroplast transformation of rapeseed plants, six media for callus induction from cotyledonary explants of rapeseed cultivars FY-1, FY-4, and FY-10 were compared under the same conditions. Calli were usually initiated at the cut edges of the explants after 7 days of culture and eventually extended all over the explants. However, distinct differences of callus appearance were seen after a period of culture among the six media used. On medium IA, the cotyledonary explants rapidly produced calli that differentiated into roots within 2 weeks (Fig. 1A-a), whereas explants cultured on medium IB formed almost no calli and turned yellow after 10–14 days (Fig. 1A-b). On medium IC containing 0.2 mg/l 2,4-D, calli formed roots within 3 weeks (Fig. 1A-c), and on medium ID with 0.4 mg/l 2,4-D, rooting was clearly inhibited but the induced calli turned yellow (Fig. 1A-d). On medium IE that contained 0.6 mg/l 2,4-D and 0.2 mg/l KT, calli displayed a light green, healthy appearance and retained this status without further differentiation for about 8 weeks (Fig. 1A-e). With the increased concentration of 2,4-D (0.8 mg/l) and KT (0.3 mg/l) on medium IF, the calli differentiated roots rapidly (Fig. 1A-f).

Comparative analyses revealed that the calli grown on medium IE showed the highest rate of dedifferentiation among six media, exhibiting a high potential for the maintenance of callus status after culture for 4 weeks (Fig. 1B, IE). On this medium, three genotypes showed dedifferentiation rates from 77.95 to 94.33% in 4 weeks, with similar efficiency for cvs. FY-4 (94.33%) and FY-10 (91.68%). Two media, ID (58.05–62.62%) and IF (46.69–64.0%), exhibited comparable rates. The remaining three media either did not form callus (IB) or quickly entered rooting (IA and IC) (Fig. 1B). The dedifferentiation rates are recognized as an evaluation factor because the longer time of maintenance for callus status after chloroplast transformation and the more probability of accumulation of homoplasmic chloroplasts are expected as there are many copies of chloroplasts in individual plant cells. It has been



**Fig. 1** Callus induction and regeneration from cotyledon explants of three rapeseed cultivars on different media. **A** Callus phenotypes induced on six induction media of cultivar FY-4. (a) Callus on medium IA. (b) Callus on medium IB. (c) Callus on medium IC. (d) Callus on medium ID. (e) Callus on medium IE. (f) Callus on medium IF. **B** Comparison of dedifferentiated calli grown on six different media. **C** Comparison of regeneration rates on three different media. Each value of percentages in Figs. **B** and **C** was the average calculated from three independent replications with standard errors. Cultivars FY-1 (black column); FY-4 (white column); FY-10 (grid column). Bar represents 5 mm. Photos were taken 4 weeks after culture

shown that a prolonged culture period of the callus generated homoplasmic transgenic chloroplasts in cauliflower (Nugent et al. 2006). Thus, the medium IE appeared optimal for the induction and maintenance of calli from cotyledonary explants of rapeseed plants. This medium (IE) was subsequently used for callus induction from cotyledon explants of rapeseed plants for chloroplast transformation.

After culture for 4 weeks on the medium IE, calli were transferred onto three different shoot regeneration media containing varied combinations of 6-BA, ZT, and KT. The results indicated that B5 medium supplemented with 1 mg/l 6-BA, 1 mg/l KT and 0.5 mg/l NAA (RC) produced the highest rate of shoots within four weeks (Fig. 1C). On this medium, all the three rapeseed genotypes displayed the highest regeneration rates, with FY-4 being 88.72% that is significantly higher than FY-1 (31.25%) and FY-10 (59.26%) (Fig. 1C). This RC medium was used for regeneration of transplastomic rapeseed plants. Based on the two comparative analyses of callus formation and regeneration, the rapeseed cultivar FY-4 consistently displayed the highest rate among three genotypes (Fig. 1B, C) and thus was chosen to produce explants for chloroplast transformation.

#### Establishment of a selection protocol

To develop an efficient system for rapeseed chloroplast transformation, an efficient selection protocol should be established firstly. Thus, the appropriate antibiotics (spectinomycin or streptomycin) and concentrations were determined for the selection of rapeseed cotyledonary tissues by culture on media containing various concentrations of spectinomycin and streptomycin. Rapeseed tissues appear to be naturally sensitive to spectinomycin and display bleaching with severe inhibition of shoot formation at a concentration of 10 mg/l spectinomycin for 4 weeks (data not shown). This concentration was used to select the transplastomic rapeseed cotyledonary explants during the first 2 months and then increased from 10 to 20 mg/l spectinomycin for the subsequent selections.

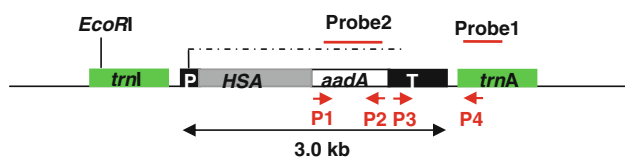
#### Construction of rapeseed chloroplast transformation vector

The rapeseed chloroplast transformation vector pCL308 targets the expression cassette into the *trnI-trnA* region of the rapeseed chloroplast genome for integration via homologous recombination. The *trnI* and *trnA* regions of the rapeseed chloroplast genome were selected for the integration. Due to the absence of rapeseed chloroplast genome sequence, *Arabidopsis thaliana* chloroplast genome was used to design *trnI* and *trnA* primers. PCR amplification of a rapeseed chloroplast genome DNA yielded two 1.5 kb fragments, *trnI* and *trnA*, which display 93 and 99% similarity to the homologs of the *Arabidopsis thaliana*, respectively. By restriction enzyme digestion, the rapeseed *trnI* fragment as left border and the *trnA* fragment as right border were constructed into pBluescript SK(–) to form an intermediate plasmid pCLLR303. The *aadA* gene expression cassette contains a 16S rRNA promoter linked

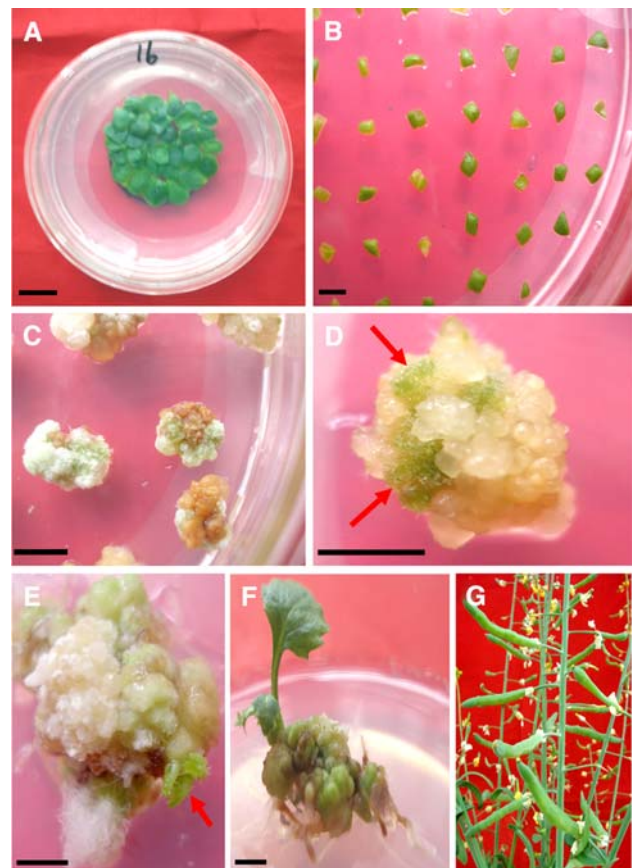
to the 5' untranslated region (UTR) of the bacteriophage T7 gene 10L (Ye et al. 2001) and a tobacco *psbA* terminator. Use of heterologous regulatory sequences limits recombination between introduced and native plastid regulatory sequences (Iamtham and Day 2000). The *aadA* gene encodes an aminoglycoside 3'-adenyltransferase, conferring resistance to spectinomycin and streptomycin. A synthesized *HSA* gene sequence was located before the selection marker *aadA* gene in the cassette and will be used for the expression of HSA protein in rapeseed chloroplast for the next step. The expression of *HSA* gene was not evaluated as the *aadA* gene was the focus of this study to establish the method for the chloroplast transformation in rapeseed plants. Both the *HSA* and *aadA* genes were located in a single expression cassette giving rise to a dicistronic transcript. The entire expression cassette, 3.0 kb, was cloned as an *SpeI-EcoRV* fragment between the *trnI* and *trnA* regions into pCLLR303, generating the vector pCL308 (Fig. 2) for the rapeseed chloroplast transformation.

#### Transformation of rapeseed chloroplasts and recovery of transplastomic plants

For selection of spectinomycin-resistant tissues, the bombarded cotyledons were cut into  $0.3 \times 0.3$  cm explants 2 days after bombardment and approximately 80 explants were placed per Petri dish (Fig. 3b) on B5 medium supplemented with 0.6 mg/l 2,4-D, 0.2 mg/l KT and 10 mg/l spectinomycin for callus induction. After 6–8 weeks of culture, spectinomycin-resistant calli started to grow (Fig. 3c, d). During the second round of selection, the shoot-producing spectinomycin-resistant calli were transferred to a shoot regeneration medium containing 15 mg/l spectinomycin and maintained for 4 weeks, generating the resistant green shoots (Fig. 3e, f). The regenerated shoots



**Fig. 2** Partial map of rapeseed chloroplast vector pCL308 and regions involved in homologous recombination. The two green boxes are homologous recombination sequences *trnI* and *trnA*. The *HSA* gene and *aadA* gene was in tandem array under control of the same *rrn* promoter (P) and *psbA* terminator (T). The red arrows with P1, P2, P3 and P4 are the regions for primer annealing for PCR amplification of the corresponding regions. A 600-bp fragment derived from *trnA* was used as probe 1 for the first Southern blot analysis, and the 800-bp whole sequence of the *aadA* gene was used as probe 2 for the second Southern blot and Northern blot analysis as described in Figs. 5 and 6, respectively. The 3.0 kb arrow line indicates the entire expression cassette



**Fig. 3** Generation of transgenic rapeseed plants after bombardment. **a** 4-day-old rapeseed cotyledons were used for chloroplast transformation by biolistic bombardment. **b** Bombarded cotyledonary tissues were cut into small pieces ( $9 \text{ mm}^2$ ) that were cultured on media containing 10 mg/l spectinomycin. **c, d** Transplastomic calli induced on selection media with green resistant calli for 6 weeks after bombardment. **e, f** Plantlets regenerated from transplastomic calli for 12 and 16 weeks after bombardment, respectively. **g** Growth phenotypes of transplastomic plants containing the *aadA* gene. Red arrows indicate the green tissues that later developed into shoots. Bar represents 5 mm

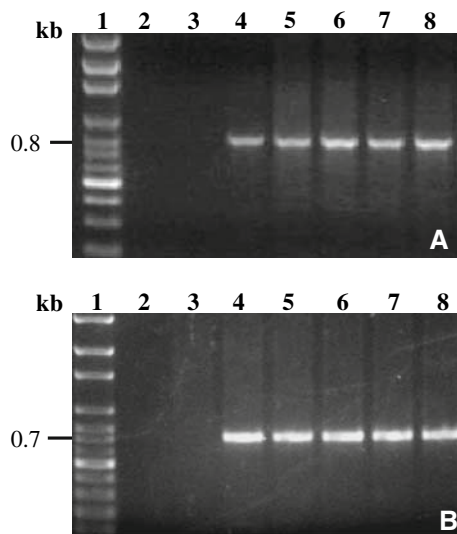
from the resistant calli were transferred to rooting medium supplemented with 20 mg/l spectinomycin for 2 weeks. Transplastomic rapeseed tissues with roots resistant to 20 mg/l spectinomycin were selected for transferring onto B5 basic medium for full plantlet development.

#### Cassette integration analysis

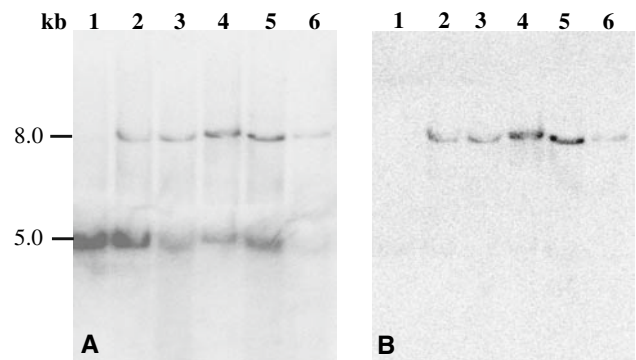
After serial selections, 73 resistant plants were obtained from 82 bombarded plates. To ascertain whether the cassette containing the *aadA* gene was integrated into the expected sites of the rapeseed chloroplast genome through homologous recombination, the spectinomycin-resistant shoots were initially screened by PCR. Based on the chloroplast transformation vector pCL308, the recombination events were expected to occur between the left and

right border sequences of the expression cassette. Thus, the first PCR with the primers P1 and P2 (Fig. 2) should detect the entire *aadA* gene sequence. As shown in Fig. 4a, an 800 bp PCR fragment was amplified from 19 samples among the 73 plants (results from 5 samples are shown), suggesting that the *aadA* gene was inserted into the rapeseed plants. A second PCR with the primers P3 (annealing to the 3' region of the *aadA*) and P4 (annealing to the 5' sequence of the *trnA*) revealed a 650 bp product in the above-PCR positive plants (Fig. 4b), further suggesting the presence of the cassette between the *trnI* and *trnA* regions in the rapeseed plant DNA samples, as expected. All the 19 PCR-positive plants were transferred into the soil and grown into maturity. All the transformed plants were fertile and set seeds.

To further verify proper integration of the PCR-positive transgenic plants, Southern blot analyses were deployed with two different probes. Total leaf DNA isolated from all 19 PCR-positive transplastomic plants after digestion with *EcoRI* was used for Southern blot hybridization first with part of *trnA* sequence (probe 1, Fig. 2). An *EcoRI* site is located upstream of left homologous recombination region revealed by our sequence analysis (data not shown; Fig. 2). The results showed that all the plants including the wild type one showed a fragment at about 5.0 kb position (Fig. 5a, lane 1), whereas all the PCR-positive transgenic



**Fig. 4** PCR analysis of transgene integration into the rapeseed chloroplast genome. PCR products were amplified from transplastomic plants and analyzed on a 1.2% agarose gel by electrophoresis. **a** Analysis of the *aadA* gene in transformed rapeseed plants using primers P1 and P2 showing an 800-bp DNA fragment. Lane 1 DNA ladder, Lane 2 water negative control, Lane 3 wild type plant, Lanes 4–8: transplastomic rapeseed plants. **b** Analysis of the expression cassette in transformed rapeseed plants using primers P3 (annealing to the 3' of the *aadA* gene) and P4 (annealing to the 5' *trnA*) yielded a 650 bp PCR fragment. Samples in different lanes were as described in **a**

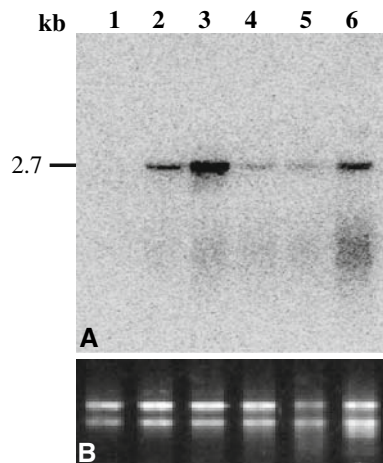


**Fig. 5** Analysis of the chloroplast vector cassette in transformed rapeseed by Southern blot hybridization. **a** The  $\alpha$ -[ $^{32}$ P]-dCTP-labeled *trnA* fragment was used as a probe 1 in the hybridization, resulting in a 5.0 kb hybridization signal from wild type plants and a 8.0 kb hybridization signal from transplastomic plants. Lane 1 wild type plant, Lanes 2–6: transplastomic plants. **b** The  $\alpha$ -[ $^{32}$ P]-dCTP-labeled *aadA* gene sequence was used as the probe 2 in the hybridization on the same membrane after stripping off the probe 1. The transplastomic lines yielded an 8.0 kb hybridization signal and the wild type had no signal

plants had an additional hybridization signal at the position of 8.0 kb (Fig. 5a, lanes 2–6, showing an example from five plants) because of the insertion of the cassette containing the *aadA* gene and the *HSA* gene within the region. These results demonstrated that the transgenic plants indeed carried the cassette integrated into the expected, specific site of the chloroplast genome, and the transgenic plants were heteroplasmic. To further corroborate the presence of the *aadA* gene in the chloroplast genome, the same membrane after stripping off the probe 1 was hybridized with the *aadA* sequence (probe 2, Fig. 2) under the same conditions. Figure 5b shows that a hybridization signal was detected only in the transplastomic plants at the expected position but not in the wild type plants, demonstrating the integration of the *aadA*-containing cassette into the rapeseed chloroplast genome.

#### The *aadA* gene expression analysis

To confirm that the *aadA* gene that is located at the 3' portion of the cassette was expressed in the transgenic plants, Northern blot analyses were carried out with the *aadA* gene as a probe. Total leaf RNA was extracted from 6 to 10 leaves of 3-week-old plants after they grown in the greenhouse from all the 19 transplastomic plants identified by PCR and Southern blot analyses and used for Northern blots. As shown in Fig. 6, the transgenic plants tested showed a hybridization signal at the position of 2.7 kb and an example from five transgenic plants is presented (Fig. 6, lanes 2–6). No signals were detected in the wild type plants (Fig. 6, lane 1). The single hybridization band with an expected size revealed the presence of a dicistronic mRNA

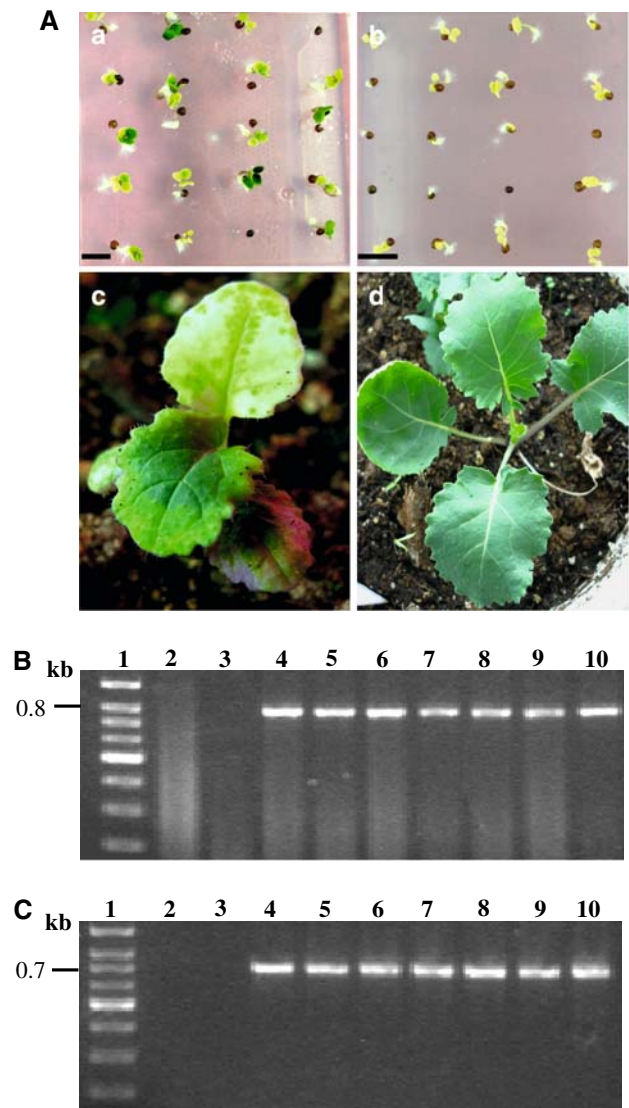


**Fig. 6** Northern blot analysis of *aadA* mRNA in the transplastomic plants. **a** A  $\alpha$ -[ $^{32}$ P]-dCTP-labeled *aadA* sequence used as a probe 2 in Fig. 5 was used in this hybridization. Lane 1 wild type plant, Lanes 2–6 transplastomic plants. **b** Ethidium bromide-stained gel showing comparatively equal loading of different RNA samples

transcribed from both *HSA* and *aadA* genes in tandem array regulated by the same set of promoter and terminator in the cassette. These results indicated that the *aadA* gene that is located at the 3' region within the integrated cassette was properly transcribed in the chloroplast genome. In the bottom of the panel, the rRNA samples from different plants displayed comparable intensity as shown by ethidium bromide staining, indicating relatively equal loading of different RNA samples. The different intensity of signals in the Northern blot may reflect the variation of the expression levels of the *aadA* gene that might be related to the copy number in their chloroplast genomes and leaf development stage.

#### Inheritance of the transplastomic *aadA* gene

To reveal whether the transgene in the heteroplasmic rapeseed plants can be inherited into the next generation, three T1 transplastomic rapeseed lines was investigated. The results showed a clear phenotypic and growth differences between the transplastomic plants and wild type controls. Most of the T1 transplastomic seeds germinate and showed green cotyledons whereas all seeds from non-transformed wild type controls either did not germinated or become yellow in 3 days under the same conditions (Fig. 7A, a, b). A small proportion (8–15%) of seeds from the transplastomic plants did not germinate or turned yellow, which implies a segregation of the heteroplasmic nature of the T1 plants. The transplastomic seedlings grown in soil displayed variegated green color of leaves with sectoring of green and yellow (Fig. 7A-c), reminiscent of a heteroplasmic character in plants. Heteroplasmic



**Fig. 7** Analysis of T1 transplastomic rapeseed plants. **A** Seed germination and seedling growth of transgenic plants (**a**) and the wild type plant (**b**) on medium containing 20 mg/l spectinomycin for 3 days, Seedlings of T1 transgenics (**c**) and wild type plant (**d**) grown in soil for 14 days in a greenhouse. **B**, **C** PCR analyses of transgenic plants with two *aadA* gene-specific primers (**B**) and two primers for the right homologous recombination region (**C**); the sizes of the resulting PCR products are as described in Fig. 4. Lane 1 DNA ladder, Lane 2 water negative control, Lane 3 wild type plant, Lanes 4–6 T1 transplastomic plants from line 1; Lanes 7–10 T1 plants from lines 2 and 3 (two from each), respectively. Bar represents 5 mm

*B. napus* seedlings exposed to spectinomycin would be expected to give rise to variegated plants as a result of plastid ribosome-loss in cells containing predominantly wild type plastids (Zubko and Day 1998). PCR analyses of seven T1 transplastomic plants revealed an 800 bp fragment of the *aadA* gene with the primers P1 and P2 (Fig. 7B) and a 650 bp DNA product derived from the right homologous recombination region with the primers P3 and



P4 (Figs. 2, 7C). These results suggested that the transgenes integrated into the chloroplast genomes in rapeseed plants were indeed inheritable.

## Discussion

A callus induction and regeneration system derived from cotyledonary tissues of elite rapeseed cultivars was developed and successfully used for rapeseed chloroplast transformation. The transformation efficiency was high with one transplastomic plant per four biolistic bombarded plates. This may represent the first known report of rapeseed chloroplast transgenic plants with molecular evidences for proper integration of the introduced cassette, expression of the *aadA* gene and its inheritance to the next generation in the transplastomic rapeseed plants.

The optimized conditions for callus induction and regeneration with green tissues derived from cotyledons appeared important for the high efficiency of chloroplast transformation. High copy number in the chloroplast genome requires a long period of culture to allow accumulation of more transgenic copies in the genome and to generate transgenic homoplasmy of the chloroplasts. B5 medium that was optimized for efficient regeneration from rapeseed leaves (Akasaka-Kennedy et al. 2005) was chosen as a basal medium for the culture of cotyledon explants in this study. However, the cotyledonary calli cultured on this medium developed quickly into shoot regeneration (Fig. 1A-a, B-IA) and appeared not suitable for chloroplast transformation. In addition, the cotyledon explants cultured on B5 medium supplemented with 0.04% MES omitting phytohormones formed almost no callus (Fig. 1A-b, B-IB) although the addition of 0.04% MES into MS medium yielded homoplasmic plants of *Brassica oleracea* L. var. *capitata* L (Liu et al. 2007; 2008). Thus, different species within *Brassica* seem to display substantial variations in response to culture conditions and the individual species may require an optimized culture regime for their chloroplast transformation.

Initial experiments indicated that plant growth regulator 2,4-D was important for maintaining callus stage without further shoot differentiation, however, the callus turned into yellow and lacked regeneration potential. Further investigations revealed that a combination (medium IE, Fig. 1B) of 2,4-D (0.6 mg/l) and KT (0.2 mg/l) were optimal for callus maintenance for a long period (8 weeks) for cotyledon-derived tissues in rapeseed, which retained its capacity for regeneration into shoots and roots on B5 medium supplemented with 1 mg/l 6-BA, 1 mg/l KT and 0.5 mg/l NAA (medium RC, Fig. 1C). The combination of the callus induction medium IE and the regeneration medium RC, together with green tissues as target explants,

seems important for the high efficiency of the chloroplast transformation. In addition, genetic variation appeared more profound in regeneration (Fig. 1C) than callus induction (Fig. 1B). A previous report suggested that small numbers of chloroplasts in cotyledon petioles may have an effect on the low efficiency of chloroplast transformation in rapeseed, which showed no detection of the genome integration for the expression cassette by Southern blot analysis (Hou et al. 2003). In addition, the cotyledon petioles of rapeseed plants were cultured in MS medium containing 6 mg/l 6-BA for callus induction and regeneration, which varied substantially on medium compositions compared to that deployed in this study. Therefore, the culture conditions and green tissues in cotyledons carrying more chloroplasts used in this study are apparently more suitable regimes for chloroplast transformation than the culture conditions and cotyledon petioles in rapeseed used by Hou et al. (2003). This appears in accordance with those reported in tobacco (Scotti et al. 2009; Soria-Guerra et al. 2009; Svab and Maliga 1993), *Arabidopsis* (Sikdar et al. 1998), cauliflower (Nugent et al. 2006) and cabbage (Liu et al. 2007, 2008).

The regions selected for homologous recombination in this study were *trnI* and *trnA* that were amplified directly from the rapeseed cultivar FY-4 with primers designed based on the chloroplast genome sequence of *Arabidopsis thaliana*. These regions have been used in chloroplast transformation of other plants (De Cosa et al. 2001; Kumar et al. 2004a, b; Lee et al. 2006) and are different from a previous report that used *rps7* and *ndhB* regions in their rapeseed transformation as they obtained no evidence for expression cassette integration by Southern blot analysis (Hou et al. 2003). Southern blot analysis performed in this study confirmed proper integration of the cassette into the rapeseed chloroplast genome (Fig. 5) with a heteroplasmy. Nugent et al. (2006) reported the generation of homoplasmic chloroplast transformation of cauliflower with the *accD-rbcL* regions for homologous recombination, albeit a low efficiency that was probably due to a sub-optimal protoplast division frequency and thus insufficient recovery of rare transformation events. The high efficiency of chloroplast transformation from this study suggests that the rapeseed-specific *trnI* and *trnA* sequences constructed in the pCL308 vector are suitable regions for homologous recombination in rapeseed, generating proper integration events in rapeseed tissues upon bombardment. These regions have been used to generate homoplasmic plants of cabbage (Liu et al. 2007, 2008). Various reports have shown that species-specific vectors contributed to efficient plastid transformation in carrot, cotton and soybean (Dufourmantel et al. 2004; Kumar et al. 2004a, b). The current transformation efficiency, one plant per four bombarded plates, is comparable to that seen in tobacco generating one

plant per bombarded plate (Svab and Maliga 1993) but greatly higher than that observed in other plants. For instance, one transplastomic plant was generated from 20 bombarded plates in tomato (Ruf et al. 2001), 35 plates in potato (Sidorov et al. 1999) and even 100 plates in *Arabidopsis* (Sikdar et al. 1998), respectively. Heteroplasmic transgenic plants obtained in this study may be due to low antibiotics selection pressures and conditions used. This report describes the development of a method for rapeseed chloroplast transformation with cotyledons as explants. Further investigation will focus on the optimization of selection for homoplasmic plants and characterization of target gene expression.

The inheritance of the generated transgenic heteroplasmic plants in rapeseed was illustrated by typical phenotypic characters of T1 transgenic plants grown in medium and soil in comparison with the wild type controls (Fig. 7A). The observation that a small proportion of T1 transgenic seeds did not germinate and a few seedlings turned yellow under spectinomycin selection pressure (Fig. 7A, a, b) suggested a genetic segregation of heteroplasmic *addA* gene in their chloroplast genomes. This apparently reflects the maintenance of the heteroplasmy nature in the plants as shown by PCR and Southern blot analyses in their previous generations (Figs. 4, 5). The molecular basis of the transgenes in the survived seedlings was revealed through PCR analysis with gene-specific primers (Fig. 7B, C), suggesting that the transgenes in the chloroplasts are inheritable as soon as they are integrated into the genome via homologous recombination. Through prolonged culture time as described for cauliflower plants (Nugent et al. 2006), it might be possible to generate transgenic homoplasmy of rapeseed plants using the method developed in this study. More stringent conditions for spectinomycin selection would also facilitate this homoplasmic selection.

The present results demonstrate optimized culture conditions for cotyledonary tissues and methods for generation of transplastomic plants from an elite rapeseed cultivar with high efficiency. This chloroplast transformation method may serve as the basis of a method to investigate further integration of genes into the chloroplast genome of rapeseed to breed cultivars with improved agronomic characters.

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