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An efficient *Agrobacterium*-mediated transformation method using hypocotyl as explants for *Brassica napus*

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Abstract Rapeseed (*Brassica napus*) is an important oil crop that supplies a considerable amount of global vegetable oil production. Genetic transformation system is important to gene functional analysis and molecular breeding. Here, an efficient *Agrobacterium*-mediated transformation protocol using hypocotyl of rapeseed as explants is described. To develop this protocol, we compared several essential factors that would affect the transformation efficiency, such as *Agrobacterium* strains, selection marker genes, and genotypes of rapeseed. Comparison of different *Agrobacterium* strains showed that the GV3101 had higher transformation efficiency than that of C58C1 and EHA105. *HPTII*, *NPTII*, and *RePAT* were used as selection marker genes in tissue culture. The results showed that the transformation efficiency was 3.7–4.8%, 2.2–22.5%, and 1.6–5.9% when the hypocotyl of Westar was infected by GV3101 and screened under hygromycin, kanamycin, and basta, respectively. The transformation efficiency of Westar was the highest and ZS11 was the lowest when five different genotypes of rapeseed (Westar, ZS9, ZS11, GY284, and WH3417) were infected by GV3101. Using this protocol, it will take 8–10 weeks to obtain transgenic plants. This protocol has been used to study gene function in several genotypes of rapeseed in our laboratory. These results indicate that it is efficient to obtain transgenic plant of rapeseed using this protocol.

Keywords Rapeseed · Transformation method · Hypocotyl · Agrobacterium · Transformation efficiency

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Introduction

Rapeseed (*Brassica napus*, AACC, 2n = 38) is one of the most important oil crops and it produces approximately 15% of edible oil globally (Gracka et al. 2016; Yu et al. 2016). It originates from a spontaneous hybridization between *Brassica rapa* (AA, 2n = 20) and *Brassica oleracea* (CC, 2n = 18) (Chalhoub et al. 2014; Rahman et al. 2017). Conventional breeding of rapeseed is labor resource intensive and time consuming, which takes eight to ten generations to develop a new variety (Mason and Snowdon 2016). Genetic transformation technology is a key tool to study gene function and provides new genetic resource for molecular breeding, such as herbicide and disease resistance that is hardly achieved by conventional breeding (Bhalla and Singh 2008). Moreover, most traits introduced by gene-transfer method are dominant. Thus, an efficient gene-

transfer system suitable for different genotypes of rapeseed would shorten the length of downstream breeding process, and develop a commercial product when using agronomic inferiority model varieties. Additionally, this would help reveal gene function in rapeseed.

Many DNA-transfer methods have been explored for plant transformation, including PEG-mediated DNA uptake, electroporation, microinjection, and particle bombardment (Poulsen 1996). PEG-mediated or electroporation-mediated protoplasts transformations have been attempted in *Arabidopsis*, *B. oleracea*, and *B. napus* (Bergman and Glimelius 1993; Eimert and Siegemund 1992; Mukhopadhyay et al. 1991; Radchuk et al. 2002; Yoo et al. 2007). However, regeneration of plant from the protoplast of *Brassica* is genotype dependent (Hansen et al. 1999). In addition, the use of protoplast requires a longer tissue culture period, which will lead to a rising risk of contamination and undesirable somaclonal variations (Bhalla and Singh 2008; Davey et al. 2005). Thus, application of PEG-mediated or electroporation-mediated protoplasts transformation method for regenerating transgenic plants is limited. Exogenous DNA or constructs also can be delivered into explant generated from microspores of rapeseed by microinjection and particle bombardment technology. The particle bombardment method involves propelling DNA-coated gold particles into intact plant tissues or cells followed by regeneration of transgenic plants (Kikkert et al. 2005). However, it leads to fragmentation of DNA during bombardment, insertion of backbone vector DNA, and insertion of multiple gene copies (Bhalla and Singh 2008). Multiple copies of the transgene can lead to gene silencing, and integration of vector DNA is undesirable, especially in the present regulatory and consumer environment (Bhalla and Singh 2008).

Agrobacterium-mediated transformation is widely used in many crop species (Tzfira and Citovsky 2006), and it has become the most common method for *Brassica* transformation (Bhalla and Singh 2008). The floral-dip is a classic *Agrobacterium*-mediated transformation method which has been successfully used in *Arabidopsis* and *Camelina* (Sitther et al. 2018; Clough and Bent 1998), which is also suitable for rapeseed transformation (Verma et al. 2008). However, the floral-dip method is difficult to expand in rapeseed because of low transformation efficiency and limited space for plant growth. The ability to regenerate transgenic plants from *Agrobacterium*-mediated transformed cells is vital for successful transformation. Hypocotyl and cotyledon explants have been used to obtain transgenic *B. napus* and *B. oleracea* (Bhalla and Singh 2008). However, most of the transformation protocols reported are relatively specific to a model cultivar such as Westar, which is not agronomically desirable (Rani et al. 2013).

Here, we described an efficient *Agrobacterium*-mediated transformation method for rapeseed using hypocotyl as explants. Following this protocol, thirteen constructs carrying hygromycin or kanamycin or basta selection marker gene were transformed into different genotypes of rapeseed independently. The results indicate that this transformation method has acceptable transformation efficiency for different genotypes of rapeseed, and it is suitable for different constructs containing different selection markers. It will benefit gene functional study and molecular breeding of rapeseed.

Materials and methods

Preparation of stock reagent

2,4-D (1.0 mg/mL) Dissolve 50 mg 2,4-D (Sangon Biotech, Cat. No. A600166-0100) in 1 mL ethanol. Add millipore water to 50 mL, then filter the solution using a 0.22-μm syringe filter. Stock at − 20 °C.

Kinetin (0.3 mg/mL) Dissolve 15 mg kinetin (Sigma, Cat. No. A600745-0025) in 1 mL 1 M HCl. Add millipore water to 50 mL, then filter the solution using a 0.22-μm syringe filter. Stock at − 20 °C.

Zeatin (1 mg/mL) Dissolve 100 mg zeatin (Sangon Biotech, Cat. No. A600748) in 1 mL 1 M NaOH. Add millipore water to 100 mL, then filter the solution using a 0.22-μm syringe filter. Stock at − 20 °C.

Indole-3-acetic acid (IAA, 0.5 mg/mL) Dissolve 25 mg IAA (Sangon Biotech, Cat. No. A600723-0025) in 1 mL 95% ethanol. Add millipore water to 50 mL, then filter the solution using a 0.2-μm syringe filter. Stock at − 20 °C.

Acetosyringone (AS, 100 mM) Dissolve 0.92 g acetosyringone (Sangon Biotech, Cat. No. A601111) in 50 mL dimethyl sulfoxide (DMSO). Stock at − 20 °C.

Timentin (300 mg/mL) Dissolve 15 g timentin (Goldbio, Cat. No. T-104-100) in 40 mL millipore water, then add millipore water to 50 mL and filter the solution using a 0.22-μm syringe filter. Stock at − 20 °C.

Silver thiosulfate (STS, 15 mM) Slowly pour $AgNO_3$ solution (Sinopharm, Cat. No. 10018461) (dissolve 509.61 mg in 30 mL millipore water) into the prepared $Na_2S_2O_3$ solution (Sinopharm, Cat. No. 10021218) (dissolve 1.74 g in 70 mL millipore water) with stirring. Filter the mixed solution using a 0.22-μm syringe filter after the reaction finished. Stock at − 20 °C.

Indole-3-butyric acid (IBA, 0.5 mg/mL) Dissolve 25 mg IBA (Sangon Biotech, Cat. No. A600725-0025) in 1 mL 95% ethanol. Add millipore water to 50 mL. Stock at room temperature.

Kanamycin (50 mg/mL) Dissolve 2.5 g kanamycin (BBI, Cat. No. A600286) in 50 mL millipore water. Filter the solution using a 0.22-μm syringe filter. Stock at − 20 °C.

Basta (5 mg/mL) Dissolve 250 mg glufosinate (Phyto technology laboratories, Cat. No. P679) in 50 mL millipore water. Filter the solution using a 0.22-μm syringe filter. Stocked at − 20 °C.

Preparation of work reagent

LB media (liquid) Dissolve 10 g tryptone (OXOID, Cat. No. LP0042), 5 g yeast extract (OXOID, Cat. No. LP0021), and 10 g NaCl (Sinopharm, Cat. No. 10019318) in millipore water to make up a total volume of 1 L, pH 7.0, autoclave (103.4 kPa, 121.0 °C 20 min). Recommended concentration of antibiotic is added to screen the *Agrobacterium* before the media using.

LB media (solid) Dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in millipore water to make up a total volume of 1 L, pH 7.0. Addition of 10 g agar powder (Biofroxx, Cat. No. 8211GR500) into liquid LB media before it autoclaves (103.4 kPa, 121.0 °C 20 min). Recommended concentration of antibiotic is added to screen the *Agrobacterium* before the media using.

Seed germination media (M_0) Dissolve 2.2 g MS powder (4.4 g/L, Duchefa Biochemie, Cat. No. P16438.01) in millipore water to make up a total volume of 1 L. Adjust the pH to 6.0 before addition of 10 g agar powder. Autoclave (103.4 kPa, 121.0 °C 15 min).

Infection and Agrobacterium suspension media (DM) Mix 4.4 g MS powder and 30 g sucrose in millipore water to make up a total volume of 1 L. Adjust pH to 5.8 and autoclave (103.4 kPa, 121.0 °C 15 min). Addition of 100 μM AS into the media before it uses.

Cocultivation media (M_1) Mix 4.4 g MS powder, 30 g sucrose, 18 g mannitol (Biofroxx, Cat. No. 69-65-8), and 6 g agarose (Tsingke, Cat. No. TSJ001) in millipore water to make up a total volume of 1 L. Adjust pH to 5.8 before addition of agarose, and then autoclave (103.4 kPa, 121.0 °C 15 min). Addition of 1 mg/L 2,4-D, 0.3 mg/L kinetin, and 100 μM AS into the media before it solidifies.

Callus induction media (M_2) Mix 4.4 g MS powder, 30 g sucrose, 18 g mannitol, and 6 g agarose in millipore water to make up a total volume of 1 L. Adjust pH to 5.8 before addition of agarose, and then autoclave (103.4 kPa, 121.0 °C 15 min). Addition of 1 mg/L 2,4-D, 0.3 mg/L kinetin, 30 μM STS, 300 mg/L timentin, and 25 mg/L kanamycin (or 5 mg/L basta, or 20 mg/L hygromycin for different antibiotic screening) into the media before it solidifies.

Shoot initiation media (M_3) Mix 4.4 g MS powder, 10 g glucose (Sinopharm, Cat. No. 10010518), 0.25 g xylose (Sinopharm, Cat. No. 63012037), 0.6 g MES (Sangon Biotech, Cat. No. A610341-0100), and 6 g agarose in millipore water to make up a total volume of 1 L. Adjust pH to 5.8 before addition of agarose, and then autoclave (103.4 kPa, 121.0 °C 15 min). Addition of 2 mg/L zeatin, 0.1 mg/L IAA, 300 mg/L timentin, and 25 mg/L kanamycin (or 5 mg/L basta, or 20 mg/L hygromycin for different antibiotic screening) into the media before it solidifies.

Root initiation media (M_4) Mix 4.4 g MS powder, 10 g sucrose, 0.5 mg/L IBA, and 10 g agar powder in millipore water to make up a total volume of 1 L. Adjust pH to 5.8 before addition of agar powder, and then autoclave (103.4 kPa, 121.0 °C 15 min). Addition of 25 mg/L kanamycin (or 5 mg/L basta, or 20 mg/L

hygromycin for different antibiotic screening) into the media before it solidifies.

Plant materials and growth conditions

Different genotypes of rapeseed including inbred spring-type Westar and semi-winter type GY284, WH3417, ZS9 (Zhongshuang 9), and ZS11 (Zhongshuang 11) were used in this study. Seeds were collected from self-crossed plant which were bagged during it flowering every generation in the field. The rooted plantlets from tissue culture were transplanted into the field, or the pot (12 cm × 15 cm) in growth room under the condition of 16 h of light and 8 h of dark at 25 °C.

Statistics and calculation

Each construct was transformed 2–8 times independently. The green seedling acquisition rate is calculated as number of final green seedlings on M4 / number of initial explants. The transformation efficiency is calculated as number of PCR positive seedlings / number of initial explants. The genome editing efficiency is calculated as number of edited plants / number of PCR positive plants.

Agrobacterium and vector

Three *Agrobacterium* strains—C58C1, EHA105, and GV3101—were used for infection. Five kinds of binary vectors with different resistance screening marker such as kanamycin (pKYLX71 (Hong et al. 2008), p35S-FAST (Lu et al. 2019), pKSE401 (Xing et al. 2014), and pCAMBIA1300 (Lu et al. 2013)) and hygromycin (pMDC83 (Curtis and Grossniklaus 2003)) carrying various genes were transformed into the competent cells of *Agrobacterium* by electric shock. For pCAMBIA1300S construction, the sequence of *hygromycin phosphotransferase II* (*HPTII*) was cloned and linked to pCAMBIA1300 to replace the *neomycin phosphotransferase II* (*NPTII*) gene. The *RePAT* gene was amplified from the vector of pU130 (Cui et al. 2016). The product was linked to pCAMBIA1300S, and then the *HPTII* gene was deleted. Finally, the vector of pCAMBIA1300S-1 was constructed. The pKSE401-GENE-sgRNA vectors were constructed as previously described (Yang et al. 2017). The modified vectors carrying various genes were transformed into the competent cells of *Agrobacterium* by electric shock. The primers used for testing the vectors were listed (Table S1). The confirmed *Agrobacterium* stain was propagated and stored at − 80 °C.

Procedure of transformation

Overview of the steps of *Agrobacterium*-mediated transformation of rapeseed is shown in Fig. 1. The detail transformation procedure is as follows.

Seed germination and preparing of hypocotyl explant The rapeseed (Westar, GY284, WH3417, ZS9, and ZS11) seeds are sterilized by 75% ethanol for 1 min, and washed by sterile water for 3 times. Then, the seeds are followed sterilizing by 0.15% $HgCl_2$ for 15 min, and washed by sterile water for 3 times. The sterilized seeds are transferred onto M_0 solid media in a Petri dish (diameter = 6 cm). The Petri dish without cover is moved into a sterilized transparent box and kept in a dark room for 7 days under 25 °C. The hypocotyl is picked out (remove the cotyledons and roots carefully) and cut into pieces of 0.6–0.8 cm length in a Petri dish (diameter = 9 cm) with 20 mL DM media. Usually, there are 150–200 pieces of explants in one Petri dish.

Preparation of Agrobacterium *Agrobacterium* stain introduced with vector is kept on a solid LB plate with suitable antibiotic. Single colony is picked and put into a glass bottle with 5 mL liquid LB and suitable antibiotic after the seed germination of 5th day. The bottle is fixed in a constant temperature shaker with 200 rpm at 28 °C. Usually, the OD value of the culture solution will reach 0.6–0.8 in 36–48 h.

Infection Cultured *Agrobacterium* is transferred into a 10-mL sterilized tube and centrifuged at 6000 rpm for 10 min. Discard the supernatant, resuspend, and wash the pellet twice by adding 5 mL DM media. Addition of 2 mL suspension solution above into the Petri dish (diameter = 9 cm) which contains prepared explants and 20 mL DM solution. The explants are infected for 30 min with shaking one time every 10 min.

Cocultivation Discard the DM solution after finishing the infection step above. Absorb the residual liquid using sterilized filter paper. The explants are transferred onto M_1 media for 2–3 days in a dark room at 25 °C.

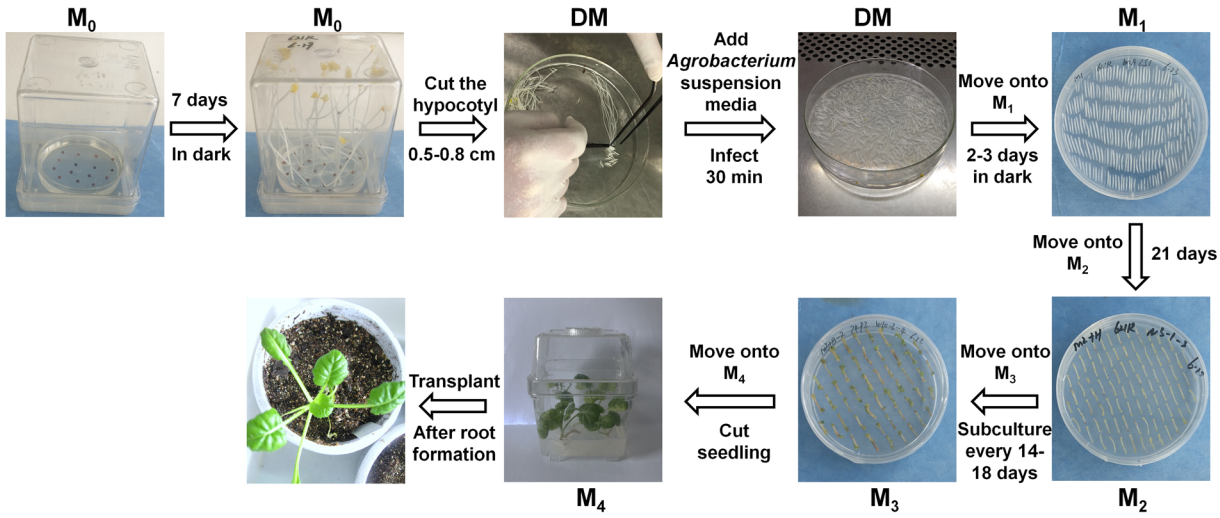

Fig. 1 Flow chart of rapeseed transgenic procedure

Callus induction The explants are transferred onto M_2 media and kept in the tissue culture room for 21 days, at the condition of 16 h day/8 h dark, 26/22 °C.

Shoot differentiation The explants are transferred onto M_3 media and kept in the tissue culture room for 14 days, at the condition of 16 h day/8 h dark, 26/22 °C. Subsequently, the explants are transferred onto a fresh M_3 media every 14 days until the shoots come out.

Root initiation and seedling transplantation Green shoot is cut from the junction of the explant, removed the extra dead tissue, and transplanted into the sterilized transparent box containing M_4 media. The box is put in the tissue culture room at 26/22 °C under 16 h day/8 h dark. Usually, the root will grow well in half a month.

DNA extraction and identification of transgenic plants by PCR

Cut a small piece of fully expanded leaf and extract the DNA using shorty buffer (Lu et al. 2016). Transgenic plants are identified by PCR using gene-specific primers. The PCR-confirmed transgenic plants are transplanted into the pots with soil in the growth room, or the field. Further, RNA and protein can be extracted for evaluation of target gene expression by semi-quantitative PCR, real-time PCR, or immunoblotting at this stage if necessary (Lu et al. 2013).

Screening of transgenic plants using antibiotics or basta

The seeds generated from T_0 plants can be screened using antibiotics or basta. For kanamycin resistance screening, the seeds are germinated on wetted filter paper for 2–3 days until the embryonic axis exposed from seed coat. Then, the germinated seeds are transferred onto filter paper wetted with 1.2 mg/mL kanamycin solution and cultured at 26/22 °C 16 h day/8 h dark for 7 days. The cotyledon color is green if the seedling is kanamycin resistance, while the cotyledon of negative transgenic plants is yellow or purple. For hygromycin resistance screening, the seeds are directly germinated on filter paper wetted with 150 μg/mL hygromycin solution and cultured at 26/22 °C 16 h day/8 h dark for 7 days. The color of cotyledon is green if the seedling has hygromycin resistance. For basta resistance screening, the seeds are sown in the field, and commercial basta (Bayer, www.premeo.de) under recommended concentration is sprayed on leaf during 5–6 leaf period. The plant grows well and the color of leaf is green if it has basta resistance.

Results

GV3101 *Agrobacterium* strain is more efficient for rapeseed transformation

We compared the transformation efficiency of three *Agrobacterium* strains, C58C1, EHA105, and GV3101. Two different vectors, pKYLX71-AtPLDα3

and p35S-FAST-AtPLDε with *NPTII* selection marker gene, were transformed into these three *Agrobacterium* strains, respectively. Then, the explants from Westar were infected by each *Agrobacterium* strain. As shown in Table 1, it was hardly to obtain green seedling after the explants were infected by C58C1. About 0–17 green seedlings were obtained from 161 to 678 explants after transfected by EHA105. The green seedling acquisition rate was 0.5–3.7% and 0–2.3% of pKYLX71-AtPLDα3 and p35S-FAST-AtPLDε, respectively (Table 1). Previous study reported that the green seedlings in M_4 media were not all real positive transgenic plants (Zhang et al. 2020). Thus, the green seedlings were further confirmed by PCR using gene-specific primers. The results showed that the transformation efficiency was 0–0.15% and 0–0.16% of pKYLX71-AtPLDα3 and p35S-FAST-AtPLDε, respectively (Table 1). Meanwhile, the green seedling acquisition rate was 16.2–31.3% and 7.2–28.6%, and the transformation efficiency was 2.7–15.1% and 2.2–7.0% when explants were infected by GV3101 carrying the same constructs (Table 1). These results indicate that GV3101 is more efficient for rapeseed transformation than the other two *Agrobacterium* strains.

Hygromycin, kanamycin, and basta resistance are efficient selection markers for rapeseed transformation

The *HPTII* and *NPTII* genes are two major selection markers in plant transgenic research (Berg et al. 1975; Gritz and Davies 1983). The *HPTII* and *NPTII* genes confer transgenic plant resistance to hygromycin (Hyg) and kanamycin (Kan), respectively. In plant, Hyg and Kan normally inhibit protein synthesis by binding to ribosomes (Davey et al. 2010). Glufosinate ammonium (basta) is a chemical reagent that can inhibit the activity of glutamine synthetase. Two glufosinate-resistant genes of *BAR* and *PAT*, encoding a phosphinothricin N-acetyltransferase, can detoxify glufosinate ammonium by acetylation of the amino group, which are also widely used as selection markers in transgenic plant research (Davey et al. 2010; Donn and Köcher 2002; Cui et al. 2016). Recently, expression of a novel PAT coding gene (*RePAT*) in rice was reported to improve plant resistance to basta (Cui et al. 2016). Thus, the *NPTII* gene in pCAMBIA1300 was replaced by *HPTII* and *RePAT* genes, and renamed as pCAMBIA1300S and pCAMBIA1300S-1, respectively (Supplemental Figure 1).

The constructs of pCAMBIA1300S-BnKCS (Hyg), pMDC83-BnlncRNA (Hyg), p35S-FAST-BnPLDα1 (Kan), p35S-FAST-BnPLDδ (Kan), pCAMBIA1300-AtPLDα1 (Kan), and pCAMBIA1300S-1-RePAT (basta) were introduced into *Agrobacterium* GV3101, which were used to infect Westar hypocotyls. Finally, 25–236 of green seedlings were obtained from 286 to 599 explants by Kan selection. The green seedling acquisition rate was 9.6–60.1%, and the transformation efficiency was 2.1–22.5% (Table 1). For Hyg screening, 38–60 of green seedlings were obtained from 732 to 1530 explants. The green seedling acquisition rate was 3.9–5.5%, and the transformation efficiency was 3.7–4.8% (Table 1). Furthermore, 25–83 of green seedlings were obtained from 321 to 768 explants after basta screening. The green seedling acquisition rate and the transformation efficiency was 7.8–16.7% and 1.6–5.9%, respectively (Table 1). Although the highest transformation efficiency was 22.5% using *NPTII* as selection marker, the transformation efficiency of *HPTII* and *RePAT* was acceptable. These results suggest that *HptII*, *NptII*, and *RePAT* are suitable as the selection marker genes for rapeseed transformation (Fig. 2).

Varied transformation efficiency of different genotypes of rapeseed

The spring-type Westar is commonly used for rapeseed transformation because of the high transformation efficiency (Bhalla and Singh 2008; Zhang et al. 2005). To test this transformation procedure which is also applied to other genotypes of rapeseed, hypocotyls from four semi-winter genotypes of GY284, WH3417, ZS9, and ZS11 were infected by GV3101 containing the vector of pKSE401 or pCAMBIA1300. Two sgRNAs target gene-specific region of fatty acid elongase 1 (*FAE1*), fatty acid desaturase 2 (*FAD2*), and ketoacyl-CoA synthase (*KCS*) and were amplified and ligated to pKSE401 before it introduced into GV3101, respectively (Tables 1 and 2). The results showed that the green seedling acquisition rate of GY284, WH3417, ZS9, and ZS11 was 3.9–5.8%, 1.1–3.9%, 0–3.8%, and 0, respectively (Tables 1 and 2). The transformation efficiency was 3.7–5.6%, 1.1–3.5%, 0–0.87%, and 0 for GY284, WH3417, ZS9, and ZS11 (Tables 1 and 2). pKSE401 has been used as a genome editing toolbox in plants (Tang et al. 2018; Yang et al. 2017). Further, we tested the genome editing efficiency of pKSE401-BnFAE1, pKSE401-BnFAD2, and pKSE401-BnKCS

Table 1 Comparison of the transformation efficiency of different *Agrobacterium*, different genotypes of rapeseed, and different constructs

Agrobacterium tumefaciens	Vector	Resistance	Recipient	Gene	Times	Number of explants	Number of green seedlings on M_4	The green seedling acquisition rate (%)	PCR positive plants	Transformation efficiency (%)
C58C1	pKYLX71	Kanamycin	Westar	*AtPLDα3*	3	192–460	0	0	-	-
	p35S-FAST	Kanamycin	Westar	*AtPLDε*	3	181–356	0–1	0–0.3	0	-
EHA105	pKYLX71	Kanamycin	Westar	*AtPLDα3*	5	161–678	3–17	0.5–3.7	0–1	0–0.15
	p35S-FAST	Kanamycin	Westar	*AtPLDε*	3	174–625	0–7	0–2.3	0–1	0–0.16
GV3101	pKYLX71	Kanamycin	Westar	*AtPLDα3*	4	148–252	24–79	16.2–31.3	4–38	2.7–15.1
	p35S-FAST	Kanamycin	Westar	*AtPLDε*	4	232–570	25–109	7.2–28.6	5–40	2.2–7.0
	p35S-FAST	Kanamycin	Westar	*BnPLDα1*	8	354–599	134–236	22.4–44.8	25–40	6.7–7.1
	p35S-FAST	Kanamycin	Westar	*BnPLDδ*	5	296–528	75–235	24.4–60.1	15–119	5.1–22.5
	pCAMBIA1300	Kanamycin	Westar	*AtPLDα1*	3	286–322	25–31	9.6–10.8	6–12	2.1–3.7
	pCAMBIA1300	Kanamycin	ZS9	*AtPLDα1*	3	232–344	0–13	0–3.8	0–3	0–0.87
	pCAMBIA1300	Kanamycin	ZS11	*AtPLDα1*	3	308–612	0	0	-	-
	pCAMBIA1300S-1	Basta	Westar	*RePAT*	7	321–768	25–83	7.8–16.7	5–45	1.6–5.9
	pCAMBIA1300S	Hygromycin	Westar	*BnKCS*	6	732–1530	38–60	3.9–5.5	35–59	3.8–4.8
	pMDC83	Hygromycin	Westar	*BnlncRNA*	2	873–990	39–43	4.3–4.5	35–37	3.7–4.0

The green seedling acquisition rate is calculated as number of final green seedlings on M4 / number of initial explants. The transformation efficiency is calculated as number of PCR positive seedlings / number of initial explants

Fig. 2 M4-agar plate for selection of transgenic rapeseed plants. The image shows the transgenic seedlings grew on M_4-agar plate with Kan antibiotics. The red arrow indicates the negative transgenic plant

by PCR products sequencing. The results indicated that the genome editing efficiency of pKSE401-BnFAE1, pKSE401-BnFAD2, and pKSE401-BnKCS was 23.3–24.0%, 9.5–22.2%, and 13.2–52.6% (Table 2), which was consistent with previous reports (Tang et al. 2018; Yang et al. 2017). These results suggest that the transformation efficiency of rapeseed is genotype dependent, which is similar to previous research (Bhalla and Singh 2008).

Confirmation of the positive transgenic plants

To investigate the transgenic-positive efficiency of regenerated plantlets, genomic DNA was extracted from these putative transgenic plantlets. PCR was performed to detect target genes by using gene-specific primers (Table S1). As expected, most of the putative transgenic plants presented gene-specific bands (Fig. 3a). Western blot was also performed to check protein expression of target gene (e.g., *BnPLDα1*) in the seedlings (Fig. 3b). Furthermore, the T_1 seeds from transgenic lines were screened by antibiotics or basta. The results displayed that the seedling growth from negative transgenic plants was inhibited and the color of its cotyledon observed yellow under Kan (Fig. 4a) and Hyg screening (Fig. 4b). The non-transgenic plants were killed by basta in the field (Fig. 4c). In brief, some regenerated green plantlets are false transgenic plants, and molecular or chemical methods need to be done to confirm their reliability.

Discussion

Due to its important position in agronomy, rapeseed has become a hot spot in scientific research, and rapeseed biotechnology has also made considerable progress. Previously, our transformation method had been used to study gene function and generate rapeseed with improved agronomic characteristics (Lu et al. 2013; Lu et al. 2016; Tang et al. 2018; Yang et al. 2017). However, the detail information of the transformation procedure is not described. In addition, most of researches use the model genotype of Westar as transformation receptor. The factors that influence the transformation efficiency such as type of *Agrobacterium*, selection marker, and genotype of rapeseed have not been compared (Lu et al. 2013; Lu et al. 2016; Tang et al. 2018; Yang et al. 2017). Here, we comprehensively described and compared multiple factors that affect the transformation efficiency by transforming different constructs into five genotypes of rapeseed via three *Agrobacterium* strains. The results showed that transgenic plants could be easily

Table 2 Comparison of the transformation efficiency of different genotypes of rapeseed using CRISPR/Cas9 vectors

Recipient	Target gene	Times	Number of explants	Number of green seedlings on M_4	The green seedling acquisition rate (%)	PCR positive plants	Transformation efficiency (%)	Number of editing plants	Editing efficiency (%)
GY284	*BnFAE1*	2	511–980	20–47	3.9–4.8	19–40	3.7–4.1	3–4	10.0–15.8
WH3417	*BnFAE1*	2	482–2200	19–64	2.9–3.9	17–60	2.7–3.5	3–9	15.0–17.6
GY284	*BnFAD2*	2	623–1080	27–63	4.3–5.8	25–60	4.0–5.6	6–14	23.3–24.0
WH3417	*BnFAD2*	2	836–2920	9–70	1.1–2.4	9–63	1.1–2.4	2–6	9.5–22.2
Westar	*BnKCS*	3	284–321	30–40	9.3–15.0	29–38	10.2–11.8	5–20	13.2–52.6

The green seedling acquisition rate is calculated as number of final green seedlings on M4 / number of initial explants. The transformation efficiency is calculated as number of PCR positive seedlings / number of initial explants. The genome editing efficiency is calculated as number of edited plants / number of PCR positive plants

obtained in four genotypes of rapeseed, suggesting that this protocol is efficient for rapeseed transformation.

Agrobacterium-mediated transformation is the most successful method for producing of genetically modified plants (Tzfira and Citovsky 2006). It has been used for transformation of many plant species (Herrera-Estrella et al. 2004). The transformation has been tested in a variety of explant types such as bud, cotyledon, hypocotyl, embryo, and callus (Bhalla and Singh 2008; Ishida et al. 2007; Zhang et al. 2005; Clough and Bent 1998; Raineri et al. 1990). Transgenic plant of rapeseed can be obtained from hypocotyl and cotyledon which are infected by *Agrobacterium* (Bhalla and Singh 2008; Rani et al. 2013). These studies reported that the transformation efficiency was 7.7–68.1%, and 5.3–50.1% after *Agrobacterium* infected cotyledon and hypocotyl from five different rapeseeds, respectively (Bhalla and Singh 2008; Zhang et al. 2005). However, the transformation efficiency was calculated by the number of shoots on selection media divided by total explant number (Bhalla and Singh 2008; Zhang et al. 2005). This statistical method cannot accurately reflect the real transformation efficiency, because not all the green shoots will generate green seedlings (Zhang et al. 2020), or survive on the rooted media (M_4 media). Additionally, the false-positive transgenic plants cannot be eliminated by antibiotic screening (Fig. 3a). We presented two values to evaluate the transformation

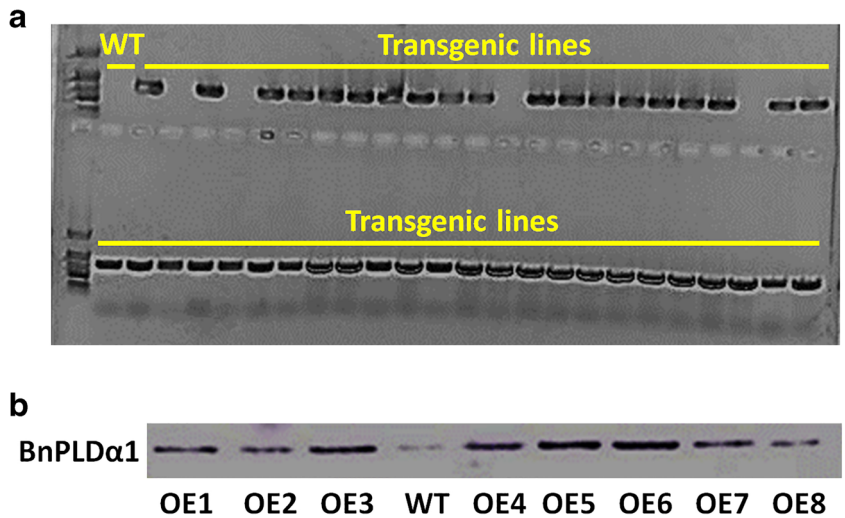

Fig. 3 The positive transgenic plants were confirmed by PCR and western blot. **a** The image shows the transgenic plants confirmed by PCR using vector-specific primers. **b** The image shows the transgenic plants confirmed by western blot using PLDα1-specific antibody. Ten micrograms of total protein of each line was loaded and separated by 8% SDS-PAGE, and then transferred to a polyvinylidene difluoride membrane (PVDF membrane). PLDα1 was detected by PLDα1-specific antibody (Lu et al. 2013). The band in the lane of WT was the coloration of endogenous PLDa1 protein

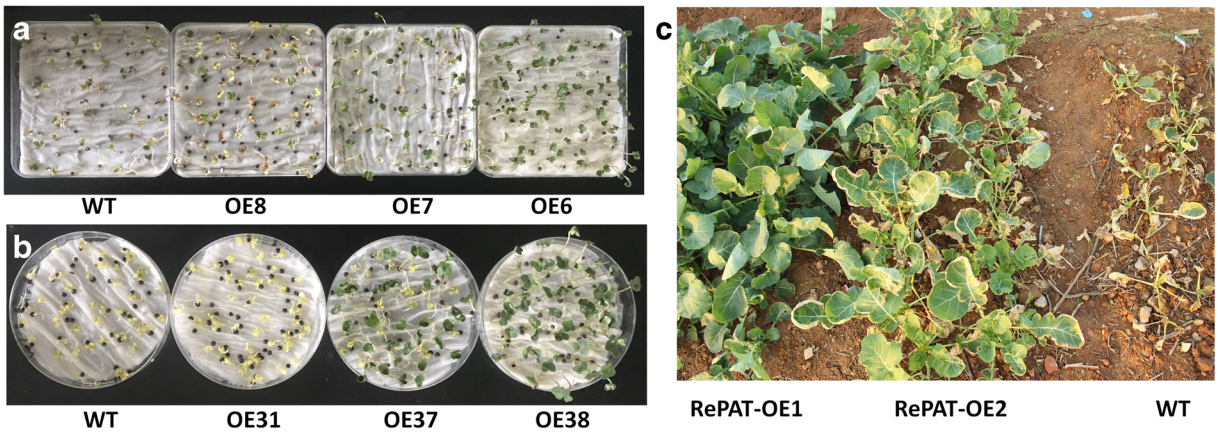

Fig. 4 The antibiotic screening in T_1 generation. **a** The image shows the seeds from transgenic plant screened by Kan in lab. **b** The image shows the seeds from transgenic plants screened by Hyg in lab. **c** The image shows the seeds from transgenic plants screened by basta in the field

efficiency, including the green seedling acquisition rate (number of final green seedlings on M_4 divided by number of initial explants) and transformation efficiency (PCR positive seedlings divided by number of initial explants). In fact, the transformation efficiency reported by previous studies is equivalent to our green seedling acquisition rate. Strictly speaking, our statistic of transformation efficiency is more rigorous. Previous studies showed that the highest transformation efficiency was 21.9% and 33.1% after the hypocotyl and cotyledon of Westar infected by LAB4404 containing *NPTII* gene, respectively (Zhang et al. 2005). In this study, the highest ratio of green seedling acquisition was 60.1%. The highest ratio of green seedling acquisition is 2–3 times than that of previous study, indicating that our transformation method is more efficient to obtain transgenic plants.

The transformation method using hypocotyl as explant can greatly reduce the number of seed consuming compared with that using cotyledon. One seedling only provides two cotyledon explants, but can provide more than ten hypocotyl explants (0.6–0.8 cm) at least. In addition, the operation of cotyledon transformation requires the incision of each explant dipping into *Agrobacterium* liquid for 10–30 s, and then keeping the incision inserting into the solid media vertically (Bhalla and Singh 2008; Zhang et al. 2005). These operations are complex and time consuming. Moreover, it takes 10–14 weeks to obtain transgenic plants (Bhalla and Singh 2008), which is longer than our transformation procedure (8–10 weeks). Together, hypocotyl transformation is time-saving and easy to operate compared with previous cotyledon transformation (Bhalla and Singh 2008; Zhang et al. 2005).

Regeneration of rapeseed in transformation is highly genotype dependent. A study reported that the variation range of regeneration rate was from 0 to 91% in 100 cultivars of rapeseed (Ono et al. 1994). We found that the regeneration rate of Westar was the highest among five genotypes of rapeseed. Westar is a disease-susceptible cultivar, and ZS11 is a highly disease-resistant cultivar. Higher resistance of ZS11 might repress the *Agrobacterium* infection and reduce the transformation efficiency, suggesting that the regeneration of callus is affected by plant resistance. In addition, the *Agrobacterium* strain is found to be a critical factor for the successful transformation (Rani et al. 2013). We compared the transformation efficiency among three *Agrobacterium* stains including C58C1, EHA105, and GV3101. The efficiency of obtaining green seedlings and transformation efficiency of GV3101 was 7.2–31.3% and 2.2–15.1%, which were much higher than that of C58C1 and EHA105. It is more efficient than that of using LBA4404 stain infected hypocotyl of Westar in previous study (Zhang et al. 2005). Taken together, GV3101 is more suitable for the transformation of rapeseed.

Screening gene of antibiotic resistance is an essential factor for improving transformation efficiency (Rani et al. 2013). Efficient selection depends on kinds of antibiotics employed and their concentration used. The green seedling acquisition rate of using *NPTII* and *RePAT* as selection gene was higher than that of using *HPTII* (Table 1). However, the ratio of positive

transgenic plants in green seedlings confirmed by PCR was 92.1–100.0% when using *HPTII* as selection gene. It was higher than that of using *NPTII* and *RePAT*. Usually, the transformation efficiency was not much different among these three selection markers, suggesting that *HPTII*, *NPTII*, and *RePAT* are suitable as selection genes for rapeseed transformation. Although the transformation efficiency was acceptable when *HPTII*, *NPTII*, and *RePAT* were used as selection genes, the variation of transformation efficiency was still greatly among different experiments, even the recipient material and construct were the same (Table 1 and Table 2). The reason might be due to this system project that was done by different researchers in different time, which would influence the transformation efficiency, such as the different operation of different person and the different situation of explants and *Agrobacterium* strains of different experiments.

In summary, we describe an efficient *Agrobacterium*-mediated transformation method using hypocotyl as explants, which is applicable to various genotypes of rapeseed. It takes approximately 8–10 weeks to complete the procedure from seed germination to obtaining of rooted plantlets. This method is highly efficient and time-saving. In addition, compared with the transformation method using cotyledon as explants, the explants are easier to obtain and the operation is simpler in our method. Together, this method will benefit gene functional study, especially in high-throughput molecular biology research in rapeseed.

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Compliance with ethical standards

Competing interests The authors declare that they have no conflict of interest.

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