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Original article – Thematic Issue

Approaches to improve the transgenic efficiency and to rescue seedlings from hyperhydricity for rapeseed (*Brassica napus*)

Yongqiang Ding, Dingyu Zhang, Guoying Yin and Wenjing Wang

Key Laboratory of Tobacco Pest Monitoring Controlling & Integrated Management, Tobacco Research Institute, Chinese Academy of Agricultural Sciences, Qingdao, People's Republic of China

Summary

Transgenic techniques play an important role in functional genomics research of *Brassica napus*, but an optimized, efficient transformation system has not been developed for this specie. The major difficulties come from the long regeneration period, low regeneration rate, and high hyperhydricity rate. In this study, we established a method with modifications on the callus-induction step, which was performed at the absence or low level of selective antibiotic in previous approaches. By adding selective antibiotic in callus-induction medium, the new method greatly enhanced the elimination of untransformed explants and shortened the period of seedling regeneration. We also established a convenient solution to rescue transgenic seedlings from hyperhydricity, a serious problem during the process of rapeseed transformation. The application of this method could produce transgenic seedlings ready for transplantation in less than eight weeks. Taken together, we have established an efficient, simple transformation system to produce transgenic rapeseed seedlings rapidly and efficiently.

Keywords

Agrobacterium-mediated transformation, tissue culture, explant, transgenic seedlings

Significance of this study

What is already known on this subject?

- *Agrobacterium*-mediated transformation is the most frequently applied technique for transformation of *B. napus*. But the efficiency to obtain transgenic *B. napus* seedlings is still at a pretty low level. The major difficulties come from the long regeneration period and low regeneration rate. Another big problem is the high hyperhydricity rate.

What are the new findings?

- We established an improved transformation method for *B. napus* that can significantly shorten the period for obtaining transgenic seedlings, and developed a simple treatment to rescue hyperhydrated seedlings of transgenic *B. napus*, which can effectively convert hyperhydrated seedlings into normal, healthy plants.

What is the expected impact on horticulture?

- These new methods can rapidly improve the transformation efficiency of rapeseed, which can accelerate the development of functional genomics of rapeseed, and it can also be used as an important reference for other horticultural plants.

Introduction

Brassica napus, or rapeseed, is one of the world's four major oil crops and also an important source of edible vegetable oil and protein. Functional genomics is a rapid progressing research aspect in this specie, in which transgenic technology plays a critical role. Currently, two main techniques are used for genetic transformation of *B. napus*. One technique is *Agrobacterium*-mediated transformation; the other is direct introduction of exogenous genes via particle bombardment, electroporation, laser microbeam irradiation, microinjection, pollen-tube pathway or polyethylene glycol-mediated methods (Bergman and Glimelius, 1993; Herve et al., 1993; Chen et al., 1994; Jones-Villeneuve et al., 1995; Radchuk et al., 2002; Sparrow et al., 2007; Liu et al., 2012). In recent years, *Agrobacterium*-mediated transformation has become the most frequently applied technique for transformation of *B. napus* owing to the low-cost and feasible application. Following the first successful application of *Agrobacterium*-mediated transformation to *B. napus* by Ooms et al. (1985), the transgenic technology has greatly progressed (Moloney et al., 1989; Gupta et al., 1993; Wang et al., 2003; Zhang et al., 2005; Sparrow et al., 2007; Boszoradova et al., 2011; Liu et al., 2012, 2015). But, the efficiency to obtain transgenic *B. napus* seedlings with current technologies is still at a low level (Bhalla and Singh, 2008; Liu et al., 2015). The major difficulties come from the long regeneration period and low regeneration rate, which strikingly increased the degree of difficulty in obtaining transgenic seedlings. Another big problem in developing transgenic seedling of *B. napus* is the high hyperhydricity rate (Cardoza and Stewart, 2003; Sheng et al., 2016). Hyperhydricity, also known as vitrification, is a common phenomenon during plant tissue culture and has harmful effects on callus regeneration (Debergh et al., 1981; Sheng et al., 2016), which seriously hinders tissue culture of genetically transformed *B. napus*. Although many studies have revealed the causes and mechanism of hyperhydricity, no effective approach has been established to eliminate hyperhydricity in *B. napus*. The above problems are technical barriers to the application of transgenic technology to *B. napus* and the high-throughout functional genomics research on this specie. In this study, we established an improved transformation method for *B. napus* that can significantly shorten the period for obtaining transgenic seedlings, and developed a simple treatment to rescue hyperhydrated seedlings of transgenic *B. napus*, which can effectively convert hyperhydrated seedlings into normal, healthy plants. The method developed in this study could quickly and rapidly generate transgenic plants of *B. napus*.

Materials and methods

Plant material, plasmid vector, and *Agrobacterium* strain

The plant material used to develop transformation method for rapeseed was *Brassica napus* cv. Zhongshuang11. A fragment of *BrMYC2* gene (NCBI accession: XM_013880351) was amplified with primer 5'-CGACGATAACGCCTCTATGA-3' and 5'-CATCCCAAACACTCCTCCTT-3', and cloned into pHZPRi–Hyg vector to generate *BrMYC2*-silencing vector as described previously (Zhang et al., 2012). Then, this vector was introduced into *Agrobacterium* strain LBA4404, and *Agrobacterium*-mediated method was used to develop transgenic plants of *B. napus*.

Medium preparation

The base tissue culture medium in this study was MS (Murashige and Skoog, 1962) medium with 3% (w/v) sucrose and the pH was adjusted to 5.8. For solid medium, 2.6 g Phytagel L^{-1} was added. Other supplements to the medium as described in Table 1 were added as following: $AgNO_3$ and phytohormones were added before autoclaving, and acetosyringone and antibiotics were added after autoclaving. For *Agrobacterium* culture, YEB medium (1 g yeast extract L^{-1}, 5 g tryptone L^{-1}, 5 g beef extract L^{-1}, 5 g sucrose L^{-1}, 0.5 g $MgSO_4 \cdot H_2O$ L^{-1}, pH 7.2) was prepared.

Explants preparation

Seeds of *B. napus* cv Zhongshuang11 were surface-sterilized in disinfectant (400 µL commercial bleach and 5 µL TWEEN 20 per 10 mL sterilized water) for 7 min. Approximately 20 sterilized seeds were placed in tissue culture bottles containing 50 mL germination medium (MS medium containing 30 g sucrose L^{-1} and 2.6 g Phytagel L^{-1}, pH 5.8). The bottles were maintained at 25°C under a 14 h light/10 h dark photoperiod for 8–10 days. Hypocotyls that had grown 6–8 cm long were cut into 1 cm long pieces and placed on a clean surface. These hypocotyl pieces were cultured for 3 days on MSp medium (Table 1) for use as explants in *Agrobacterium*-mediated transformation.

Agrobacterium-mediated transformation and regeneration

A single colony of *A. tumefaciens* strain LBA4404 containing the *BrMYC2*-silencing vector was inoculated to 5 mL of liquid YEB medium containing 50 mg kanamycin L^{-1} and 50 mg rifampicin L^{-1} at 28°C overnight under shaking. Cultured bacteria with an OD_{600} of 0.6–0.8 were collected by centrifugation at 6,500 *g* for 2 min and gently suspended in 70 mL of *Agrobacterium*-inoculation co-cultivation (MSc) medium. Three independent infections were performed for each transformation. For each infection, approximately 100 explants were incubated with 50 mL *Agrobacterium* suspension by immerging hypocotyls pieces into the suspension for 7 min with gentle shaking. After infection, the bacterial suspension was removed, and the explants were air-dried on sterilized paper towels and subsequently transferred onto MSc medium plates for 2 days co-cultivation in growth room under dark conditions.

Following co-cultivation, explants were transferred to plate containing MSi medium at a density of 50 explants per plate and cultured for at least 14 days until obvious calli were induced. Then, the explants were subcultured onto MSd medium, and transferred onto fresh MSd plate every 2 weeks until shoot buds regenerated. The regenerated hygromycin-resistant shoots of 2 cm in height were excised from the explants and transferred into bottles with MSr medium to root. Regenerated plantlets with well-developed roots except the hyperhydrated seedlings were transferred into flowerpots and maintained in a greenhouse at 23°C under a 14 h light/10 h dark photoperiod. To maintain suitable moisture, the seedlings were covered with Saran Wrap at the first week until the seedlings grown were healthy and robust.

Method for rescuing of hyperhydrated seedlings

Reverse of hyperhydrated seedlings were performed in a laminar flow clean bench with vertical air flow. After cleaning with 75% alcohol, the bench was disinfected by ultraviolet irradiation. Bottles with hyperhydrated seedlings were placed inside the clean bench, with caps removed, and ventilated for 10 min in the air-flow to evaporate the excessive

Table 1. Composition of tissue culture medium used in this study.

Medium	Medium components
MS	MS (Murashige and Skoog) base medium with 3% (w/v) sucrose, pH 5.8
MSp (pre-cultivation medium)	MS basal salts, 30 g L^{-1} sucrose, 2 mg L^{-1} 2,4-D[z], 1 mg L^{-1} 6-BA[y], 2.6 g L^{-1} Phytagel, pH 5.8
MSc (co-cultivation medium)	MS basal salts with 30 g L^{-1} sucrose, 2 mg L^{-1} 2,4-D, 1 mg L^{-1} 6-BA, 2.6 g L^{-1} Phytagel, pH 5.8. And, 20 mg L^{-1} acetosyringone was added after autoclaving.
MSi-0 (unselective callus-induction medium)	MS basal salts with 30 g L^{-1} sucrose, 2 mg L^{-1} 2,4-D, 1 mg L^{-1} 6-BA, 2.6 g L^{-1} Phytagel, pH 5.8. And, 250 mg L^{-1} cefotaxime was added after autoclaving.
MSi (callus-induction medium)	MS basal salts with 30 g L^{-1} sucrose, 2 mg L^{-1} 2,4-D, 1 mg L^{-1} 6-BA, 2.6 g L^{-1} Phytagel, pH 5.8. And, 250 mg L^{-1} cefotaxime and 15 mg L^{-1} hygromycin were added after autoclaving.
MSd (differentiation medium)	MS basal salts with 30 g L^{-1} sucrose, 1 mg L^{-1} 6-BA, 2 mg L^{-1} [x]ZT, 5 mg L^{-1} $AgNO_3$, 2.6 g L^{-1} Phytagel, pH 5.8. And, 250 mg L^{-1} cefotaxime and 15 mg L^{-1} hygromycin were added after autoclaving.
MSr (rooting medium)	1/2 MS basal salts with 15 g L^{-1} sucrose, 2 mg L^{-1} [w]NAA, 4 g L^{-1} Phytagel, pH 5.8. And, 250 mg L^{-1} cefotaxime was added after autoclaving.

[z] 2,4-D, 2,4-dichlorophenoxyacetic acid.
[y] 6-BA, 6-benzyladenine.
[x] ZT, *trans*-zeatin.
[w] NAA, naphthaleneacetic acid.

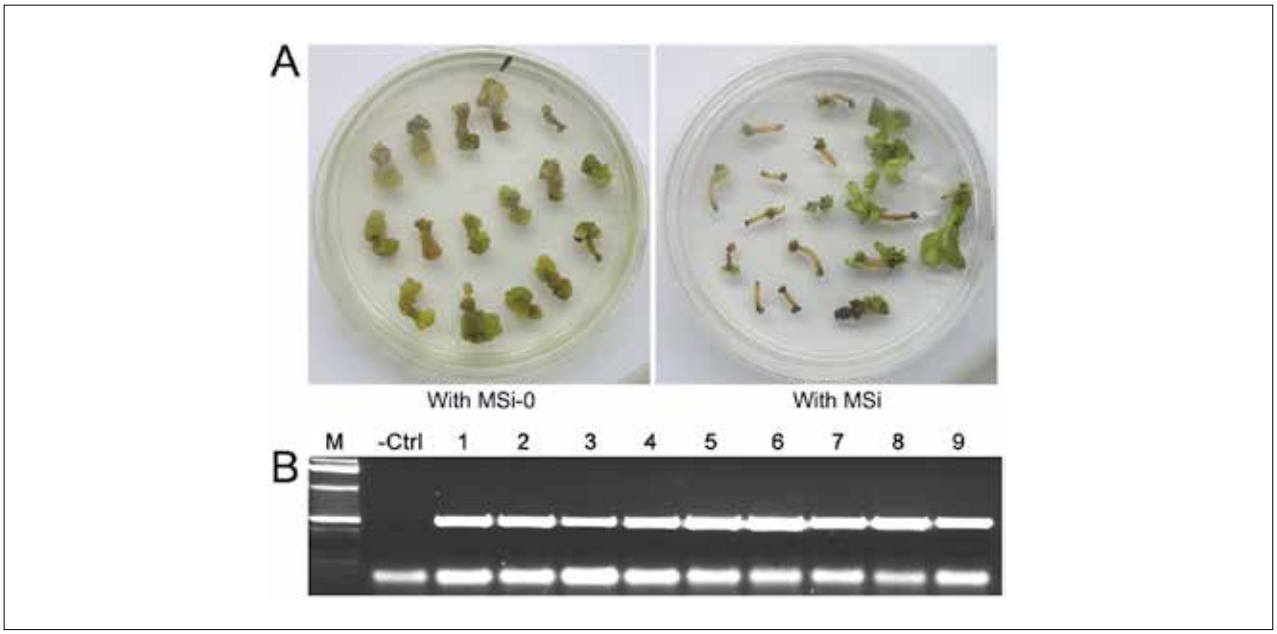


Figure 1. Callus induction and seedling regeneration. (A) Calli and regenerated shoots 6 weeks after the co-cultivation. The calli formed on MSi medium are smaller and tighter than those on MSi-0 medium and also started seedling regeneration much earlier. With MSi-0, calli formed in the method with MSi-0 as callus-induction medium (left). With MSi, calli and shoots formed in the method with MSi as callus-induction medium (right). (B) Identification of transgenic seedling by PCR amplification of genomic DNAs. All identified plants are true transgenic plants, and showing is a representative PCR amplification result. 20 seedlings derived from each transformation methods were determined, and none false transgenic seedling was found. M indicates DNA marker, -Ctrl indicates an untransformed control plant, and numbers indicate different lines transformed with the target vector.

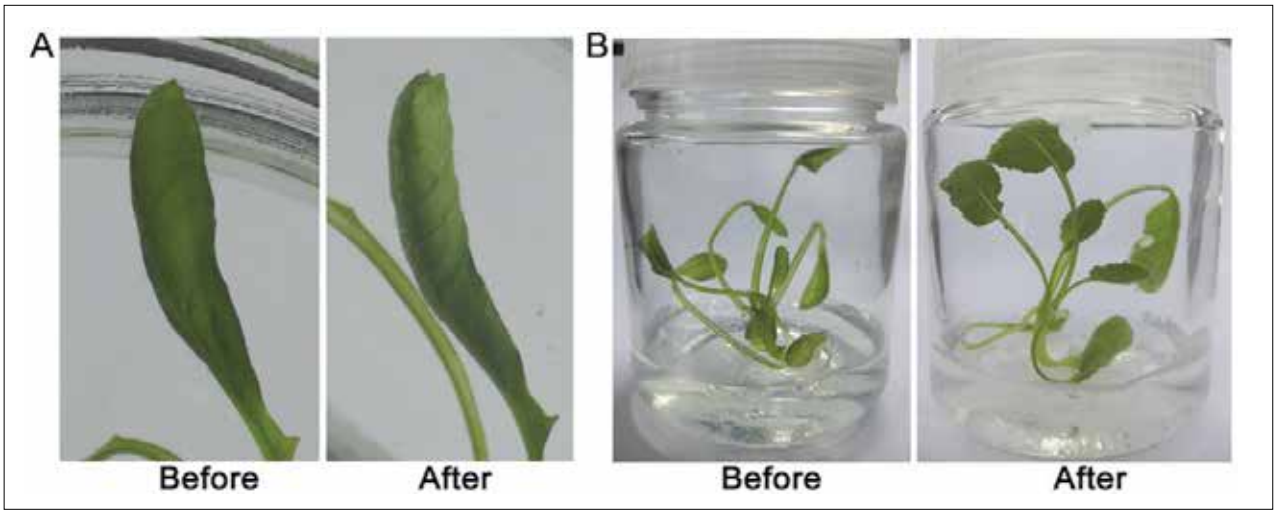


Figure 2. Rescue of hyperhydrated seedlings. (A) Appearance change of hyperhydrated leave after once ventilation treatment, which looks turning back to normal. Before, a hyperhydrated leaf. After, showing the hyperhydrated leaf after once ventilation treatment. (B) Appearance change of hyperhydrated seedling after four times ventilation treatments, which is already a complete normal plant. Before, showing a hyperhydrated seedling. After, showing the hyperhydrated seedling after four times ventilation treatments.

Table 2. Comparison of transformation efficiencies between different methods with hypocotyl explants of *B. napus* cv. Zhongshuang11.

Callus-induction medium in the methods	Time to start regeneration	Time to complete regeneration	Seedling regeneration rate (%)	True transgenic seedlings (%)	Hyperhydricity rate (%)
MSi-0	>8 weeks	~20 weeks	~5	100	19±4
MSi	4 weeks	<12 weeks	>10	100	21±3

moisture in the seedlings. The hyperhydrated seedlings were subjected to ventilation once per day or once every other day. After 4 rounds of such treatment, the hyperhydrated seedlings could be recovered to healthy and robust seedlings. The seedlings could then be transferred into the flowerpot for further growth.

Identification of transgenic seedlings by PCR amplification

Genomic DNA of the transgenic seedlings was extracted with CTAB method as previously described (Zhang et al., 2012). Gene transformation of transgenic plants was confirmed by PCR amplification using vector-specific primer 5'-AGCATTCTACTTCTATTGCAGC-3' and *BrMYC2*-specific primer 5'-CATCCCAAACACTCCTCCTT-3'.

Results and discussion

Improvements in callus induction and seedling regeneration of *B. napus*

Hypocotyl explants, which could be obtained in large amount, were widely used in *Agrobacterium*-mediated transformation of *B. napus*. In most previously developed approaches, a step of callus induction without selective antibiotic was included after the co-cultivation to promote the formation of calli from explants (Liu et al., 2015; Bhalla and Singh, 2008; Boszoradova et al., 2011), i.e., inducing calli on MSi-0 medium (Table 1). In fact, we observed that the period of seedling regeneration from these calli is strikingly long and over three fourths of these calli could not generate transgenic seedlings. Thus, we suspected that these induced calli might have over-dedifferentiated and still contain a large untransgenic portion. To solve this problem, we established a method in which the callus induction was performed on medium with normal level of selective antibiotic, i.e., the MSi medium (Table 1) that contains 15 mg L^{-1} hygromycin. Every two weeks, the explants were replaced with fresh callus-induction medium and the obvious bad or dead explants were discarded. The hypocotyl explants started to form calli after two weeks culture on MSi medium, and the calli are notably smaller and tighter than that formed on unselective callus-induction medium MSi-0 (Figure 1A). The explants with induced calli were transferred onto differentiation medium MSd (Table 1) and replaced with fresh MSd medium every two weeks. Strikingly, the explants handled in the new method started seedling regeneration after two weeks growth on MSd medium, while those from unselective callus-induction medium MSi-0 were still in loose calli (Figure 1A). And, it routinely took more than one month for them turning into tight seedling regenerating calli. When the regenerated seedlings grew to 2 cm tall, they were transferred onto rooting medium MSr in which the selective antibiotic hygromycin was removed. From approximate 300 explants, over 30 seedlings were regenerated in 12 weeks, and 20 of them were randomly selected for transgene identification. Identification of transgenic seedlings with PCR amplification suggested that all seedlings regenerated in the new method are true transgenic plants (Figure 1B). Transformation with the new method could generate transgenic seedlings in two months and about 10% of the initial explants could go through to seedling regeneration in this period, however, it took more than three months for the method using MSi-0 as callus-induction medium and the regeneration rate was less than 3% in three months (Table 2). We speculate the major advantage in the new method is that the addition of normal level of selective antibiotic in callus-induction medium suppressed the formation of untransgenic calli and specifically allowed the formation of transgenic calli. Therefore, the transgenic calli didn't have to compete with the untransgenic ones and could reach a better status for seedling regeneration in shorter time. Previously, scientists ever tried to use lower level of selective antibiotic in both callus-induction and differentiation medium in order to increase the regeneration efficiency, but less than 20% of the regenerated seedlings were true transgene plants (Boszoradova et al., 2011). The earlier application of normal level of selective antibiotic should be the key factor enhancing the ratio of true transgene plants.

Rescuing hyperhydrated seedlings

In developing transgenic rapeseed, hyperhydricity often happens during the seedling regeneration stage, and the hyperhydrated seedling could grow on medium but not survive after being transferred into soil. The hyperhydricity of shoots in tissue culture bottles is mainly attributed to the high relative humidity (Böttcher, 1988). Presumably, the excessive moisture in seedlings made them too weak to be adapted to natural environment. Previous practices suggested that hyperhydricity can be prevented more effectively by reducing relative humidity than by changing the composition of the medium (Rutledge and Douglas, 1988; Castro-Coucha et al., 1990; Cardoza and Stewart, 2003; Sheng et al., 2016), implying that reduction of the excessive moisture is of great importance for the survival of hyperhydrated seedlings. However, this problem has not yet been solved.

In this study, we established a method that could efficiently rescue the hyperhydrated seedlings. In this approach, the hyperhydrated seedlings, with their growth bottles uncapped, were placed inside the clean bench and ventilated for 10 min in the air-flow to evaporate the excessive moisture once per day or once every the other day. As shown in Figure 2A, leaves of the hyperhydrated seedlings would turn into a normal appearance after one ventilation treatment. Generally, the hyperhydrated seedlings could be recovered to healthy and robust seedlings after 4 rounds of this treatment. And, the rescued seedlings could survive in soil as normal regenerated seedlings.

Transplantation of the transgenic seedlings

After the transgenic seedlings, including the rescued hyperhydrated seedlings, had formed healthy roots, they were transplanted into soil and covered with Saran wrap for 10 days in the greenhouse for acclimatization to the controlled environment. Over 95 percent of the seedlings could grow into healthy plants.

In this study, we have established a simple, efficient method for transformation of *B. napus* that can quickly and efficiently yield a high percentage of positive transgenic plants. In addition, we have developed a simple method to eliminate hyperhydricity, and this technique is able to effectively convert hyperhydrated seedlings into normal healthy seedlings. By improving transformation efficiency, the method developed in this study should contribute to the functional genomics research in *B. napus*.

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Addresses of authors:
Yongqiang Ding, Dingyu Zhang, Guoying Yin and Wenjing Wang*
Tobacco Research Institute of Chinese Academy of Agricultural Sciences, No.11 Forth Longitudinal Keyuan Rd., Laoshan District, Qingdao 266101, P.R. China
* Corresponding author; E-mail: wangwenjing@caas.cn