A two-step protocol for shoot regeneration from hypocotyl explants of oilseed rape and its application for *Agrobacterium*-mediated transformation

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Abstract

A two-step protocol for improving the frequency of shoot regeneration from oilseed rape (*Brassica napus* L.) hypocotyl explants was established. The protocol consists of a pre-culture on callus induction medium (CIM) and a subsequent shoot regeneration on shoot induction medium (SIM). The SIM was Murashige and Skoog medium supplemented with different concentrations of 6-benzylaminopurine (BA; 2 - 5 mg dm^{-3}) and naphthaleneacetic acid (NAA; 0.05 - 0.15 mg dm^{-3}). Maximum frequency of shoot regeneration (13 %) was on the SIM medium containing 4 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA, but it increased to 24.45 % when 20 μM silver thiosulphate (STS) was added. Strikingly, an extremely high frequency of shoot regeneration up to 96.67 % was reached by a two-step protocol when hypocotyl explants had been pre-cultured for 7 d on a CIM medium containing 1.5 mg dm^{-3} 2,4-dichlorophenoxyacetic acid. In addition, the shoot emergence was also 7 d earlier than that observed by use of the one-step protocol. The two-step protocol was also applied for regeneration of transgenic plants with *cZR-3*, a nematode resistance candidate gene. As a result, 43 plants were generated from 270 shoots and from these 6 plants proved to be transgenic.

Additional key words: auxins, benzylaminopurine, *Brassica napus*, callus induction, plant tissue culture.

Introduction

Oilseed rape (*Brassica napus* L.) produces highly valuable oil for human nutrition as well as for biofuels. It is also getting more and more importance as a model crop plant for functional genome analysis. For instance, more than 70 % gene transformation experiments reported in *Brassica* so far were either for identification or for functional analysis of novel genes (Sparrow *et al*. 2004).

The *Agrobacterium*-mediated transformation had been widely applied for generation of transgenic plants of various *Brassica* species including oilseed rape (Cardoza and Stewart 2003, Lee *et al*. 2004, Cho *et al*. 2008), but the efficiency strongly relies on shoot regeneration frequency (Akasaka-Kennedy *et al*. 2005). Generally, there are two strategies leading to shoot regeneration: one is *via* somatic embryogenesis and the other one is *via* organogenesis (Hanson and Wright 1999, Dibax *et al*. 2010). In case of oilseed rape, various explants can be used for shoot regeneration, including cotyledon (Narasimhulu and Chopra 1988, Tang *et al*. 2003), hypocotyl (Khehra and Mathias 1992, Tang *et al*. 2003, Jonoubi *et al*. 2005), flowering internodes (Klimaszewska and Keller 1985, Tang *et al*. 2003), stem sections (O'Neill *et al*. 1996), immature cotyledons (Turgut *et al*. 1998), leaves (Akasaka-Kennedy *et al*. 2005) and as well thin cell layer (Shu and Loh 1991, Ghnaya and Charles 2008).

Cotyledon explants of oilseed rape were most frequently used for shoot regeneration because they are easy to regenerate (Tang *et al*. 2003). However, the transformation efficiency of cotyledon explants by *Agrobacterium*-mediated transformation is extremely low. For instance, Mukhopadhyay *et al*. (1992) reported that 2 chimeric transformed shoots could be obtained from

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Abbreviations: BA - 6-benzylaminopurine; CIM - callus induction medium; CTAB - cetyltrimethyl ammonium bromide; 2,4-D - 2,4-dichlorophenoxyacetic acid; GUS - β-glucuronidase; MS - Murashige and Skoog; NAA - naphthaleneacetic acid; SIM - shoot induction medium; STS - silver thiosulphate; X-Gluc: 5-bromo-4-chloro-3-indolyl β-D-glucuronide.
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more than 10 000 cotyledons treated by *Agrobacterium*. Besides, the transformation efficiency was strongly genotype-dependent (Sparrow *et al*. 2004). In contrast, hypocotyl explants of oilseed rape are easily prepared and as well transformed by *Agrobacterium*. But, they often suffered from poor shoot regeneration frequency (Mukhopadhyay *et al*. 1992).

In this paper, we report a two-step protocol, which can significantly improve shoot regeneration frequency from hypocotyl explants. Its potential for generation of transgenic plants by *Agrobacterium*-mediated transformation is demonstrated.

Materials and methods

Semi-winter type oilseed rape (*Brassica napus* L. cv. Zheshuang 758), kindly provided by the Zhejiang Academy of Agricultural Science, P.R. China, was used for shoot regeneration and *Agrobacterium*-mediated transformation experiments. Seeds were first surface-sterilized in 70 % ethanol for 0.5 min and then submerged in 10 % calcium hypochlorite including 2 drops of *Tween-20* for 25 min, and then rinsed 4 to 5 times with sterilized distilled H_2O. The seeds were sown in 150 cm^3 triangular flask containing 50 cm^3 half-strength Murashige and Skoog (1962; MS) phytohormone-free germination medium supplemented with 20 g dm^{-3} sucrose and 8 g dm^{-3} agar (pH 5.8). Phytohormones, MS basal salts and antibiotics were purchased from *Sigma* (Hamburg, Germany). All media were sterilized by autoclaving at 120 °C for 20 min. Phytohormones, silver thiosulphate (STS) and antibiotics were filter-sterilized by passing through a 0.2 μm syringe filter and added to the cooled (70 °C) autoclaved media.

Shoots were regenerated by use of one- and two-step protocol, respectively. By one-step protocol, hypocotyl segments (5 - 10 mm) from 5-to 6-d-old seedlings were cultured only on the MS medium supplemented with different combinations of 6-benzylaminopurine (BA; 2 - 5 mg dm^{-3}) and naphthalene acetic acid (NAA; 0.05 - 0.15 mg dm^{-3}) with or without 20 μM STS (Table 1). By two-step protocol, hypocotyl segments were first pre-cultured on callus induction medium (CIM) complemented with 2,4-dichlorophenoxyacetic acid (2,4-D; 0.5 - 1.5 mg dm^{-3}) for 3 or 7 d and then transferred onto the shoot induction medium (SIM) with 4 mg dm^{-3} BA and 0.10 mg dm^{-3} NAA with or without 20 μM STS solution (Table 2). In all cases, ten explants were placed in one 90 mm Petri dish and at least forty explants were prepared. Cultures were incubated in a culture room at temperature of 25 ± 1 °C and 16-h photoperiod (irradiance of 100 μmol m^{-2} s^{-1}). After 3 - 4 weeks of culture, the adventitious shoots that formed on the explants were counted. Shoot regeneration frequency [(number of explants with shoots / total number of explants) × 100 %] was averaged from 3 to 4 replicates. *SPSS 11.0* software (*SPSS Inc*., Chicago, USA) was used for statistical analysis.

For plant transformation, a recombinant binary vector pAMcZR-3 was used, which contains the full-length cDNA of *cZR-3* (Tian *et al*. 2004), a sugar beet resistance gene candidate (accession number: DQ907613). In addition, the binary vector carries the GUS reporter gene and a selectable marker, neomycinphosphotranferase gene (*nptII*) for kanamycin resistance. The plasmid DNA of pAMcZR-3 was transformed into *Agrobacterium* strain GV3101 by electroporation (Shen and Forde 1989). The hypocotyl segments (5 - 10 mm) were directly immersed in bacterial suspension with gentle shaking for 10 min. The segments were subsequently blotted on sterile filter paper and transferred in the 90 mm Petri dish containing fresh CIM to co-culture with bacteria for 2 d. After co-cultivation the segments were washed 3 times with liquid CIM containing 500 mg dm^{-3} carbenicillin to kill the excessive *Agrobacterium*. The segments were blotted on the sterilized filter paper and placed on the CIM containing the antibiotic for 5 d. After, the segments were transferred on the new SIM containing 500 mg dm^{-3} carbenicillin and 50 mg dm^{-3} kanamycin for selection till the putative green shoots appeared. The whole segments including green shoots were transferred onto solid half-strength MS medium combined with 10 g dm^{-3} sucrose, 9 g dm^{-3} agar and antibiotics for shoot elongation. After 3 weeks, the green shoots were transferred on the same shoot elongation medium to develop the roots. The plantlets were transferred to soil in greenhouse and vernalized at 4 °C for 40 d. The plants were grown in plastic bags until the flowering.

For histochemical GUS assays, 10 cm^3 50 mM Na_3PO_4 buffer (pH 7.0), 0.2 mg cm^{-3} 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) and two drops of *Triton-100* was added in the Petri-dish which contained the putative transgenic and negative control leaves. The leaves were covered with X-Gluc solution and incubated at 37 °C for 16 h. After it, the leaves were cleared using 70 % ethanol to fade the chlorophyll. The GUS staining signals were evaluated under a stereomicroscope (*Stemi SV 11*, *Zeiss*, Jena, Germany).

For polymerase chain reaction (PCR) the genomic DNA from young leaf tissue was extracted using CTAB method as described by Rogers and Bendich (1985). The gene- specific primers used for amplification are: cZR-3A: 5'-AGTTATTGATAGGGCTATGG-3' and cZR-3 B: 5'-ATACTTGAAGCAGTCAGG-3' resulting in a fragment (cZR3-A/B) of 410 bp in size (Lein *et al.* 2007). PCR was performed as follows: DNA denaturation at 94 °C for 3 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 53 °C and 1.3 min at 72 °C. A final extension cycle was at 72 °C for 10 min. The PCR products were visualized by electrophoresis on a 1.2 % agarose gel stained with ethidium bromide. For Southern blot analysis,

50 ng genomic DNA was restricted by restriction enzymes at 37 °C for 5 h. The digested DNA was fractioned on 0.75 % agarose gel and transferred onto a *Hybond-N*$^+$ membrane (*GE Healthcare*, Munich, USA) by capillary diffusion blotting with 0.25 M NaOH/1.5 M NaCl blotting solution overnight. Southern blots were hybridized using ^{32}P-labelled cZR-3 A/B DNA fragment as probe according to Feinberg and Vogelstein (1983) at 62 °C overnight. The blots were washed twice with 0.5× SSC, 0.2 % (m/v) sodiumdodecyl sulphate (SDS) for 30 min and together with the film exposed at -70 °C for 48 h.

Results

The MS medium was supplemented with different concentrations of BA (2 to 5 mg dm^{-3}) and NAA (0.05 to 0.15 mg dm^{-3}) and resulted shoot regeneration and regeneration frequencies were calculated (Table 1). Explants began to be swollen after being cultured in the medium for 7 d (Fig. 1*A*,*B*) and first shoots appeared after 20 d (Fig. 1*C*). Callus formation from the tip of hypocotyl explants was observed. Although adventitious shoots could be observed from the cut end of hypocotyl explants 30 d after cultivation in most cases (Fig. 1*D*), frequencies of shoot regeneration from different media varied from 0 to 13 % (Table 1). Since the maximum shoot regeneration frequency (13 %) was given in the medium supplemented with 4 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA, it was used as a basal medium for further experiments.

To increase shoot regeneration frequency from oilseed rape hypocotyl explants, a two-step protocol for shoot regeneration was tested, in which two media CIM and SIM were used. Hypocotyl explants were first pre-cultured on a CIM medium containing 2,4-D (0.5 to 1.5 mg dm^{-3}) to induce callus formation for 3 or 7 d and then transferred on a SIM medium with 4 mg dm^{-3} BA, 0.1 mg dm^{-3} NAA as well as varied 2,4-D concentration and with or without STS. Three days pre-culture of hypocotyl explants on all CIM media tested did not improve of shoot regeneration frequency, whereas 7-d pre-culture resulted in a drastic increase in shoot regeneration frequency that ranged from 21.67 to 96.67 % (Table 2). An extremely high frequency of shoot regeneration was given in the case when hypocotyl explants had been 7 d pre-cultured on CIM containing 1.5 mg dm^{-3} 2,4-D and subsequently cultured on SIM supplemented with 20 μM STS (Table 2).

The shoot regeneration frequencies were significantly increased by addition of STS either by the one-step or by the two-step regeneration protocol. In case of one-step protocol, the shoot regeneration frequency was improved from 1.11 to 16.67 % on the medium with 2 mg dm^{-3} BA and 0.05 mg dm^{-3} NAA. The shoot regeneration frequency could further increase up to 24.45 % when the medium was supplemented by 4 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA in combination with STS (Table 1).

Table 1. Effect of BA and NAA concentrations on the shoot regeneration frequency from hypocotyl explants on SIM in one-step protocol after 30-d cultivation. Means ± SE of three replicates. Means followed by the same letter indicate no significant difference (Duncan's multiple range test, $P \leq 0.05$).

BA [mg dm^{-3}]	NAA [mg dm^{-3}]	Shoot regeneration frequency [%] -STS	+STS
2	0.05	1.11 ± 1.11^{a}	16.67 ± 3.33^{bcd}
3	0.05	7.22 ± 0.28^{abc}	24.45 ± 2.22^{d}
4	0.05	3.53 ± 0.23^{ab}	16.67 ± 3.33^{bcd}
5	0.05	10.42 ± 2.08^{abc}	6.67 ± 6.67^{abc}
2	0.1	6.67 ± 1.67^{abc}	19.45 ± 6.41^{cd}
3	0.1	2.22 ± 1.11^{a}	20.00 ± 3.85^{cd}
4	0.1	13.00 ± 1.00^{abcd}	24.45 ± 2.22^{d}
5	0.1	0.00^{a}	13.33 ± 1.82^{abcd}
2	0.15	7.78 ± 1.92^{abc}	15.56 ± 2.94^{bcd}
3	0.15	1.67 ± 1.67^{a}	8.89 ± 0.88^{abc}
4	0.15	6.67 ± 2.31^{abc}	10.00 ± 3.35^{abc}
5	0.15	0.00^{a}	0.00^{a}

Table 2. Effect of 2,4-D concentration, pre-cultivation time and supplement of STS on the shoot regeneration frequency from hypocotyl explants on MS medium containing 4 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA. Means ± SE of three replicates. Means followed by the same letter indicate no significant difference.

Pre-cult. time	2,4-D [mg dm^{-3}]	Shoot regeneration frequency [%] -STS	+STS
3 d	0.5	6.67 ± 0.77^{ab}	40.00 ± 17.32^{d}
	1.0	0.00 ± 0.00^{a}	22.33 ± 6.80^{bc}
	1.5	3.33 ± 0.77^{a}	21.67 ± 2.89^{bc}
7 d	0.5	26.67 ± 5.77^{cd}	57.33 ± 11.01^{e}
	1.0	0.00 ± 0.00^{a}	71.33 ± 14.43^{e}
	1.5	10.00 ± 0.32^{ab}	96.67 ± 5.77^{f}

Furthermore, the shoot induction was more rapid in the two-step protocol in combination with addition of 20 μM STS in the SIM medium. The explants started to be swollen 5 d after transferring on the SIM, callus appeared clearly around the cut end of hypocotyls and potential shoots emerged about 10 d after the transferring on the SIM. In average, the shoot emerging time was 7 d earlier compared to that observed by use of one-step protocol.

The two-step regeneration protocol was applied for generation of oilseed rape transgenic plants with *Agrobacterium*-mediated transformation (Fig. 2). Two days after co-cultivation hypocotyls on CIM with *Agrobacterium* cells, the segments were subjected to two-step protocol for regeneration. In total, 270 shoots

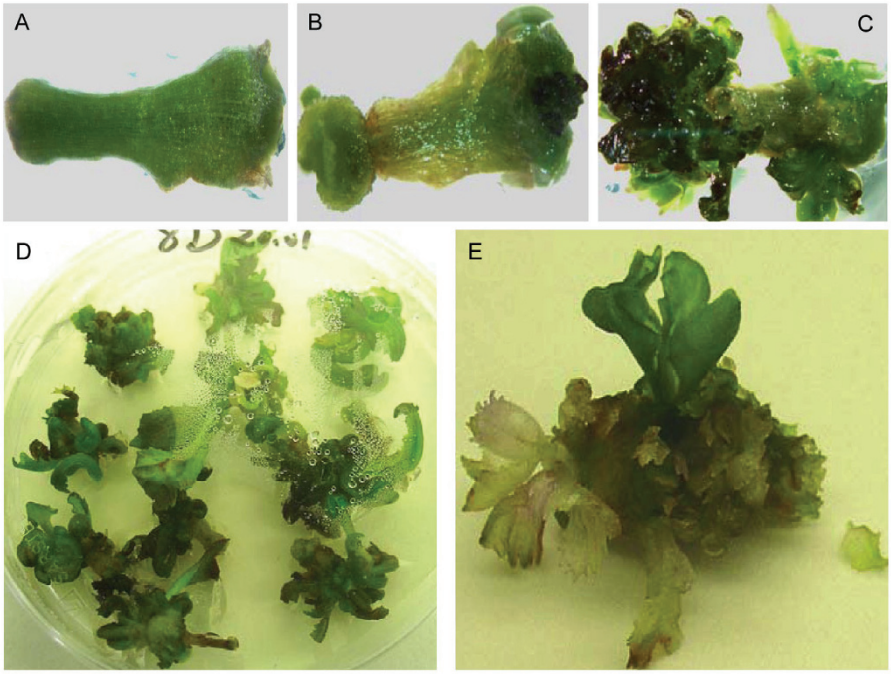

Fig. 1. Shoot regeneration from oilseed rape hypocotyl explants by a two-step protocol. Development of shoots regenerated from hypocotyl explants 5 d (*A*), 10 d (*B*), 20 d (*C*) and 30 d (*D*) after cultivation on SIM medium supplemented with 4 mg m^{-3} BA, 0.1 mg dm^{-3} NAA and 20 μM STS. The hypocotyl explants were pre-cultured for 7 d on the CIM medium with 1.0 mg dm^{-3} 2,4-D. For *Agrobacterium*-mediated transformation experiments, the vitrificated shoots (*E*) after cultivation in the medium containing 500 mg dm^{-3} carbenicillin and 100 mg dm^{-3} kanamycin sulphate were demonstrated.

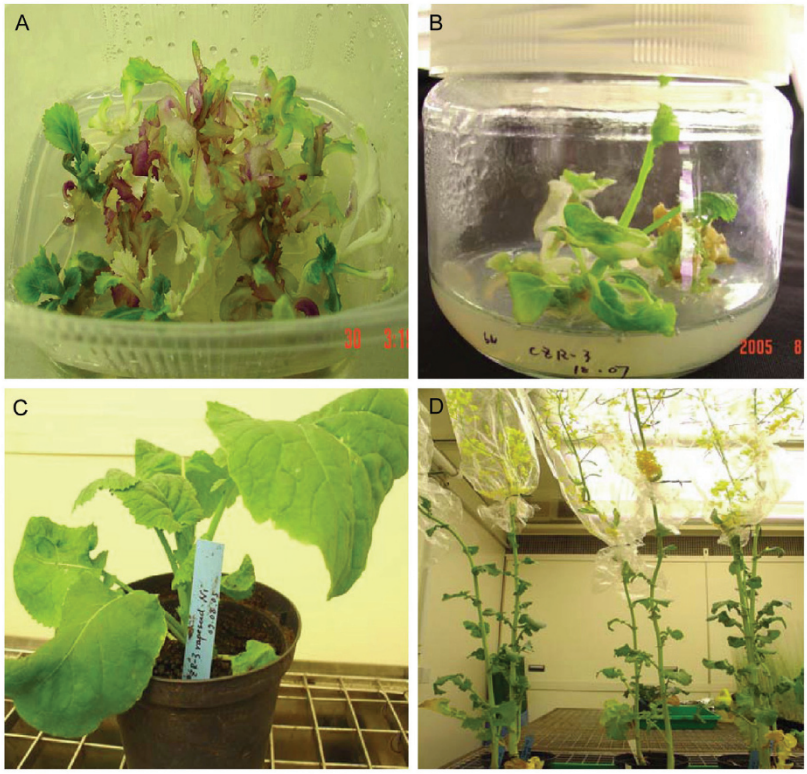

Fig. 2. Generation of transgenic plants from hypocotyl explants of *B. napus* cv. Zheshuang758. *A* - Shoots regenerated from hypocotyl explants on SIM containing 500 mg dm^{-3} carbenicillin and 50 mg dm^{-3} kanamycin sulphate; *B* - the elongated shoots on SIM, *C* - the transgenic plantlet transferred into the soil, *D* - transgenic oilseed rape plants in a growth chamber.

were regenerated, from which 43 putative transgenic plants were obtained (Fig. 2).

To verify the transgenic nature, all of 43 putative transgenic plants were subjected to histochemical GUS assays (Fig. 3*A*), to *cZR-3*-specific PCR (Fig. 3*B*) and as well to Southern-blot hybridization experiments using a *cZR-3*-specific DNA fragment as a probe (Fig. 3*C*). As a result, 6 of 43 plants (about 14 %) proved to be *cZR-3*-transgenic, giving a strong GUS staining, the cZR-3-specific PCR amplification and the *cZR-3*-specific Southern-hybridizing signal.

Discussion

In this paper, we reported a two-step shoot regeneration protocol for oilseed rape (*B. napus* L.) and demonstrated that the shoot regeneration frequency from hypocotyl explants of oilseed rape can be greatly improved by using this protocol. The maximum shoot regeneration frequency reached 96.67 %, and the shoot appearance was accelerated. We conclude that the duration of pre-cultivation on CIM and the supplementation with STS were, in addition to 2,4-D concentration, main factors influencing shoot regeneration frequency in our experiments. This result is in coincidence with observation made by Tang *et al*. (1999).

An important finding in this study is that addition of ethylene inhibitor STS into SIM medium increased shoot regeneration frequency either in one-step or two step protocols. A similar observation had been reported in different regeneration systems, *e.g*. with cotyledonary explants of *B. campestris* (Chi *et al*. 1991), hypocotyl explants of *B. juncea* (Pua and Chi 1993), cotyledon, leaves and thin cell layer explants of *B. napus* (Tang *et al*. 2003, Akasaka-Kennedy *et al*. 2005, Ghnaya and Charles 2008). In consistence with the observation of Khan *et al*. (2003), we observed that most of transformed shoots were necrotic on the medium not supplemented by STS (data not shown). The 2,4-D concentration in medium had been reported to have the promoting effect on the callus quantity whereas the callus quality is often influenced by the duration of pre-cultivation on CIM. Application of 2,4-D into the CIM can induce excessive callus growth during the subsequent shoot regeneration.

Even though the *Agrobacterium*-mediated transformation has been applied for rapeseed breeding and gene function analysis since Fry *et al*. (1987), most of transformation experiments on *B. napus* had been restricted to a few mostly spring genotypes (Cardoza and Stewart, 2003). We demonstrated in this paper that the two-step protocol can be successfully used for regeneration of transgenic plants from a semi-winter cultivar Zheshuang 758. About 14 % of plants regenerated by use of this protocol proved to be transgenic, demonstrating its potential in this context. However, comparing to a high shoot regeneration frequency with the two step protocol, the transformation efficiency obtained in this study was low. Only 2.2 % of regenerated shoots were transgenic. We found that some of explants regenerated in the selection medium containing 50 mg dm^{-3} kanamycin, but failed to develop when shoots were subjected to shoot

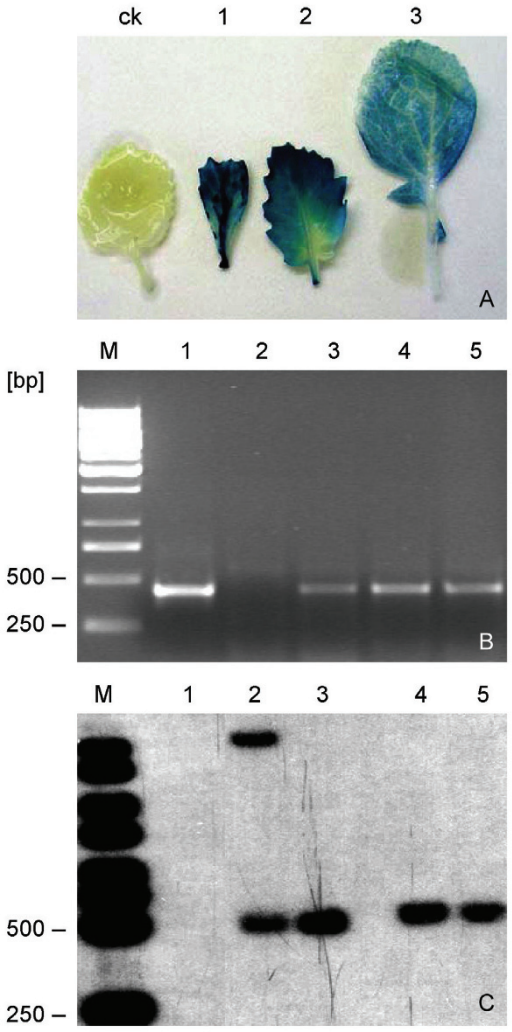

Fig. 3. Molecular characterization of transgenic oilseed rape plants. *A* - Histochemical GUS assays with leaves of an untransformed oilseed rape plant that served as a negative control (ck) and of transgenic oilseed rape plants (1, 2, 3); *B* - PCR analysis with *cZR-3*-specific primers with the *cZR-3*-recombinant plasmid DNA (1) that served as a positive control, an untransformed plant (2) that served as a negative control and transgenic oilseed rape plants (3, 4 and 5); *C* - Southern blot hybridization with an untransformed plant (1) that served as a negative control and transgenic oilseed rape plants (2, 3, 4, 5). The *cZR-3*-specific DNA fragment (A/B) was used as a probe.

elongation medium containing 100 mg dm^{-3} kanamycin. Obviously, they were not able to develop roots and therefore became vitrified (Fig. 1*E*). Thus, improvement of transformation efficiency for *B. napus* still remains a great challenge.

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