A novel in vitro transformation of *Lepidium draba* L. using rapid direct shoot regeneration

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Abstract The present research is carried out to study *Lepidium draba* gene transformation for the first time, using direct shoot explants. As a prerequisite for gene transformation, the regeneration conditions in *L. draba* were optimized. We achieved an efficient and reproducible protocol for successful direct shoot regeneration without intervening callus formation. The results indicate that *L. draba* is the insistent species of Brassicaceae in direct shoot regeneration. Various explants of *L. draba* were genetically transformed with different strains of *Agrobacterium tumefaciens*, viz., LBA4404, GV3850, GV3101, and EHA105, using the vector pBI121. Expression of GUS reporter protein was assayed by histochemical staining. In addition, using the PCR method with specific primers proved the integration of GUS gene into the plants. The highest transformation efficiency was achieved with *Agrobacterium* strain GV3850. Moreover, we found that infected hypocotyl and root explants of *L. draba* interestingly yielded higher transformation efficiency, so that in hypocotyls on average exceeded 70% of the explants. This study showed that *L. draba*, in addition to the numerous desirable traits, has a high potential for gene transfer.

Keywords *Agrobacterium*-mediated · Gene transformation · β -Glucuronidase · Direct shoot regeneration · *Lepidium draba*

Introduction

Genetic engineering technologies are essential for the improvement of crops and result into the development of plants with improved yield and quality (Kaikavoosi et al. 2015; Thorat et al. 2017; Soltani Nejad et al. 2015). Transformation procedures have a significant influence on some basic research in biology and agricultural biotechnology (Khatami et al. 2015b). Gene transformation in plants involves the integration of DNA into plant cells and following regeneration of transformed plants. Therefore, plant transformation includes the integration of a new gene into plant genome and the ability of cells carrying transgene to regeneration (Gelvin 2003; Yang et al. 2016; Mohan et al. 2017; Mortazavi et al. 2017; Khatami et al. 2015a).

Transformation in plants is studied by a number of methods like direct gene transfer by absorption and biolistic transformation (Gene gun), microinjection, nanoparticles, liposome method, chemical method, infiltration, shoot tip method, and silicon carbide mediated and *Agrobacterium*-mediated transformation (Tripathi and Tripathi 2003; Rao et al. 2009; Zare et al. 2017; Nejad et al. 2016; Nejad et al. 2017; Azizi et al. 2016; Khatami et al. 2017c).

Agrobacterium-mediated transformation is widely used for gene transfer in plants (Khatami et al. 2016a) due to its high transformation efficiency and relatively stable integration and expression of transgene, reduced copy number of transgenes, reduced co-suppression, and repeated recovery of plants with usual phenotypes (Enríquez-Obregón et al. 1999; Hansen et al. 1997; Park et al. 1996).

Proliferation and tissue culture regeneration protocol are proved absolutely necessary for the development of transgenic plants. Transgenic plants are taken from transformed

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tissues, using regeneration methods that are usually highly time-consuming (Maheshwari et al. 2016; Alam et al. 2017). These methods mostly show the mutated morphogenetic traits. In addition, plants regenerated are often abnormal in appearance and can exhibit a loss in fertility. Hence, transgenic plants that regenerate in a long process may not be useful for studying the expression of a foreign gene. Therefore, transformation procedures that avoid long-term tissue culture would be highly advantageous (Valvekens et al. 1988).

Lepidium draba L. (Hoary cress) is a small flowering plant of mustard family, Brassicaceae (Cruciferae) that reproduces by seeds and horizontal creeping roots (Jacobs 2007). Hoary cress is indigenous to southwestern (Caucasus region) and central Asia (Talmaciu et al. 2010).

Its young leaves and shoots are used in salads or cooked as a potherb, and it has even been recommended as having health benefits and medicinal properties (Facciola 1998; Guil Guerrero and Torija Isasa 1997; Fahey et al. 2002, 2013; Khatami et al. 2017a). The plant contains large amount of glucoraphanin that can produce sulforaphane (SFN)—an anti-cancer agent—through myrosinase hydrolysis activity (Fahey and Talalay 1999).

According to the previous studies, *L. draba* has different ploidy levels (Mulligan 2002; Mulligan and Frankton 1962). Previously, researchers described the development of polymorphic microsatellite markers that revealed the polyploidy of this plant (Khatami and Pourseyedi 2015).

Hoary cress has high regeneration efficiency and excellent capability to produce direct shoot in that is necessary and very useful for gene transfer process and reducing the required time for transformation in vitro (Ghotbzadeh et al. 2015; Zinhari et al. 2016; Khatami et al. 2016b, 2017b).

There have been numerous reports describing the transformation of protoplasts or wounded tissue from a variety species of Brassicaceae, using either *Agrobacterium*-mediated or direct gene transfer techniques (Damm et al. 1989). However, there are no reports of gene transfer to *L. draba*.

Most of transformation reports are related to callus-based organogenesis and somatic embryogenesis (Damm et al. 1989; Radchuk et al. 2002; Alvarez and Ordás 2013). In some cases, relatively long period of time required for regeneration, high level of somaclonal variation, and restricted growth were disadvantages (Cheng et al. 2001; Zinhari et al. 2016).

As part of our experiments on transformation of *L. draba*, we attempted to regenerate shoots directly from various explants. Here, the effective and rapid production of large numbers of transgenic plants in *L. draba* is described via direct shoot regeneration. Therefore, the problems of long-time regeneration and somaclonal

variation were greatly reduced compared to intervening callus in the regeneration process. Direct shoots usually allow the regeneration of more stable and uniformly transformed plants in comparison to those arising from the indirect shoot regeneration (Annappurna and Rathore 2010; Ghimire et al. 2010; Liu et al. 2010).

In this investigation, we describe an efficient method for direct shoot regeneration from cotyledon, hypocotyl, and root explants of *L. draba* from germinating seedlings. In addition, we report *L. draba* transformation for the first time, through a procedure utilizing four strains of *A. tumefaciens* harboring the binary vector pBI121. We have targeted the various explants of *L. draba* and have used the cotyledons of *Nicotiana tabacum* var *samsun* and *Brassica napus* as controls for transformation. Our intention was to evaluate and compare the efficiency of GUS gene transfer into these plants.

Materials and methods

Plant material and sterilization

Mature seeds of Hoary cress, which are collected in Kerman, were classified in Herbarium of Shahid Bahonar University of Kerman, Iran. We used *N. tabacum* var *samsun* and *B. napus* as evidence for transformation. All seeds were first washed with running tap water followed by shaking in liquid dishwashing detergent solution for 15 min. Washed seeds were surface sterilized using 70% ethanol for 1 min, followed by 2.5% (v/v) sodium hypochlorite solution for 15 min, and then rinsed with sterile deionized water three times for 5 min each.

Germination

The seeds were germinated in several sterile flasks on 30 ml of 1/2 X MS medium with 15 g l⁻¹ sucrose and 5 g l⁻¹ agar. The pH was adjusted to 5.8 before autoclaving (Murashige and Skoog 1962). All the cultures were maintained at 26 °C under a 16 h light and 8 h dark cycle with fluorescent light. For the regeneration test and genetic transformation, the seedlings were used when the cotyledons had expanded fully and were several millimeters long.

Culture conditions and direct shoot regeneration

To develop an efficient protocol for direct shoot regeneration in *L. draba*, we cultured various explants on MS medium containing 6-Benzylaminopurine (BAP; 0, 0.5, 1, 2, 3, or 4 mg l⁻¹) and naphthalene acetic acid (NAA; 0, 0.2, 0.5, 1, or 2 mg l⁻¹). The cotyledons, hypocotyls, and roots from 14 days old of *L. draba* seedlings grown in light

were used as explants. MS used in this study was supplemented with 30 g l⁻¹ sucrose and 8 g l⁻¹ agar, pH 5.8, with different concentrations of BAP and NAA. The entire petioles were removed under laminar flow hood. The wounded cotyledons were horizontally placed on the regeneration medium, while they were immersed in the medium only from the cut ends. Hypocotyls and roots were cut into 10 mm pieces and placed on the regeneration medium after removing the root tip and plant crown where the stem meets the root. After culturing explants on MS medium supplemented with different concentrations of plant growth regulators, cultures were incubated at 26 °C in 16 h light and 8 h dark conditions and were observed regularly for direct shoot formation. The explants were transferred to fresh medium after 2 weeks. Data was collected after 1 month of incubation as the mean performance of three replicated plates per treatment.

Shoot rooting and development of plantlets

Depending on the produced response (calli or shoot buds), the tissues that regenerated direct shoots larger than 20 mm were excised and transferred to rooting medium consisting of MS supplemented with 0.2 mg l⁻¹ NAA for root regeneration. Rooted shoots were placed on basal MS medium and maintained in the same photoperiod and temperature conditions for development. Then, the plantlets were transferred to the soil in pots and acclimatized under the normal greenhouse conditions of 28 ± 4 °C temperature and 60–70% relative humidity.

Kanamycin-resistance test

To develop a rapid and efficient *Agrobacterium*-mediated transformation method for *L. draba*, we assayed the action of different concentrations of kanamycin in direct shoot regeneration from different explants. Explants were cultured on the basal MS medium, supplemented with appropriate concentrations of BAP and NAA for direct shoot regeneration of each explant, 200 mg l⁻¹ cefotaxime, and kanamycin in concentrations of 0, 15, 25, 50, 75, and 100 mg l⁻¹.

Binary vector and bacterial strains

Four strains of *A. tumefaciens* including LBA4404, GV3850, GV3101, EHA105 harboring the binary vector pBI121, which contains kanamycin resistance (*nptII*) gene and the β -glucuronidase (GUS) gene, were used for transformation.

Agrobacterium tumefaciens culture

For transformation, the bacteria were grown at 28 °C in 10 ml of LB medium containing 50 mg l⁻¹ from each kanamycin and rifampicin. After 24 h, 2.5 ml of bacterial cultures were incubated at 28 °C in 50 ml of LB medium containing 50 mg l⁻¹ rifampicin about 6–8 h to a concentration around 0.8 at OD₆₀₀. After centrifuging at 4500 rpm for 10 min, we re-suspended the pellet in a mixture containing liquid MS medium and LB medium at a ratio of 3:1, as the inoculation solution.

Agrobacterium-mediated transformation

Cotyledons, hypocotyls, and roots from 14-day-old *L. draba* and cotyledons from 8-day-old *B. napus*, and 20-day-old *N. tabacum* seedlings were used as explants. The explants were dipped in the inoculum suspension for about 5 min and dry-blotted on filter paper and co-cultured on the co-culture medium. The co-cultivation medium contained the basal MS medium, and was supplemented with suitable concentrations of BAP and NAA, at pH 5.8, containing 0.5 mg l⁻¹ BAP and 0.2 mg l⁻¹ NAA for cotyledons and 1 mg l⁻¹ BAP for hypocotyls and roots of *L. draba* to direct shoot regeneration. *B. napus* cotyledons were cultured in 4.5 mg l⁻¹ BAP to generate direct shoot (Kahrizi et al. 2010). 1 mg l⁻¹ BAP and 0.1 mg l⁻¹ NAA for cotyledons of *N. tabacum* were used for direct somatic embryogenesis (Stolarz et al. 1991). The plates were wrapped with aluminum foil and incubated at 26 °C. After 48 h of co-cultivation, the bacterial cells were washed off with sterile deionized water, and the explants were dry-blotted on filter paper and placed on the selection medium, which was the same as the co-culture medium. However, they were supplemented with 200 mg l⁻¹ cefotaxime and 25 mg l⁻¹ kanamycin and then maintained at 26 °C under a 16 h light and 8 h dark. The explants were transferred to the fresh selection medium after 2 weeks.

Confirmation of the transformed plants

Histochemical assay for GUS

Using the histochemical GUS staining procedure of Jefferson (1987), we performed a histochemical assay to detect GUS activity on kanamycin-resistant shoots. Samples were incubated overnight at 37 °C with X-Gluc in phosphate buffer; the stained tissues were rinsed several times with 70% ethanol to remove chlorophyll (Jefferson 1987).

PCR analysis

Total DNA samples from 0.5 g of fresh young tissues of the putative transgenic and control plants were isolated using the method of Maroof et al. (1994).

For PCR analysis, we used two specific primers sets (Table 1). The primers used were: (1) for the GUS gene, 35S-F and NOS-R, yielding a 2098 bp product to verify integration of the transgene; (2) for non-transferable part of pBI121, PBI-F, and PBI-R, yielding a 709 bp product to ensure that no DNA contamination of *A. tumefaciens* containing pBI121 was in plant tissues.

Data analysis

In regeneration experiment, the frequency of direct shoot was calculated by dividing the number of explants that produced direct shoot to the total number of explants used. The average number of shoots per explant was used to evaluate the shoot induction. The transformation frequency was calculated through dividing the number of explants that produced transgenic shoots by the total explants used for *Agrobacterium* infection. All the results were analyzed in a factorial experiment based on a completely randomized design. For analysis, ANOVA and Duncan's test were performed using the SPSS Statistics 22.

Results

Direct shoot regeneration

It is important to optimize the regeneration of *L. draba* to increase the transformed plants successfully. Therefore, growth regulators cytokinin (BAP) and auxin (NAA) combined at different levels and were evaluated for their direct shoot regeneration ability on the cotyledon, hypocotyl, and root explants of *L. draba*.

Initially, the explants were cultured on MS medium containing different concentrations of BAP and NAA. After 1 month, the number of explants that had produced

direct shoot and the number of shoots per explant were recorded.

Explants cultured on MS medium supplemented with growth regulators started swelling after 4–6 days followed by the formation of either callus or shoots. Shoots differentiated subsequently from the 7th day to the 14th. Direct shoot regeneration occurred without callus formation in all explants (Fig. 1). However, in some cases, there was also callusing on the explant along with shoot bud development from the cut margin of the explants.

BAP alone or with low level of NAA in the medium caused desirable direct shoot regeneration in *L. draba*. In all treatments on various explants of *L. draba* together, about 93% showed the appearance of direct shoots. The in vitro direct shoot responses of *L. draba* explants to different concentrations of growth regulators are enumerated in Table 2. According to our observations, shoot buds from hypocotyls and roots were initiated in the first week, but cotyledons exhibited a delayed response, while complete direct shoots were obtained at the end of the fourth week regardless of type of explants.

A two-factor ANOVA revealed a significant interaction between the effects of BAP and NAA in direct shoot regeneration and the average number of shoots. The combinations of BAP with NAA gave a wonderful result in direct shoot regeneration of *L. draba*. However, among the various explants tested, roots segments were the most regenerative. The analysis of the results showed that the root explants were the best choice for direct shoot regeneration (Table 3) and most of the treatments did not show significant differences on direct shoot regeneration frequency for roots (Table 2). The frequency of direct shoot regeneration in 8 treatments reached 100% when using roots as explants; for example in 0.5 or 1 mg l⁻¹ BAP (Table 2).

Direct shoot regeneration occurred considerably in concentrations of BAP more than NAA in the roots, particularly when NAA was below 2 mg l⁻¹. In this study, the number of direct shoots in root explants was more than other explants (Table 3), especially in 1 mg l⁻¹ BAP where the average number of shoots per explant was 16 (Table 2). These results suggest that 1 mg l⁻¹ BAP is

Table 1 Details of primers used in transgenic event characterization

Sr. no.	Primer ID	Primer sequence (5'–3')	Target	Product size (bp)
1	35S-F	GATGTGATATCTCCACTGAC	β -Glucuronidase (GUS) gene that is located between 35S promoter and NOS terminator from pBI121	2098
	NOS-R	TGATAATCATCGCAAGACCG		
2	PBI-F	TTGCCCTCATCTGTTACGC	Downstream of T-DNA left border of pBI121 (non-transferable part of pBI121)	709
	PBI-R	TTTCATGCCCTGCCCTAG

Fig. 1 Direct shoot regeneration from various explants of *L. draba*. **a** Direct shoots from roots; **b** direct shoots from hypocotyls; **c** direct shoots from cotyledons

suitable and effective for in vitro shoot regeneration of root explants of *L. draba*.

The results showed that, when using the different concentrations of BAP in the absence of NAA, shoot formation that occurred from hypocotyl explants was high (Table 2). Therefore, depending on the BAP concentration, direct shoot regeneration ranges from 40% in 2 mg l⁻¹ BAP to 93% in 1 mg l⁻¹ BAP. When NAA was increased from 0.2 to 2 mg l⁻¹ with different concentrations of BAP, all treatments resulted in poor or no shoot regeneration in hypocotyls (Table 2). The shoots from hypocotyls were developed directly from the cut ends. In the MS medium with 2 mg l⁻¹ BAP and 0.5 mg l⁻¹ NAA, also in 1 mg l⁻¹ BAP alone, direct shoot number was highest (8 on average) (Table 2). Therefore, 1 mg l⁻¹ BAP is considered as an appropriate direct shoot induction medium in hypocotyls.

When culturing cotyledons on the MS medium with 0.5 mg l⁻¹ BAP and 0.2 mg l⁻¹ NAA, the highest frequency of direct shoot regeneration (73%), in this explant, was obtained. The average number of direct shoot in this treatment was at a desirable level (7 on average). While the highest number was observed in the MS medium supplemented with 0.5 mg l⁻¹ BAP and 1 mg l⁻¹ NAA (13 on average) for cotyledon explants (Table 2), the shoot frequency results suggested a requirement of 0.5 mg l⁻¹ BAP and 0.2 mg l⁻¹ NAA for optimal shoot regeneration response in cotyledons.

Adventitious roots could be seen forming within 1 week after the regenerated shoots were placed on root induction medium. Frequency of root formation was nearly 100%. For optimal elongation of shoots and growth of plantlets, development in basal MS medium was found to be essential. Finally, the rooted plants were transferred to pots and maintained under greenhouse conditions. Rooted

plants were successfully acclimatized in pre-sterilized soil with more than 80% survival.

Kanamycin resistance test

In the kanamycin resistance test, the effect of 0, 15, 25, 50, 75, and 100 mg l⁻¹ kanamycin was studied on direct shoot regeneration in *L. draba* explants. The cultures became yellowish in the second week. In this test, the regeneration frequency decreased with increase in kanamycin concentration. Since the reduction of regeneration frequency between 25 and 100 mg l⁻¹ kanamycin concentrations did not show significant difference, therefore, we chose 25 mg l⁻¹ kanamycin concentration for the gene transformation experiment.

Agrobacterium-mediated transformation

Using the appropriate direct shoot regeneration protocol for *L. draba*, we carried out a good number of *Lepidium* transformations. Derived explants from the in vitro germinated seedlings of Hoary cress, tobacco, and canola were inoculated with *Agrobacterium* cells and co-cultivated on co-cultivation medium for 2 day in the dark. The preparation and treatment of explants were performed carefully to avoid damage that reduces the subsequent regeneration frequency. After co-cultivation, the explants were sub-cultured in regeneration medium containing 200 mg l⁻¹ cefotaxime and 25 mg l⁻¹ Kanamycin to select transgenic cells. When *L. draba* explants were co-cultivated with *Agrobacterium*, no decline in the shoot regeneration efficiency was observed. Direct shoot regeneration in the explants of *L. draba* started to occur in 2–3 weeks after *Agrobacterium* infection. While the most shoot regeneration happened between 3 and

Table 2 Effect of different concentrations of BAP and NAA on direct shoot regeneration from explants of *L. draba*

Concentration (mg l ⁻¹)		% of explants showing direct shoot regeneration (mean ± SE)			Average number shoot per explants ± SE		
NAA	BAP	Cotyledons	Hypocotyls	Roots	Cotyledons	Hypocotyls	Roots
0	0 (control)	13 ± 2 ^{cde}	7 ± 6 ^{fg}	40 ± 4 ^f	2 ± 1 ^{ef}	2 ± 1 ^{fg}	4 ± 1 ^l
	0.5	13 ± 2 ^{cde}	87 ± 5 ^b	100 ± 0 ^a	1 ± 0 ^{fg}	4 ± 1 ^{ef}	13 ± 1 ^b
	1	20 ± 5 ^{bcd}	93 ± 2 ^a	100 ± 0 ^a	5 ± 0 ^{de}	8 ± 1 ^{ab}	16 ± 2 ^a
	2	13 ± 4 ^{cde}	40 ± 6 ^{cd}	60 ± 6 ^{def}	1 ± 1 ^{ef}	4 ± 0 ^{ef}	4 ± 1 ^j
	3	27 ± 6 ^{bcd}	80 ± 2 ^b	100 ± 4 ^a	4 ± 0 ^e	6 ± 1 ^{bcd}	12 ± 2 ^{bc}
0.2	4	7 ± 3 ^{de}	73 ± 3 ^b	60 ± 1 ^{def}	1 ± 1 ^{fg}	1 ± 0 ^{gh}	2 ± 1 ^m
	0	13 ± 7 ^{cde}	20 ± 7 ^{def}	73 ± 2 ^{cde}	1 ± 1 ^{fg}	1 ± 1 ^{gh}	3 ± 1 ^m
	0.5	73 ± 3 ^a	47 ± 5 ^c	87 ± 7 ^{bc}	7 ± 0 ^{bcd}	1 ± 0 ^{gh}	4 ± 1 ^j
	1	7 ± 8 ^{de}	20 ± 4 ^{def}	87 ± 7 ^{bc}	2 ± 1 ^{ef}	3 ± 1 ^{fg}	4 ± 0 ^k
	2	13 ± 7 ^{cde}	20 ± 7 ^{def}	100 ± 0 ^a	1 ± 0 ^{fg}	1 ± 1 ^h	5 ± 0 ^{hi}
0.5	3	20 ± 6 ^{bcd}	33 ± 5 ^{def}	80 ± 4 ^{cd}	4 ± 1 ^e	1 ± 0 ^{gh}	3 ± 1 ^l
	4	7 ± 5 ^{de}	33 ± 3 ^{de}	100 ± 0 ^a	1 ± 0 ^{fg}	4 ± 1 ^{ef}	6 ± 0 ^{gh}
	0	27 ± 2 ^{bcd}	40 ± 6 ^{cd}	93 ± 2 ^{ab}	2 ± 0 ^{ef}	3 ± 1 ^{fg}	4 ± 1 ^k
	0.5	7 ± 8 ^{de}	13 ± 8 ^{efg}	100 ± 0 ^a	0 ± 0 ^{fg}	1 ± 0 ^{gh}	3 ± 1 ^m
	1	13 ± 6 ^{cde}	0 ± 0 ^g	100 ± 0 ^a	3 ± 0 ^{ef}	0 ± 0 ^h	5 ± 0 ^{gh}
1	2	7 ± 7 ^{de}	20 ± 7 ^{def}	93 ± 2 ^{ab}	2 ± 0 ^{ef}	8 ± 1 ^a	6 ± 0 ^{gh}
	3	27 ± 5 ^{bcd}	27 ± 6 ^{def}	87 ± 4 ^{bc}	3 ± 0 ^{ef}	5 ± 1 ^{cde}	6 ± 1 ^{fg}
	4	20 ± 6 ^{bcd}	0 ± 0 ^g	100 ± 0 ^a	4 ± 1 ^e	0 ± 0 ^h	7 ± 0 ^{fg}
	0	13 ± 7 ^{cde}	33 ± 5 ^{de}	87 ± 3 ^{bc}	3 ± 1 ^e	7 ± 0 ^{bc}	5 ± 0 ⁱ
	0.5	27 ± 5 ^{bcd}	0 ± 0 ^g	80 ± 5 ^{cd}	13 ± 1 ^a	0 ± 0 ^h	7 ± 0 ^{ef}
2	1	7 ± 6 ^{de}	7 ± 2 ^{fg}	60 ± 0 ^{def}	6 ± 1 ^{cd}	0 ± 0 ^h	5 ± 1 ^{hi}
	2	7 ± 5 ^{de}	27 ± 3 ^{def}	47 ± 5 ^{ef}	3 ± 1 ^{ef}	4 ± 1 ^{de}	8 ± 0 ^{ef}
	3	13 ± 4 ^{cde}	20 ± 3 ^{def}	73 ± 3 ^{cde}	2 ± 0 ^{ef}	4 ± 1 ^{ef}	8 ± 1 ^{ef}
	4	33 ± 2 ^{bc}	7 ± 7 ^{fg}	80 ± 4 ^{cd}	9 ± 1 ^{abc}	1 ± 0 ^{gh}	6 ± 1 ^{gh}
	0	0 ± 0 ^e	7 ± 4 ^{fg}	67 ± 6 ^{def}	0 ± 0 ^g	0 ± 0 ^h	5 ± 0 ^{hi}
	0.5	33 ± 3 ^{bc}	0 ± 0 ^g	93 ± 2 ^{ab}	10 ± 1 ^{ab}	0 ± 0 ^h	7 ± 0 ^{ef}
	1	47 ± 3 ^b	20 ± 3 ^{efg}	40 ± 6 ^f	5 ± 0 ^{cd}	2 ± 1 ^{fg}	6 ± 0 ^{fg}
	2	13 ± 7 ^{cde}	7 ± 5 ^{fg}	87 ± 4 ^{bc}	6 ± 1 ^{bcd}	2 ± 1 ^{gh}	6 ± 1 ^{fg}
	3	33 ± 8 ^{bc}	0 ± 0 ^g	53 ± 5 ^{def}	9 ± 1 ^{abc}	0 ± 0 ^h	11 ± 2 ^{cd}
	4	27 ± 7 ^{bcd}	7 ± 8 ^{fg}	60 ± 0 ^{def}	7 ± 1 ^{bcd}	2 ± 1 ^{gh}	12 ± 1 ^{bc}

L. draba explants were cultured on MS medium supplemented with the different concentrations of NAA and BAP. Data were recorded after 1 month. Variability around the mean was represented as ±SE. Data having the same letter in a column were not significantly differed by Duncan's multiple comparison test ($P < 0.05$)

Table 3 Influence of various explants on direct shoot regeneration from *L. draba*

Explant type	Shoot induction (%) ±SE	No. of shoots per explants ±SE
Cotyledon	19 ± 3 ^b	3 ± 1 ^b
Hypocotyl	26 ± 5 ^b	2 ± 0 ^c
Root	80 ± 3 ^a	6 ± 1 ^a

Various explants were cultured on MS medium supplemented with different concentrations NAA and BAP. Data were recorded after 1 month. Variability around the mean was represented as ±SE. Data having the same letter in a column were not significantly differed by Duncan's multiple comparison test ($P < 0.05$)

Fig. 2 Regeneration of various transformed explants on Kanamycin antibiotic selection medium. **a** *L. draba* cotyledons, **b** *L. draba* hypocotyls, **c** *L. draba* roots, **d** *B. napus* cotyledons, and **e** *N. tabacum* cotyledons

4 weeks (Fig. 2a–c), this regeneration lasted about 1 month. Direct shoot regeneration in *B. napus* occurred after 2–3 weeks (Fig. 2d) and in 1–2 months developed. After 2 weeks in *N. tabacum*, direct somatic embryogenesis was observed. Development of somatic embryos was progressed in the 4th week and mature plants were obtained after 1–2 months (Fig. 2e).

The rooting percentage of transgenic lines on the rooting medium (MS medium supplemented with 0.2 mg l^{-1} NAA) was close to 100%. Rooting of the transgenic lines could happen automatically on the proliferation medium in some cases, especially in root explants of *L. draba*. When the expansion of the roots in MS medium was slow or stopped, the possibility of escape was very high. All rooted plants were shifted in pots and the analysis was performed to confirm the transgenic nature. The establishment of plantlets in greenhouse was nearly 70%. The plants which grew well on the selection medium were considered as transgenic lines and thus used for confirmation of transformation. In these plants, the putative transgenic lines were first verified by GUS staining before sufficient amount of plant materials was obtained for PCR analysis. The β -glucuronidase enzyme activity was detected histochemically as described. The strong GUS expression was

observed in the tissues of the putative transgenic lines, while no blue color was observed for non-transgenic as control (Fig. 3).

Besides the histochemical GUS assay that confirmed the transfer and expression of GUS, the integration of GUS gene was also carried out by PCR analysis. Purified genomic DNA samples of putatively transformed and non-transformed plants were subjected to PCR analysis using specific primers. The expected amplicon size of 2098 bp of the GUS gene was observed on agarose gel from transformed plants only. Please confirm the section headings are correctly identified (Fig. 4a).

For detection of possible contamination of DNA templates with *A. tumefaciens* containing pBI121, another PCR analysis was performed on the samples with forward (PBI-F) and reverse (PBI-R) primers of in plasmid. We showed that all randomly selected transformants were PCR positive for GUS gene; no one detected any contamination with DNA of *A. tumefaciens* containing pBI121 (Fig. 4b). Therefore, in the first step with 35S-F and NOS-R primers, PCR analysis indicated that the tested plants were positively transformed with GUS transgene. In the next step with PBI-F and PBI-R, primers proved that the transformed plants have no bacterial contamination.

Fig. 3 GUS histochemical analysis of transformed and non-transformed plants: **a** non-transgenic *L. draba* control; **b** transgenic lines of *B. napus* (left) and *N. tabacum* (right); **c** transgenic Lines of *L. draba*,

d the appearance of blue color in the tissues of transgenic *L. draba* ($\times 4$); **e** the appearance of blue color in the cells of transgenic *L. draba* ($\times 400$)

Transformation frequency was calculated for different explants of three plants transformed with four *Agrobacterium* strains. Inoculated explants yielded transformation with an efficiency between 21% in *B. napus* infected by LBA4404 and 92% in hypocotyls of *L. draba* infected by GV3850 (Table 4). Means-comparison test revealed that

transformation frequency achieved with *A. tumefaciens* strain GV3850 (58%) was highest, while no significant difference was observed in transformation frequency with LBA4404, GV3101, and EHA105 (Table 4).

Among the different explants of these three plants, hypocotyl (77%) and root (67%) explants of *L. draba* were

Fig. 4 PCR analysis: **a** screening for the presence of GUS gene via 35 SF and NOS-R primers: SM = Marker (100–3000 bp); PCR amplification of GUS gene in putative transgenic plants regenerated from: 1 = *L. draba* cotyledons, 2 = *L. draba* hypocotyls, 3 = *L. draba* roots, 4 = *N. tabacum* cotyledons, 5 = *B. napus* cotyledons; 6 = positive control (isolated plasmid DNA from bacterial cells); 7 = non-transformed *L. draba*; 8 = negative control. **b** Screening for

detecting possible contamination of samples with *Agrobacterium* DNA via PBI-F and PBI-R primers: SM = Marker (100–3000 bp); 1 = positive control (isolated plasmid DNA from bacterial cells); PCR amplification from the transgenic lines regenerated from 2 = *L. draba* cotyledon, 3 = *L. draba* hypocotyl, 4 = *L. draba* root, 5 = *N. tabacum* cotyledon, 6 = *B. napus* cotyledon; 7 = negative control

found to be best for transformation, because their frequencies were highest. Transformation efficiency recorded in cotyledons of *L. draba* was more than *N. tabacum*. However, they were not significantly different. The lowest frequency was observed in *B. napus* (Table 4). Thus, hypocotyls and roots of *L. draba* were highly susceptible to *Agrobacterium*-mediated gene transfer compared with other explants. Infected root segments displayed very high direct shoot regeneration rates, often with numerous shoots per explants as a consequence.

Discussion

In this study, optimal culture conditions to regenerate direct shoots of *L. draba* in vitro were assessed. Shoot formation without any callusing stage in some plants was studied (Bhat et al. 2010; Cheng et al. 2001; Nagori and Purohit 2004). We believe that the development of an

optimized regeneration system would greatly facilitate genetic transformation and other molecular studies in this plant. The effect of BAP and NAA on in vitro regeneration has been widely reported in various plant species (Ghnaya et al. 2008; Guo et al. 2005; Ray et al. 2011). The beneficial effects of the combination of BAP and NAA in direct shoot induction were reported recently in *L. draba* (Ghotbzadeh et al. 2015; Zinhari et al. 2016). Therefore, to determine the suitable levels of BAP and NAA for direct shoot regeneration of *L. draba*, combination of different concentrations of these growth regulators was investigated. The evaluation of shoot induction was based on the frequency of direct shoot regeneration in each treatment and the average number of shoots per explant. In this study, the results indicated that BAP treatment with low level of NAA was probably required for the optimal shoot formation from explants of *L. draba*. Therefore, the presence of BAP has a special role in regeneration of direct shoots in this plant. Moreover, in direct shoot regeneration of *B.*

Table 4 Transformation efficiency of transformed different explants of *L. draba*, *N. tabacum*, and *B. napus* with four different strains of *A. tumefaciens*

<i>Agrobacterium</i> strains	Explants				
	Cotyledons of <i>L. draba</i>	Hypocotyls of <i>L. draba</i>	Roots of <i>L. draba</i>	Cotyledons of <i>N. tabacum</i>	Cotyledons of <i>B. napus</i>
LBA4404	42 ^{ef}	83 ^{ab}	83 ^{ab}	37 ^{efg}	21 ^g
GV3850	58 ^{bcd}	92 ^a	50 ^{def}	54 ^{cd}	50 ^{def}
GV3101	58 ^{bcd}	83 ^{ab}	67 ^{abc}	29 ^{fg}	29 ^{fg}
EHA105	46 ^{ef}	50 ^{def}	67 ^{abc}	50 ^{def}	25 ^{fg}
	51 ^b	77 ^a	67 ^a	43 ^b	31 ^c

The means were compared using Duncan's test at $P \leq 0.05$

oleracea, BAP is needed for high frequency which considerably increased the number of shoots produced per explants (Cheng et al. 2001). It is obvious that high auxin concentrations induce callus formation, but exclude shoot regeneration (Ivarson et al. 2013). Organogenesis in vitro depends not only on the type of plant growth regulators, but also on the ability of tissues to respond to growth regulators changes (Akbaş et al. 2009). High dependence of regenerative capacity on tissue was reported by Cheng et al. (2001). In our investigation, direct shoot formation was observed from all types of *L. draba* explants after they were cultured on MS medium supplemented with BAP and NAA, but with different frequencies. Interestingly, explants of *L. draba* in direct shoot induction medium displayed a short regeneration period. Therefore, we were able to produce complete plants quickly. This means saving the time for the production of transformed plants. The entire plant regeneration protocol from explant to complete plant took about 1.5–2 months.

The root explants were found to be more regenerative than the hypocotyls and cotyledons for shoot induction. Researchers showed that among the four tested explants of *B. napus* (peduncles, hypocotyls, cotyledons, and leaf petioles), cotyledons produced the highest shoot regeneration frequency (56.67%), Ghnaya et al. (2008) reported that the hypocotyls of *B. napus*, compared with the ability of different explants, exhibited a high rate of rapid shoot regeneration via caulogenesis (Tang et al. (2003).

In this study, shoot buds were developed from all over the root segments, but in hypocotyls observed commonly in the cut ends. In cotyledon explants, direct shoot formation occurred in the region where the petiole was cut and margins of cotyledons that were in contact with medium. Shoot buds first appeared at the cut ends of cotyledons after 10 days in vitro. This is in line with the findings of Cheng et al. (2001). However, researchers reported that direct shoot regeneration was observed only at the cut ends of cotyledonary petioles of *B. rapa* and the hypocotyls did not form shoots. These results indicate that a significant difference exists in shoot regeneration from specific explants in species of Brassicaceae.

In our study, supplementing the medium with 1 mg l^{-1} BAP without NAA significantly increased the frequency of direct shoot regeneration and the number of shoots formed per explants in roots. On this medium, 100% of explants were induced to produce an average of more than 16 shoots. In addition, 1 mg l^{-1} BAP induced the maximum shoot regeneration frequency and a relatively high level of production of shoots per explant in hypocotyls. The highest direct shoot regeneration frequency could occur from cotyledons in 0.5 mg l^{-1} BAP and 0.2 mg l^{-1} NAA, while the maximum shoot regeneration frequency from cotyledons of *B. napus* was obtained in MS medium

supplemented with 3 mg l^{-1} BAP and 0.15 mg l^{-1} NAA (Tang et al. 2003). In shoot regeneration of *B. napus* that is reported previously, 4.5 mg l^{-1} BAP alone was proved to be essential for direct shoot regeneration of cotyledons.

Therefore, in *L. draba*, the best concentrations for direct shoot regeneration were 1 mg l^{-1} BAP without NAA when using hypocotyls and roots as explants and 0.5 mg l^{-1} BAP and 0.2 mg l^{-1} NAA in cotyledons. Thus, these concentrations were chosen for the subsequent transformation experiments.

Occurrence of roots in the regenerated shoots on NAA-supplemented media was remarkably successful. The presence of NAA in the root induction medium indicated the percentage root formation to nearly 100%. In *B. oleracea*, the percentage of root formation was increased from 34% in control to almost 90% in the root induction medium supplemented with NAA (Cheng et al. 2001).

We have developed an in vitro system for rapid- and high-frequency generation of *L. draba*. As this system produces shoots directly and quickly without the involvement of a callus phase, it greatly reduces somaclonal variation which is a more desirable method for gene transfer.

In this experiment, *L. draba* has been more successful both in transformation with *A. tumefaciens* and produces a complete transgenic plant. However, any gene transfer has not been reported to this plant so far. In addition to *L. draba*, the cotyledons of *N. tabacum* and *B. napus* were infected by *A. tumefaciens* as controls. Various explants are used for transformation experiments in *B. napus* (Cardoza and Stewart 2003; Charest et al. 1988). In this study, for comparison with *L. draba*, cotyledons of *B. napus* were chosen as explants for further optimization of in vitro direct shoot regeneration (Kahrizi et al. 2010).

This transformation protocol is reproducible and simple, and enables us to produce transgenic plants at a frequency on average exceeding 70% in the hypocotyls of *L. draba* inoculated with *A. tumefaciens*, which is considerably higher than in the previous reports on *Agrobacterium*-mediated transformation. The data from the study suggest that these plants were readily transformed with *A. tumefaciens* strain GV3850. On the basis of visual analysis, transformed plants with this strain revealed strong blue coloration in all transformed tissues. This is in agreement with some previous studies which reported variability in GUS protein expression of plants upon transformation with different strains of *A. tumefaciens* (Alvarez and Ordás 2013; Wroblewski et al. 2005; Yadav et al. 2014). The analysis of the number of transgenic plants among all regenerated plants showed that the infected hypocotyl and root explants from *L. draba* considerably yielded high transformation efficiency compared to various explants tested in this study. Similar results are reported which hypocotyls explants are

suggested as the best explants in regeneration and transformation in canola (Zhang and Bhalla 2004). In addition, researchers showed that root explants are successfully used to develop a reliable *A. tumefaciens*-mediated transformation *Arabidopsis thaliana* (Valvekens et al. (1988). Because of its importance as an oilseed, there are many reports of gene transformation of Brassicaceae, especially *B. napus*. Compared with our results, frequency of transformation in these reports was relatively lower. Cardoza and Stewart (2004) report 17–25% of transformation frequency of hypocotyl explants obtained from 8- to 10-day-old seedlings of *B. napus* using callus induction. Transformation frequency of canola reported by Ramzan Khan et al. (2003) is 48%. Ray et al. (2004) shows that transformation frequency of *B. juncea* is between 3.8 and 10.2%. Transformation of 5-day-old cotyledons of *B. rapa* reported only 9% by Wahlroos et al. (2003). Ivarson et al. (2013) show that gene transformation frequency of 6-day-old hypocotyls of *L. compestre* is 6%. However, gene transformation frequencies reported by different researchers cannot be compared because of the various conditions. The type of explant is important for enhancing transformation rates. Although variations were observed among genotypes, different studies detect a higher transformation frequency from hypocotyl explants. Root explants of *L. draba* have a high potential for rapid shoot generation. Fertile plants can be generated reproducibly within 1.5–2 months. The high occurrence of shoot establishment and the histological simplicity of *L. draba* root segments make this a desirable system to study initiation and development of shoots (Zinhari et al. 2016). By cutting root explants at short intervals during regeneration, the tissues giving rise to shoot can be identified. We incorporated this regeneration method with *A. tumefaciens* infection to develop an efficient and rapid transformation procedure. According to this study, we were able to transform *L. draba* successfully using hypocotyl and root explants. Due to high and easily regeneration and efficient transformation, *L. draba* could be ideally suited for cloning genes by implement complementation experiments. It has the potential to become a viable host system for foreign protein production. This plant is edible; therefore, it can be used to produce recombinant proteins, which are taken orally. With the successful conclusion of the model plants like *Arabidopsis* and rice, an important role of these plants in development of many basic studies has been found. Specific features of some plants are impossible to study fully in the available models. *L. draba* has a short generation time, small size, easily grown at high density in glasshouse or culture room, and produces large number of seeds, and ability to produce direct shoot in vitro (Jacobs 2007). This plant has also different ploidy levels (Bon et al. 2005; Mulligan 2002; Mulligan and Frankton 1962). It is

easy for transformation efficiently by *A. tumefaciens* based on this research. These features are similar to those of *Arabidopsis*. Consequently, *L. draba* can be considered to be a model plant, provided that the complementary experiments perform to assess the feasibility of the model system. Therefore, for the first step, we have developed an efficient transformation protocol.

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Author contributions ZZ and ShP developed the regeneration experiments, produced the transformed plants, confirmed the transgenic lines, analyzed the data, and wrote the manuscript, ShP conceived the project, contributed to the data analysis, and edited the final manuscript; JZ just helps to prepare the transformed bacteria.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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