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Research

Keywords: DsRed, agrobacterium-mediated hypocotyl transformation, visual screen, Brassica napus

Posted Date: December 31st, 2019

DOI: https://doi.org/10.21203/rs.2.19547/v1

A Novel Method for High-efficiently Obtaining the Transgenic Lines by combining a convenient, rapid and efficient Visual Screening method and an efficient genetic transformation System in *B. napus*

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Abstract

Background: *Brassica napus* is an important oilseed crop which offer a considerable amount for global vegetable oil production. The establishment of an efficient genetic transformation system with a convenient transgenic-positive screening method is of great importance for gene functional analysis and molecular breeding. However, to our knowledge, there are few such aforementioned systems available for efficient application in *B. napus*.

Results: Based on the well-established genetic transformation system in *Brassica napus*, five vectors companied with red fluorescence protein from *Discosoma sp.* (*DsRed*) were constructed and transformed via *agrobacterium*-mediated hypocotyl transformation. The average of 59.1% tissues were marked with red fluorescence by visual screening method in tissue culture medium, and 96.1% of which were amplified with the exogenous genes in eight different rapeseed varieties on average. In addition, the final transgenic-positive efficiency of the rooted plantlets was up to 90.7% from the red fluorescence marked tissues, which was much higher than that of the previous reports. Besides, *DsRed* also could be applicable to the seedlings, including of seed coats, roots, hypocotyls and cotyledons during seed germination. These results indicate that the high-efficiency genetic transformation system combining with the transgenic-positive visual screening method presented here will help us to efficiently and conveniently obtain transgenic-positive rapeseed plantlets.

Conclusion: A novel method was developed for rapidly, conveniently and high-efficiently obtaining the transgenic lines, which would help to obtain the higher proportion of transgenic positive regenerated plantlets, thereby avoiding the long period plant regeneration. This will benefit the gene functional study especially in high throughput molecular biology research.

Keywords: *DsRed*, *agrobacterium*-mediated hypocotyl transformation, visual screen, *Brassica napus*

Introduction

Brassica napus is an important oilseed crop ranking the second oilseed production worldwide [1], which was derived from the hybridization between *B. rapa* and *B. oleracea* [2]. To satisfy the increasing demand, it is essential to improve various important economically and agriculturally traits by genetic engineering techniques, which was a powerful tool for gene functional analysis and crop improvement [3]. Genetic transformation technique has promoted the improvement of crop varieties by integrating novel genes to satisfy the needs of high-yield and good-quality, such as characters with effective oil production [4], herbicide and disease-resistance [5, 6]. Now, it has been successfully used to improve some major crops, such as soya bean, maize and cotton [7-9]. And new varieties of these aforementioned plants modified by transgenic methods are now planted widely in many countries, bringing great benefits for farmers [10]. However, to our knowledge, the genetic transformation technique in rapeseed is still relatively lower in transformation efficiency and inefficient in positive screening compared with other crops. Therefore, a suitable and favorable genetic transformation system companied with highly efficient screening method is essential for rapeseed breeding and improvement.

To date, several genetic transformation methods have been reported and routinely applied into model plants and major crops, such as *Arabidopsis thaliana* and *Nicotiana Benthamiana*, rice, wheat and maize [11-15]. In *B. napus*, various technologies, including of PEG-mediated DNA uptake [16, 17], electroporation [18], particle bombardment [19], *agrobacterium*-mediated transformation and microspore transfection [20, 21], have been used to obtain the genetically modified plants. Among these technologies, *agrobacterium*-mediated transformation is the most general, reliable and effective method [22-24]. For example, Maheshwari *et al.* (2011) investigated the effect of hormonal combinations, donor plant age and explant types on the transgenic frequency and the regeneration capacity in four different rapeseed lines (nvigor 5020, Westar, Topas and its microspore derivative-Line 4079). They found that the transformation frequency was 54.2 ± 2.5% and 53.7 ± 5.3% in the cultivars of Invigor 5020 and Westar, respectively, while 16.0 ± 0.2% and 13.4 ± 4% for Topas and Line 4079, respectively. However, due to the diverse ability of genetic transformation caused by

distinct genetic backgrounds within the cultivars [14], there still remains recalcitrance in several cultivars not capable to be genetically transformed, especially in commercial varieties.

High efficiently obtaining the transgenic-positive regenerated plantlets are mainly depended on the subsequent selection. Plant regeneration, however, is a time-consuming process which requires long growth cycle, it often also results in undesired abortion and reduced fertility at the same time. Plenty of methods have been frequently used for transgenic-positive plants screening, such as antibiotic, PCR, Southern blot analysis, GFP or GUS staining [14, 25]. These methods, however, are tedious, which are usually performed with leaves, roots or siliques from the regenerated plantlets. Therefore, it is necessary to develop a novel screening method to simplify and promote the process of plant transformation. Recently, red fluorescence protein from *Discosoma sp.* (*DsRed*), similar to *Aequorea victoria* green fluorescent protein (GFP) in the secondary structure, has been applied to identify the transgenic seeds by visual screening in plants [15, 26, 27]. Both the excitation and emission wavelength of *DsRed* (554 nm and 586 nm) are longer than that of GFP (480 nm and 505 nm), which could enable the transgenic-positive screening in higher sensitivity [28, 29]. Although both *DsRed* and GFP do not affect the vegetative and reproductive growth of plant, GFP is more easily to be influenced by the intrinsic chlorophyll in plants [30, 31]. *Stuitje et al.* (2003) found that the sensitivity of *DsRed* was higher than that of GFP in *Arabidopsis* [32]. Thus, *DsRed* has been widely used to mark the transgenic pollen, leaf or seed, even extraplastidic membranes of chloroplasts for visual selection or offspring separation [32-35]. Eckert *et al.* (2005) firstly conducted the *DsRed* and another common reporter protein-GFP expression in *Leptosphaeria spp. and Oculimacula spp.* to observe the interactions between fungal pathogen species and rapeseed in vitro and *in planta* [36]. Zheng *et al.* (2019) used *DsRed*-labeled *Verticillium longisporum* to inoculated with rapeseed in the condition of greenhouse, and confirmed that potential dissemination of *V. longisporum* was systemic beginning from roots to seeds in oilseed rape [37]. However, thus far, *DsRed* hasn't been used for rapid identification neither in tissue culture, nor in the process of seed development in *B. napus*.

The objective of the present study was to construct a novel and high-efficiently method

to obtain the transgenic-positive lines by combining of a rapid, convenient and efficient transgenic-positive screening technology and a highly efficient *agrobacterium*-mediated hypocotyl genetic transformation system in *B. napus*. In this study, the transformation efficiency was higher than that of previous reports. Meanwhile, the *DsRed* was used as a visual screening marker for transgenic-positive plants screening during tissue culture and seed development, which made the screening process easier, more convenient, and highly efficient. With this visual screening method in the calli of early tissue culture stage, the transgenic-positive efficiency in the present study was achieved to 90.7% in the rooted plants, which was very efficient for high-throughput obtaining transgenic-positive lines.

Methods

Plant materials and growth conditions

Allotetraploid rapeseed genotypes used in the present study included spring varieties *B. napus* L. cv. Jia9709, semi-winter varieties *B. napus* L. cv. Jia2016, and 6 commercial winter varieties or the parents of commercial varieties, including of *B. napus* L. (cv. Zhong Shuang 8, Zhong Shuang 11, Zhong You 821, 7633, B351 and Shan3B). The seeds of Jia2016 and Jia9709 were kindly provided by Prof. Chunyu Zhang from Huazhong Agriculture University (Wuhan, China). The seeds of Zhong Shuang 8, Zhong Shuang 11, Zhong You 821 were developed and provided by Institute of oil crops, Chinese Academy of Agricultural Sciences. 7633, B351 and Shan3B were kindly provided by Prof. Dianrong Li from Hybrid Rapeseed Center of Shaanxi Province. These rapeseed varieties were cultured in pots (6 × 6 × 9 cm) in tissue culture room, and vernalized in a cooler at 4 °C for about 4 weeks after 4~6 leaves. All the plantlets were grown in pots (12 cm × 15 cm) in greenhouse with 2500 lux. During their growth, water was supplied three times a week. Aphids were controlled with imidacloprid (Jiangsu Changqing Biotechnology Co., China) and sticky coloured cards (Chunhe, China).

Vector construction and *Agrobacterium*-mediated transformation in rapeseed

For *Bna*A07g17400D and *Bna*C05g34170D over-expression vectors, the CDS fragments were amplified and then purified with QIA quick extraction kit (QIAGEN, America), then linked to pcmabia-1303 plasmid which was linearized with EcoR1 and Bste11 digestion using

in-fusion enzyme referred to In-Fusion® HD Cloning Kit User Manual. For *Bna*A07g17400D knock down vector, two regions (i.e., 364 nt - 839 nt and 575 nt - 986 nt) were designed. And for *Bna*C05g34170D knock down vector, the region of 220 nt - 691 nt was designed. The sense and antisense strands of the two fragments were amplified with R1-S-F/R, R1-A-F/R, R2-S-F/R, R2-A-F/R, R3-S-F/R and R3-A-F/R (**Table S1**), respectively. After amplified and purified, they were cloned into p35S-1390 which was linearized with Sac 1 and SnaB 1 for sense and antisense strands construction, respectively. Afterwards, the marker gene *DsRed*, amplified with designed primers *DsRed*-F/R (**Table S1**), were purified and then cloned into these reconstructed vectors digested with Hind III.

These reconstructed vectors were transformed into *agrobacterium* strain GV3101, and then to identify and select the positive clone for later plant transformation. They were further transformed into rapeseed hypocotyls via the *agrobacterium tumefaciens* mediated method referred to *Zhou et al.* (2002) [38]. Rapeseed seeds surface were cleaned with sterilized water, and sterilized with 70% ethanol for 1 min, then soaked with 50% disinfectant for 3 min, and finally rinsed 3-5 times with sterilized water in aseptic conditions. It was cultivated on 1/2 MS medium for 7-8 days in complete darkness environment. Then, *agrobacterium* was inoculated and grew overnight until the OD600 reached to 0.4 - 1.6, and 2 mL was sucked when the OD600 = 0.4. Note that the volume of *agrobacterium* solution we took should be in the same proportion, that is, 0.5 mL should be sucked when the OD600 = 1.6. It was further suspend in the infected medium (4.4 g/L MS, 30 g/L sucrose, 200 mM AS) while diluting 10 times for 10-15 min hypocotyls infection, and co-cultivated for 1-2 days on co-cultivation medium (4.4 g/L MS, 30 g/L sucrose, 18 g/L Mannitol, 1 mg/L 2,4-D, 0.3 mg/L KT, 200 mM AS and 8 g/L agar) at 25 °C in darkness. After co-cultivation, hypocotyls were transferred onto calli-induced medium (4.4 g/L MS, 30 g/L sucrose, 18 g/L mannitol, 1 mg/L 2,4-D, 0.3 mg/L KT, STS (0.1 M $Na_2S_2O_3$: 0.1 M $AgNO_3$ = 4:1), 300 mg/L timentin, 25 mg/L hygromycin and 8 g/L agar) with 3300 lux. After 20 days, the hypocotyls with embryogenic calli were then transferred into shoot-induced medium (4.4 g/L MS, 10 g/L glucose, 0.25 g/L xylose, 0.6 mg/L MES, 2.0 mg/L ZT, 0.1 mg/L IAA, 3 mg/L $AgNO_3$, 300 mg/L timentin, 25 mg/L hygromycin and 8 g/L agar) for shoots regeneration, and replaced the medium every 2-3

weeks. Regenerated shoots with 3 leaves at least were then transferred into box filled with root formation medium (4.4 g/L MS, 10 g/L sucrose, 1 mg/L IBA, 300 mg/L timentin and 8 g/L agar). Semi-winter plantlets with 4-6 leaves with well-developed roots were vernalized in a cooler at 4 °C for 2~4 weeks, and transplanted into pots as well as cultivated in greenhouse.

Fluorescence observation and imaging

The red fluorescence in the calli and shoots on the medium during the preliminary stage were observed with hand-held green fluorescent flashlight through red filter in the dark, and the pictures were then captured with camera (Nikon, Japan). The red fluorescence in the roots, seed coats, hypocotyls, cotyledons of seedlings were observed at laser confocal fluorescence microscopy FV1000 (Olympus, Japan). The pictures were processed and arranged with photoshop software.

RNA extraction and real-time qPCR

To analyze the relative expression level of *DsRed* and *Bna*A07g17400D gene, a set of roots, seed coats, hypocotyls, cotyledons and their mix samples from seedlings were collected one week after sowing the seeds on the MS medium. Total RNA was extracted using RNAprep Pure Plant Kit (polysaccharides & polyphenolics-rich) (Tiangen, China) referred to the manufacturer's protocol. Then, approximately 1 μL RNA samples were taken to measure the RNA concentration using NanoDrop 2000 spectrophotometer (Termo Scientifc, USA). The cDNA was synthesized with 1 μg RNA using a ReverTra Ace qPCR RT Master Mix kit with gDNA Remover (Toyobo, Japan) according to the manufacturer's protocol, and the cDNA was kept in -20℃.

The Realtime PCR reactions containing 20 μL were performed with SYBR Green Realtime PCR Master mix (Toyobo, Japan) on Stepone Plus (ABI, America) according to the provided protocol. Three technical replicates were analyzed. The relative expression of each gene was calculated using the ΔΔCt methods. Primers used in RT-PCR were listed in **Table S1**.

DNA extraction, PCR-based identification and Southern blotting in transgenic rapeseed plants

Calli and young leaf tissues of T0 plants were collected from greenhouse-grown, and young leaves of T3 positive transgenic plants were collected from field. They were frozen in liquid nitrogen and stored in - 80 °C. Genomic DNA was extracted with NuClean PlantGen DNA kit (CWBIO, China) referred to the manuscript.

To check the positive transgenic plant, the presence of the *Bna*A07g17400D and *Bna*C05g34170D gene were performed by PCR amplifications using the primer pair *Bna*A07g17400D - OE - F/R and *Bna*C05g34170D - OE - F/R for E1 and E2 transgenic lines, respectively, while the presence of the *DsRed* gene using the primer pair Red-F/R in three RNAi lines. The primers are available in **Table S1**.

For Southern blotting analysis in transgenic rapeseed plants, total genomic DNA was extracted using the standard CTAB method to obtain a great amount of DNA [39]. 30 μg DNA from each sample was digested with EcoR I, which was flanking the *DsRed* gene in E1, E2 and RNAi vector, respectively. The digested DNA samples were fractionated on a 0.8% agarose gel and transferred onto a nylon Hybond-N+ membrane with a membrane transfer instrument. The *DsRed* PCR product was labelled with Digoxigenin and used as probe to hybridize with the digested DNA on the membrane. The hybridization and detection steps were performed according to the instructions from the DIG High Prime DNA Labeling and Detection Starter Kit II.

Results

High Efficiency of *Agrobacterium*-mediated Hypocotyl Transformation in Rapeseed

For *agrobacterium*-mediated hypocotyl transformation in rapeseed, over-expression vector pcmabia-1303 and RNAi vector p35S-1390 were used for transformation, respectively (**Figure 1a**). Consequently, two over-expression vectors were constructed for *Bna*A07g17400D and *Bna*C05g34170D (hereafter called E1 and E2), respectively. Besides, three RNAi vectors were also designed and constructed to knock down these two genes, that is, two were for *Bna*A07g17400D (hereafter called R1 and R2) while the third one was for *Bna*C05g34170D (hereafter called R3). In E1 and E2, the expression of *Bna*A07g17400D and *Bna*C05g34170D was controlled by glycinin promoter, while in R1, R2 and R3, sense and

antisense strands of RNAi fragments were guided by CaMV35S promoter (**Figure 1a**). And the marker gene-*DsRed*, was also under the control of CaMV35S promoter (**Figure 1a**).

Then, hypocotyls from eight different rapeseed varieties were used for *agrobacterium* mediated transformation. Tissue culture for hypocotyls transformation, that is, calli formation, shoots redifferentiation, roots formation to regenerated plantlets was well performed in the present study (**Figure S1**). As is known, the ability of T-DNA integrating into plant genome was an important factor for plant transformation. Thus, the *agrobacterium* activity was carefully controlled as OD_{600} = 0.4~1.6, then diluted 10 times for hypocotyls infection (Detailed methods were in **Experimental Procedures**). Besides, the ability of the transgenic calli to develop into a complete plant is also very vital for plant regeneration. Thus, STS, which consisted of 0.1 mol/L $Na_2S_2O_3$ and 0.1 mol/L $AgNO_3$ in the proportion of 4:1, was added to calli formation medium to avoid tissue browning. And 3 mg/L $AgNO_3$ was added to shoot redifferentiation medium to promote shoots regeneration. Finally, 1 mg/L IBA was added to root formation medium for root generation (Detailed methods were in **Experimental Procedures**). In total, eight different rapeseed varieties were separately transformed via aforementioned *agrobacterium*-mediated hypocotyl transformation in *B. napus*.

DsRed for Rapid and Convenient Screening during Tissue Culture

Considering the expression of *DsRed* was in the whole plant development process, we guess the red fluorescence that existed in transgenic tissue could be observed through red filter by hand-held green fluorescent flashlight. Therefore, it is possible to utilize red fluorescence emitted by *DsRed* to screen transgenic-positive tissues in the primary stage of tissue culture. In this way, we attempted to distinguish the transgenic-positive and transgenic-negative calli and shoots in tissue culture medium. Expectedly, strong red fluorescence was observed in transgenic-positive calli and shoots compared to that of transgenic-negative ones (**Figure 1b, Figure 2c and 2e**). Moreover, red fluorescence emitted by putative transgenic-positive calli or shoots could also be clearly observed under fluorescence stereomicroscope (**Figure S2**). This strong contrast indicated the feasibility of using *DsRed* as visual screening marker in tissue culture of *B. napus*. With this method, plenty of transformed rapeseed hypocotyls were observed with red fluorescence on calli-inducing

medium (CIM) and shoot regeneration medium (**Figure 1b, Figure 2c and 2e**). By this way, the transgenic-positive and transgenic-negative shoots could be clearly distinguished, which proved that it is feasible to screen positive regenerative plantlets conveniently with visual screening method. Furthermore, we counted the calli with red fluorescence that could be observed under hand-held green fluorescent flashlight. It was found that the average calli with red fluorescence was achieved to 59.1%, that is, 56.9% (76/137) for E1, 50.6% (66/131) for E2, 66.9% (73/109) for R1, 50.8% (97/191) for R2 and 71.4% (167/234) for R3, respectively (**Table 1**). These results revealed that the visual screening method by red fluorescence provided an obvious advantage in rapid identification and convenient discrimination during the early stage of tissue culture.

DsRed for Efficient Genetic Transformation during Tissue Culture

To investigate whether the calli emitted red fluorescence were integrated with desired exogenous DNA fragments, the potential transgenic calli and shoots were randomly selected for identification by PCR amplification. Hypocotyls of three commercial rapeseed varieties (Zhong shuang 8, Zhong shuang 11, Zhong you 821), a spring rapeseed variety (Jia 9709) and a semi-winter variety (Jia 2016) were transformed with R2 vector. As expected, almost all of the commercial cultivars were with *DsRed* amplification products, i.e. 95.5% (21/22), 100% (22/22), 95.5% (21/22), 100% (22/22) and 100% (22/22) were identified with the PCR products at the length of 426 bp (**Figure S3A**). This high-efficiency genetic transformation system showed that it was a successful attempt in different rapeseed varieties with visual screening the red fluorescence from the induced calli to shoots.

Additionally, to further identify whether this transformation system was also appropriate for other rapeseed varieties with different expression vectors, hypocotyls from another three commercial rapeseed varieties (7633, B351 and Shan 3B), which were transformed with E1 vector, were also analyzed by PCR detection. It showed that 83.3% (15/18), 94.4% (17/18) and 100% (18/18) were amplified at the length of 722 bp for *Bna*A07g17400D gene (**Figure S3B**). Consequently, these results presented that different commercial rapeseed varieties and different expression vectors used in present study are competent to be transformed by *agrobacterium*-mediated hypocotyl transformation method (**Figure 2**). Furthermore, the

average of 96.1% tissues observed with red fluorescence could be indeed checked with exogenous gene in eight different rapeseed varieties. Collectively, it illuminated that the visual screening method with *DsRed* could be used to shorten the screening period and accelerate the regeneration process to obtain high-efficiency positive transgenic plantlet.

High-efficiency for Rooted Transgenic-Positive Plantlets in Rapeseed

High-efficiently obtaining transgenic-positive regenerated plantlets was of great importance for transgenic function study. Therefore, to investigate the transgenic-positive efficiency via visual screening method based on *agrobacterium*-mediated transformation in rapeseed, the plantlets transformed with E1, E2, R1, R2 and R3 vectors that regenerated from hypocotyls of "Jia2016" was collected, respectively. About 196 independent rooted transgenic plantlets were obtained. And all these positive transgenic plants obtained have normal phenotype both in vegetative growth and reproductive growth, and no sterile transgenic plants were found in the field.

Next, genomic DNA were extracted from putative transgenic rapeseed plantlets, and PCR amplification were performed to detect the integration of *DsRed*, *Bna*A07g17400D and *Bna*C05g34170D, respectively. As expected, most of the putative transgenic lines were presented with positive bands in the agarose gel (**Figure 1c**). To be specific, 80% (8/10) and 90.7% (49/54) was detected for *Bna*A07g17400D and *Bna*C05g34170D amplification, respectively (**Table 2**). In addition, 88% (22/25), 81.4% (35/43) and 89.1% (57/64) were detected for R1, R2 and R3 by *DsRed* fragment amplification, respectively (**Table 2**). The high transgenic-positive efficiency indicated that different vectors used in this study could be successfully transformed via *agrobacterium*-mediated hypocotyl transformation system.

***DsRed* for Convenient and Efficient Screening during Seed Germination**

Considering the thick tissue in *B. napus*, it is not easy to distinguish the positive transgenic seeds and seedlings with hand-held green fluorescent flashlight after obtaining T1 seeds and its regeneration plants. Red fluorescence, unlike green fluorescence, is capable to be observed under laser confocal fluorescence microscopy without the interference of chlorophyll. To verify the screening feasibility during the primary stage of seed germination,

experiments were performed using confocal microscope. For each part, i.e. seed coats, roots, hypocotyls and cotyledons, were randomly selected for further analysis (**Figure 3a** and **Figure 2h**). It was revealed that the red fluorescence could be successfully observed in all the cells of different parts of seeding (**Figure 3a**).

Furthermore, real time PCR analysis was performed to check the relative expression level of *DsRed* and *Bna*A07g17400D genes during seed germination. The result showed that the roots, seed coats, hypocotyl and cotyledons of seedling with red fluorescence were all showed strong expression of *DsRed*, respectively (**Figure 3b**). Compared to WT, the relative expression level of *DsRed* was demonstrated extremely higher in transgenic RNAi lines, and the relative expression of *Bna*A07g17400D was lower in three transgenic RNAi lines (**Figure 3b**). These results indicated that *DsRed* is a reliable method for positive screening without influencing desired RNAi events.

Gene Copy Number Identification of *DsRed* by Southern Blotting

In order to verify *DsRed* whether could be applied to identify the integrated copy number in regeneration plants, Southern blotting was used to detect the integration of *DsRed* cassettes in several transgenic plants. Three T3 plants for each line were randomly chosen for Southern blotting, and eight were confirmed to insert with *DsRed* gene (**Figure 4**). Southern blotting results revealed that the *DsRed* gene showed one - to - five copies in the transgenic rapeseed plants. To be specific, in E1 lines, all the three plants were inserted with three copies. In R1 lines, one plant was inserted with single copy and two plants were inserted with two copies, respectively. In R2 lines, two plants were inserted with three copies, and the third plant was without insertion which indicated that R2-20-10-1 was a marker-free transgenic plant (**Figure 4a**). In E2 lines, all the three plants were inserted with one copy. In R3 lines, two plants were inserted with five copies and one plant was inserted with three copies (**Figure 4b**).

It's worth noting that all E1 lines stemmed from the same parent (E1-15-21) were presented the same three T-DNA copies (**Figure 4a**). Similarly, all E2 lines from one T1 plant (E2-9) were inserted with one copy, and two R2 lines stemmed from the same T2 plants (R2-20-6) also contained three T-DNA copies (**Figure 4a**). It indicated that E1-15-21,

R2-20-6 and E2-9 could be regarded as stable and homozygous transgenic plants. However, in R1 and R3 lines, the T-DNA copy number showed diversity in different plants (**Figure 4**). And the copy number of the third R2 plant (R2-20-10-1) was also different from the previous two plants (**Figure 4a**). It was because of the different parents they stemmed from. These results demonstrated that the T-DNA copy numbers were invariable between the same T2 lines (E1-15-21 and R2-20-6) whereas variable among different T2 lines (R1-3-2, R1-3-19, R1-5-19 and R2-20-10), illustrating that stable transgenic plants could be obtained in T3 plant even if in tetraploid rapeseed plants.

Discussion

Agrobacterium-mediated rapeseed transformation was considered to be strongly genotype dependent [5, 40]. In the past thirty years, transgenic rapeseed plants were mostly obtained from a few model spring varieties, such as Westar [40-42]. After that, other spring rapeseed genotypes, such as Precocity, Kumily, Haydn and J9709, were also successfully transformed even if with low transformation efficiency [43-45]. Meanwhile, several winter cultivars have also been studied for plant transformation, such as DH12075 and Falcon [42, 46, 47]. In the present study, different commercial rapeseed varieties, different expression vectors and different genes were efficient to be transformed by *agrobacterium*. Few studies were focused on the concentration of *agrobacterium*, in this study, five different vectors were transferred into the semi-winter variety-J2016 by *agrobacterium*-mediated hypocotyl transformation method, and the OD600 of *agrobacterium* suspension solution used to transfect hypocotyls was 0.06, which was diluted 10 times from the original concentration (OD600 = 0.4-1.6). The *agrobacterium* transfection step was lasted for around 10 min. Finally, an average of 85.8% transgenic-positive rooted plantlets were obtained under the condition of 1 mg/L 2,4-D in CIM and 3 mg/L $AgNO_3$ in SIM. Compared to the previous studies, it seems that both original and final concentrate of *agrobacterium* was also an significant factor influenced transformation efficiency [5, 46, 48, 49]. Besides, there was no obvious difference in the transformation efficiency among different vectors, except for E1, which was utilized in our exploratory study for optimum experimental conditions. Therefore, the establishment of an efficient *agrobacterium*-mediated rapeseed transformation system using hypocotyls will

359 also enable genome editing of valuable genes controlling important traits.

360 Based on the well-established transformation system with high transformation efficiency,
361 an effective transgenic-positive screening technology is of great importance for shortening the
362 period of tissue culture and simplifying the transformation system in rapeseed (**Figure 2**).
363 *DsRed*, as a selectable marker gene for transgenic seed screening, is advantageous to separate
364 transgenic and non-transgenic seeds in many plants, such as *Arabidopsis*, rice and *Camelina*
365 *sativa* [15, 50, 51]. In *Arabidopsis*, *DsRed* has been used as visual selection markers to
366 promote the transgenic seeds screening [32, 52, 53]. However, considering the impediment of
367 tissue in thickness, *DsRed* hasn't been used in rapeseed for convenient screening to accelerate
368 the process of transgenic-positive plantlet selection. In the present study, the red fluorescence
369 emitted by *DsRed* marked calli and shoots could be easily observed through red filter by
370 hand-held green fluorescent flashlight. Up to 61.2% calli were observed with red fluorescence,
371 and almost all of these calli could be detected with *DsRed* gene by PCR analysis. The red
372 fluorescence in calli and rapeseed seedlings could also be observed by fluorescence
373 stereomicroscope and laser scanning confocal microscope, respectively. What's more, it's
374 worth noting that the fluorescence intensity varied from each other. This might be due to the
375 rapeseed genotypes, i.e. homo- or hemizygosity of the transgene [53]. Besides, *DsRed*, as a
376 marker for Southern blotting analysis, was also successfully applied for estimating one to five
377 copies in the transgenic offspring plants like other studies [40, 41]. These results indicated
378 that visual screening maker-*DsRed* is of great success in accelerating genetic transformation
379 system in *B. napus*.

380 In addition, several methods are alternative for transgenic-positive plants screening
381 (**Figure 2** and **Table 3**). Antibiotics screening is frequently used in most transformation
382 studies, such as kanamycin, hygromycin or herbicide [25, 49, 54]. However, the dosage of
383 these substance might influence the regeneration rate and further break the balance of
384 transgenic/non-transgenic seed. PCR amplification is time and cost consumption in plant
385 preparation and identification. Southern blotting analysis, as a traditional method for
386 identification of transgenic copy number, is costly in terms of reagents, equipment, time and
387 labor [39]. GUS expression assay is a compromise in the matter of cost. However, it usually

spends long time in tissue decoloring. What's more, GUS expression assay often expresses following the expression of target gene in plant cells or tissues [55]. As for GFP, it needs the aid of a confocal microscope. And it's usually associated with red fluorescence to detect sub-cellular localization due to the interference of chlorophyll [56]. Compared with previous methods, the present study firstly introduced visual selectable marker gene-*DsRed* to accelerate transgenic plantlets screening. It just needs a combination of light filter with hand-held fluorescent flashlight to easily distinguish the transgenic-positive and transgenic-negative plants during the primary stage of tissue culture and seed germination in rapeseed. Therefore, the application of *DsRed*, which served as a marker gene, will facilitate the screening transgenic positive lines and utilization of tissue culture or cell culture in plant biotechnology.

Conclusion

A novel method was developed combining an efficient genetic transformation system with a convenient and rapid transgenic-positive visual screening method in rapeseed. In this way, the potential transgenic-positive plants could be quickly and efficiently picked out by a convenient visual screening method, which greatly accelerate the regeneration process. Besides, the high transgenic positive efficiency could be easily achieved based on the optimized agrobacterium-mediated hypocotyl transformation, which greatly increase the number of genetic transformed plants. The aforementioned system will be a good alternative for rapeseed genetic transformation and gene functional study.

Acknowledgements

Our thanks to Prof. Chunyu Zhang from Huazhong Agriculture University and Prof. Dianrong Li from Hybrid Rapeseed Center of Shaanxi Province for their contributions to offer the seeds of different rapeseed varieties.

Author's Contributions

KZ participated in the entire experiment and wrote the manuscript. JJ carried out the transformation and identification the winter rapeseed varieties (7633, B351 and Shan3B) and modified the manuscript. LL, RD and LQ were participated in the transformation and

identification of the commercial rapeseed varieties (Zhong shuang 8, Zhong shuang 11, Zhong you 821). XC and WJ took participated in part of tissue culture experiments. CK and YL gave helpful suggestions for this study. MM and AA took part in part of PCR detection. ML and CH designed, led and coordinated the overall study. KZ and JJ contributed equally to this paper.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

The work was supported by the National Natural Science Foundation of China (31671721) and the National key research and Development Program (2016YFD0101300).

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Figure legends

Figure 1. Vectors construction for gene over-expression and knock-down. (a) Diagram of the construction for over-expression vector. The expression of *Bna*A07g17400D and *Bna*C05g34170D is driving by glycinin promoter. The N terminus of *Bna*A07g17400D and *Bna*C05g34170D is companied with a flag. (b) Diagram of the construction for RNAi knock-down vector. The expression of sense strands and antisense strands is driven by the 2x35S promoter and with a 2x nuclear localization signal (NLS) at the N terminus. *DsRed* in both vectors was also under the control of the CaMV35S promoter.

Figure 2. Overview of the novel method for high-efficiently obtaining the transgenic lines combing with a visual screening method compared with antibiotics screening in *B. napus*. (a) High-efficient genetic transformation combining with a convenient visual screening marker DsRed via agrobacterium-mediated hypocotyl transformation in *B. napus*. Hypocotyls were transformed with a vector companied with DsRed and hygromycin via *agrobacterium*- mediated transformation system. The light grey lines inside the oval represented hypocotyls from *B. napus*. (b and c) Calli formation and screening during calli-induced stage with or without visual screening method. The calli would be survived under antibiotics screening with hygromycin in b, and further picked by visual screening using a hand-held green fluorescent flashlight in c. The endpoints in the line represented the formed callus, and the red spots in the endpoints represented the red fluorescence observed in the calli. (d and e) Shoots formation in shoots-induced medium with or without visual screening method. The calli would be survived under antibiotics screening with hygromycin in d, and further picked by visual screening using a hand-held green fluorescent flashlight in e. The red spots in the shoots represented the red fluorescence observed in the cotyledons. (f and g) To high-efficiently screen the transgenic-positive rooted T0 plantlets and T1 transgenic-positive plants. Transgenic-positive rooted T0 plantlets and T1 transgenic-positive plants could be rapidly and high efficiently obtained when combining with the convenient visual screening method. (h) Visual screening in cotyledons, hypocotyls, seed coats and roots during seed germination. The seedlings could be screened with visual marker DsRed using laser confocal fluorescence microscopy.

Figure 3. Efficient and convenient screening in the primary stage of tissue culture. (a-d) n showed that the transgenic-positive and/or -negative tissues could be distinguished clearly using the convenient hand-held green fluorescent flashlight by visual screening. The middle and the right column showed that the red fluorescence could be observed clearly the calli and shoot by visual screening using the same method through the stage of tissue culture. The yellow arrows indicated red fluorescence.

Figure 4. Transgenic-positive plants identification in T0 plants. Amplification of *Bna*A07g17400D gene in E1 transformed lines, and *Bna*C05g34170D gene in E2 transformed lines, as well as *DsRed* gene in R1, R2 and R3 transformed lines by designed primers, respectively. P, plasmid. WT, wild type plant. Marker, DL 100bp ladder.

Figure 5. Convenient screening during seed germination. (a-m) Observation of the red fluorescence through the primary stage of seedling. The red fluorescence could be observed at the cotyledon (b, f and j), hypocotyl (c, g and k), seed coat (d, h and l) and root (e, i and m) for rapeseed seedling under laser confocal fluorescence microscopy FV1000, respectively. (n) Relative expression of *Bna*A07g17400D and *DsRed* in the seedlings with red fluorescence. The upper one showed that the relative expression of *DsRed* gene in the seedlings of root, seed coat, hypocotyl and cotyledon. The middle one showed the relative expression of *DsRed* gene in seedlings of different plants. The lower one showed that the relative expression of *Bna*A07g17400D gene in seedlings of different plants.

Figure 6. Southern blot analysis of *DsRed* genes for copy number identification in T3 transgenic rapeseed plants. (a) Southern blot analysis for *Bna*A07g17400D over-expression and knock down. (b) Southern blot analysis for *Bna*C05g34170D over-expression and knock down. WT, wild type; P-E1/E2, over-expression plasmid pcmabia-1303; P-R1/R2/R3, knock down plasmid p35S-1390; M: λ DNA/Hind III- Plus DNA marker.

Table 1. Transformation Efficiency with Red Fluorescence in Different Lines.

Table 2. Transformation Efficiency of Different Lines.

Table 3. Comparisons of different screening methods.

Supporting information legends

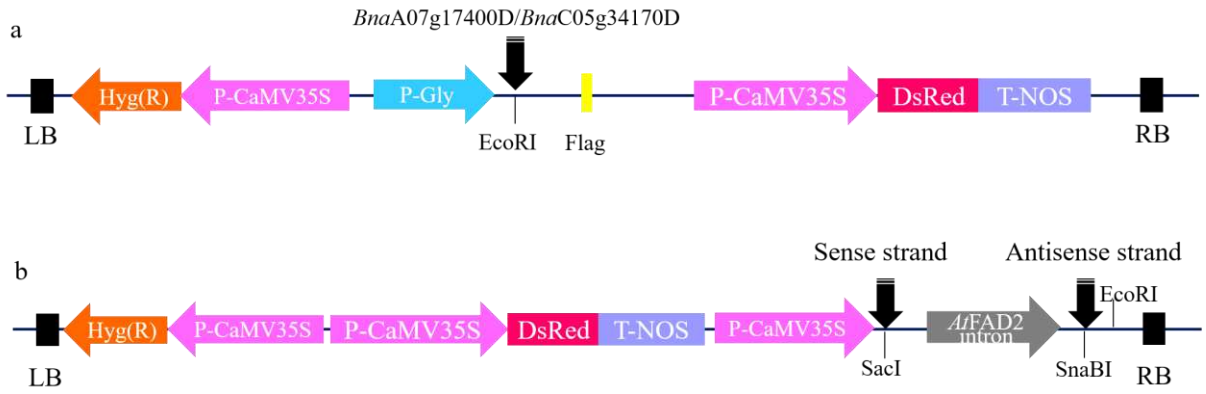
Figure S1. The formation process of hypocotyls from calli to a regenerated plantlet. (a

and b) represented calli were induced from hypocotyls, (c) represented calli were prepared to swollen to shoots, (d and e) represented shoots formation and cotyledon development companied with the appearance of growth point.

Figure S2. Visual screening for transgenic-positive and/or -negative tissues under fluorescence stereomicroscope. Bright field images for tissues in the medium (a), and dark-field images for tissues in the medium using hand-held green fluorescent flashlight in (b).

Figure S3. PCR amplification for transgenic-positive identification in calli. (a) Amplification of *DsRed* from calli of Jia9709, Jia2016, Zhong shuang 8, Zhong shuang 11 and Zhong you 821, respectively. P, plasmid. WT, wild type plant. Marker, DL 100bp ladder. (b) Amplification of *Bna*A07g17400D from calli in 7633, B351 and Shan3B, respectively. P, plasmid. N, wild type plant. Marker, DL 2000bp.

Table S1. Primers used in this study.



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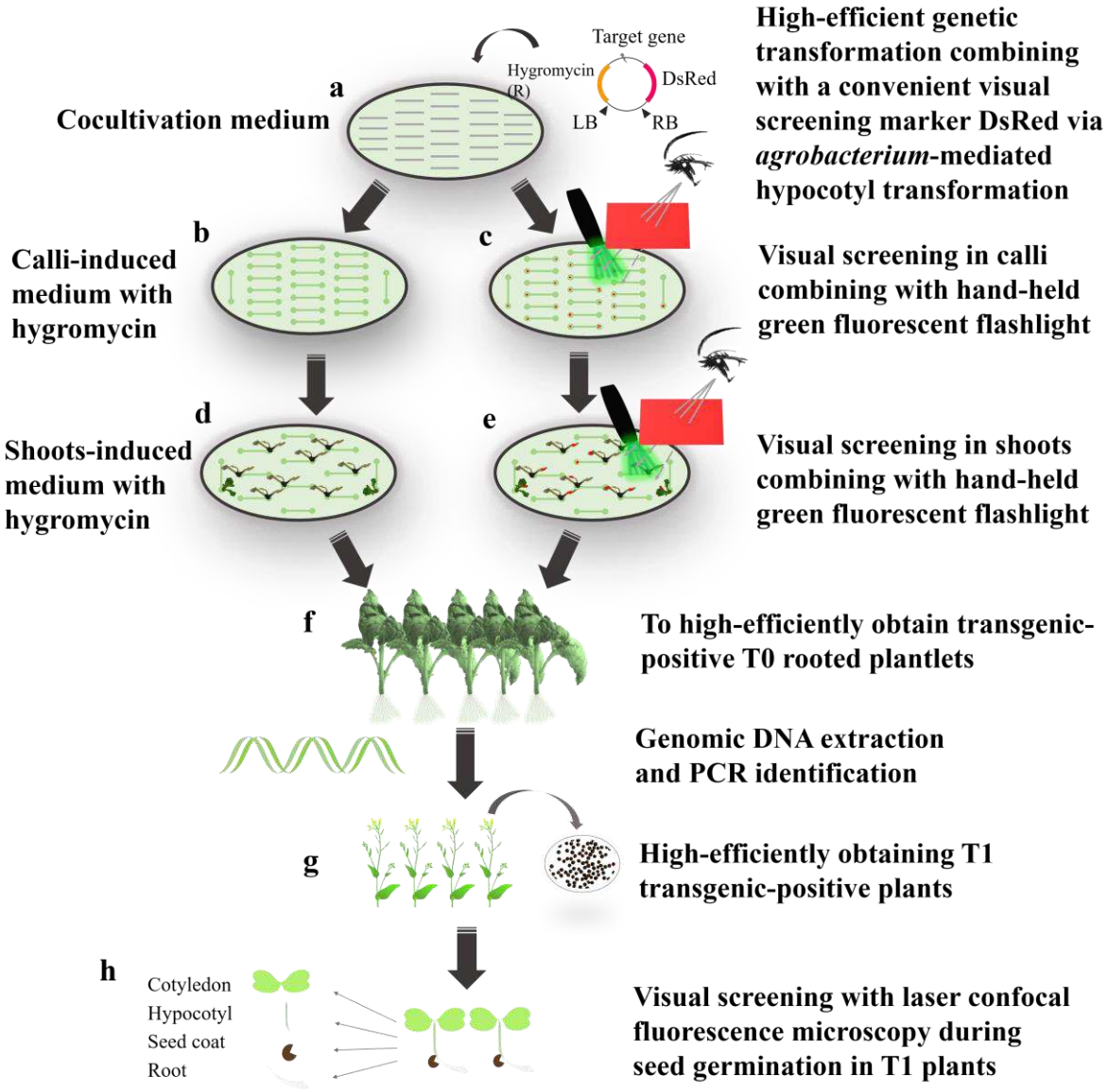

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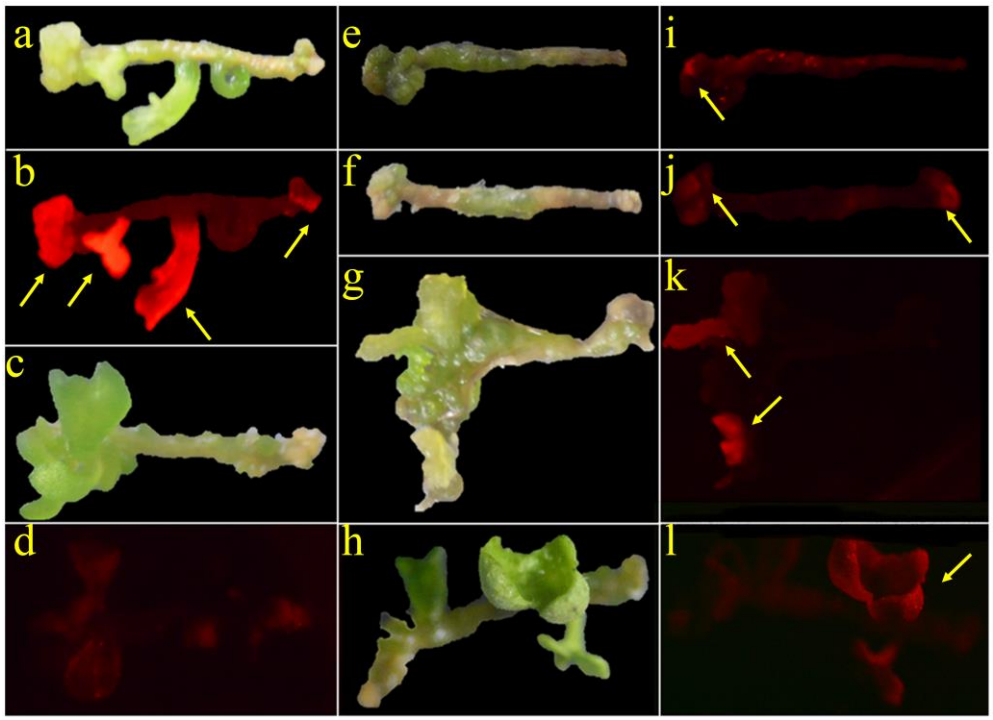

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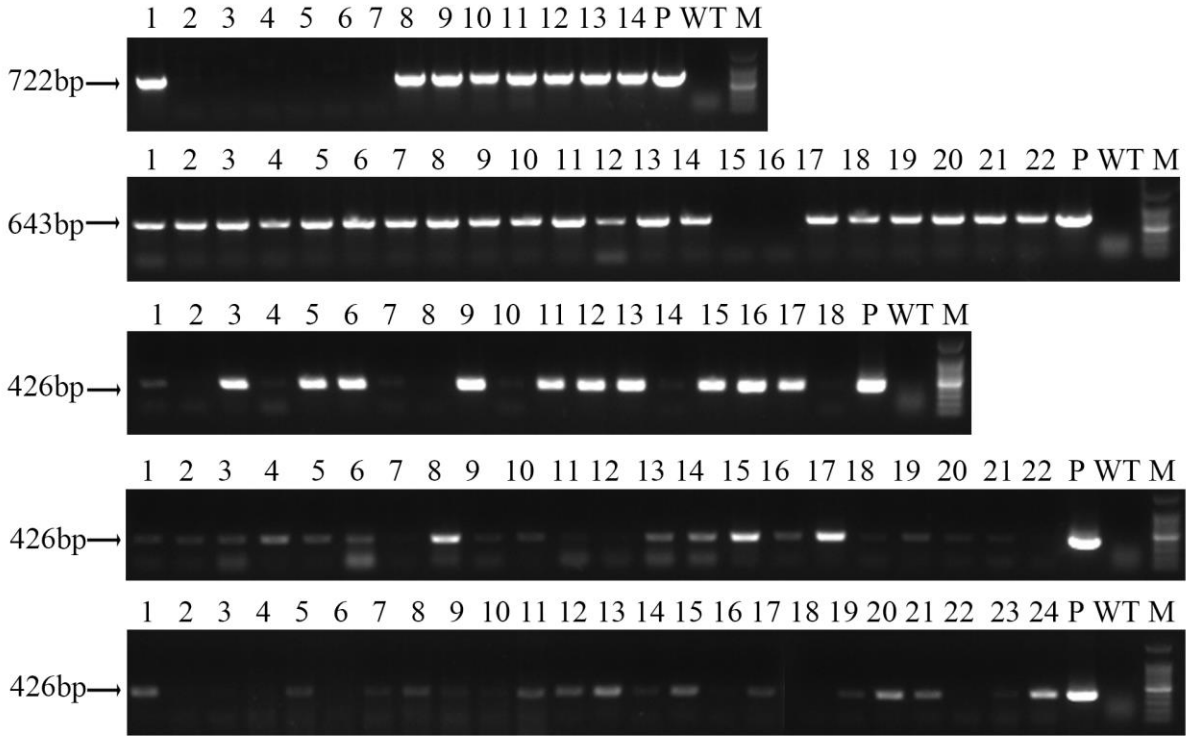

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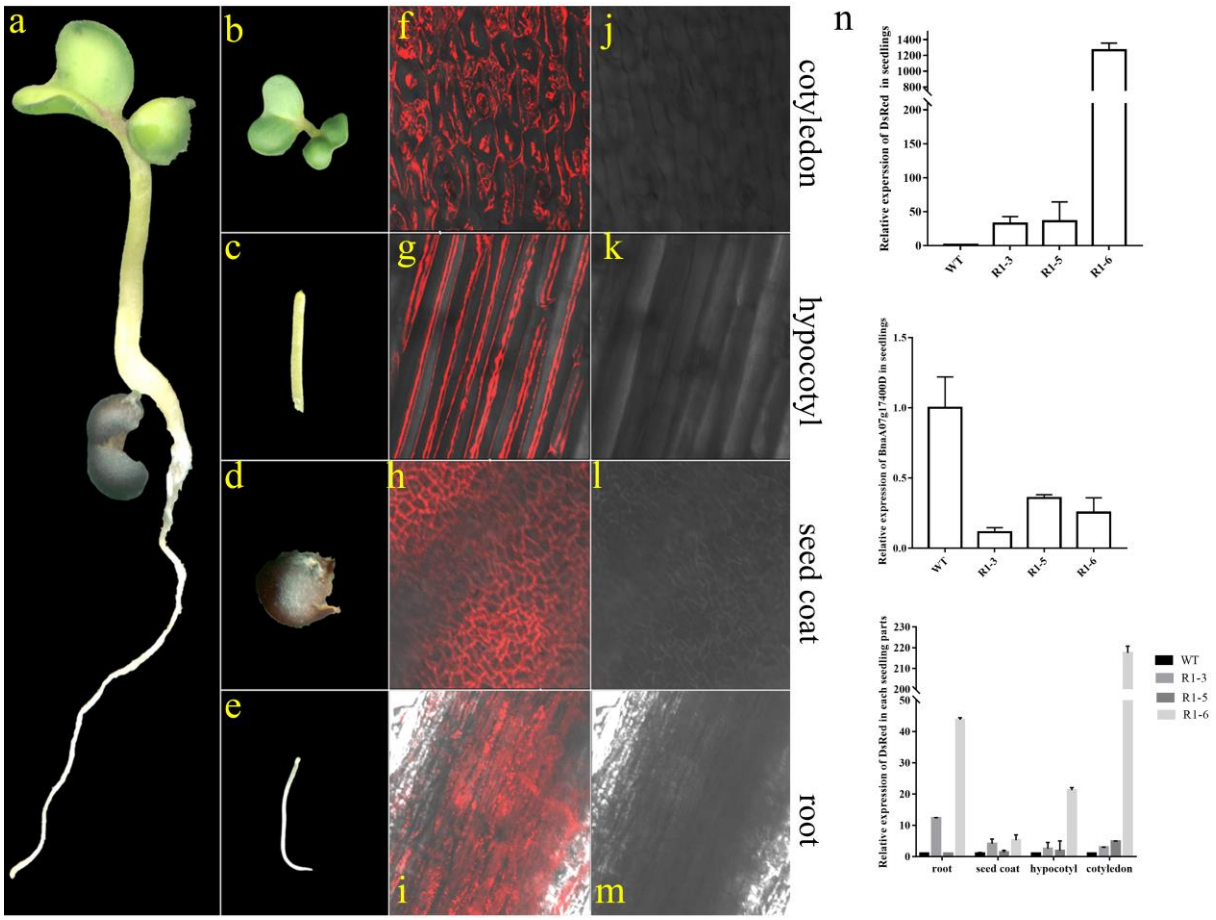

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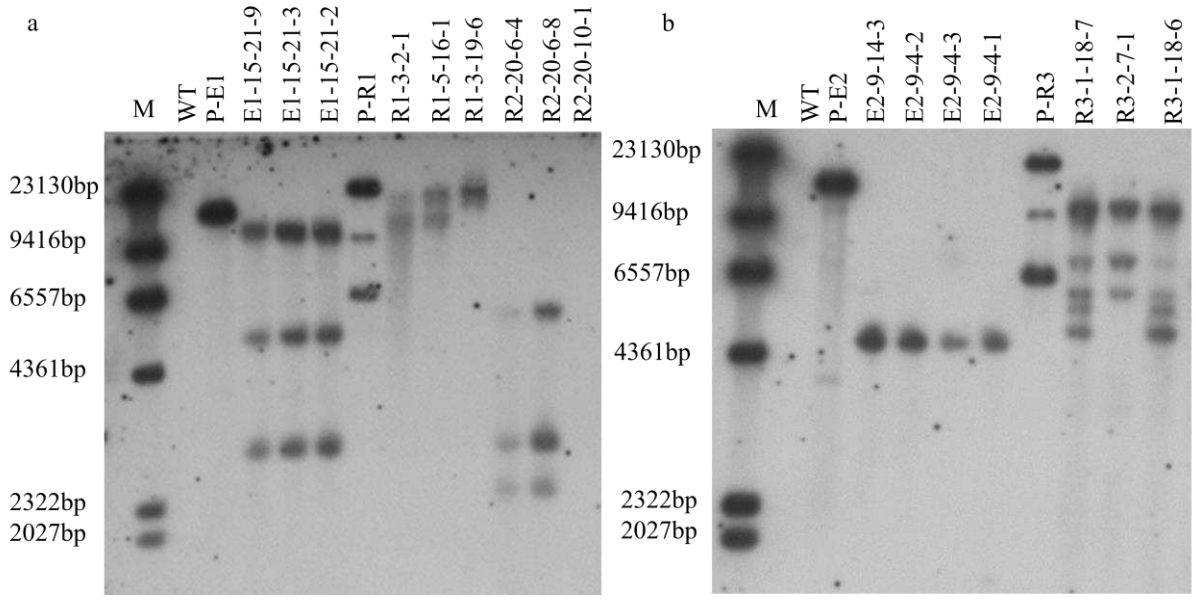

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716

Table 1. Transformation efficiency with red fluorescence in different lines

target Gene	Number of experiments	Hypocotyls with DsRed	Number of Hypocotyls transformed	Transformation efficiency
*Bna*A07g17400D	1	47	77	61.0%
	2	31	60	51.7%
*Bna*C05g34170D	1	30	57	52.6%
	2	36	74	48.6%
R1	1	35	59	59.3%
	2	38	50	76.0%
R2	1	47	92	51.1%
	2	50	99	50.5%
R3	1	109	143	76.2%
	2	58	91	63.7%

717

Table 2. Transformation efficiency of different lines

NO.	Total	transgenic positive	Transformation efficiency
*Bna*A07g17400D	10	8	80.0%
*Bna*C05g34170D	54	49	90.7%
R1	25	22	88.0%
R2	43	35	81.4%
R3	64	57	89.1%

Table 3. Comparisons of Different Screening Methods

Methods	Special Reagents	Equipment	Total Time (D) /1000 samples	Cost/200 Reaction	Labour	Injuries to Plant
PCR Ampification	DNA extract kit	Thermal Cycler	2	High	High	Leaf
Antibiotic/Herbicide Selection	Antibiotics	Box	⩾2	Low	Moderate	Seed
Southern Blot Analysis	Southern Blot Hybridization kit	Hybridization Oven	⩾7	High	High	Leaf
GUS Expression Assays	GUS Dye	Microscopy	⩾2	Moderate	Moderate	Leaf / Root
GFP	No	Laser Scanning Confocal Microscopy	⩾1	Moderate	Moderate	Leaf / Root
Visual Screening	No	Hand-Held Fluorescent Flashlight, Light Filter	⩽0.5	Zero	Low	No

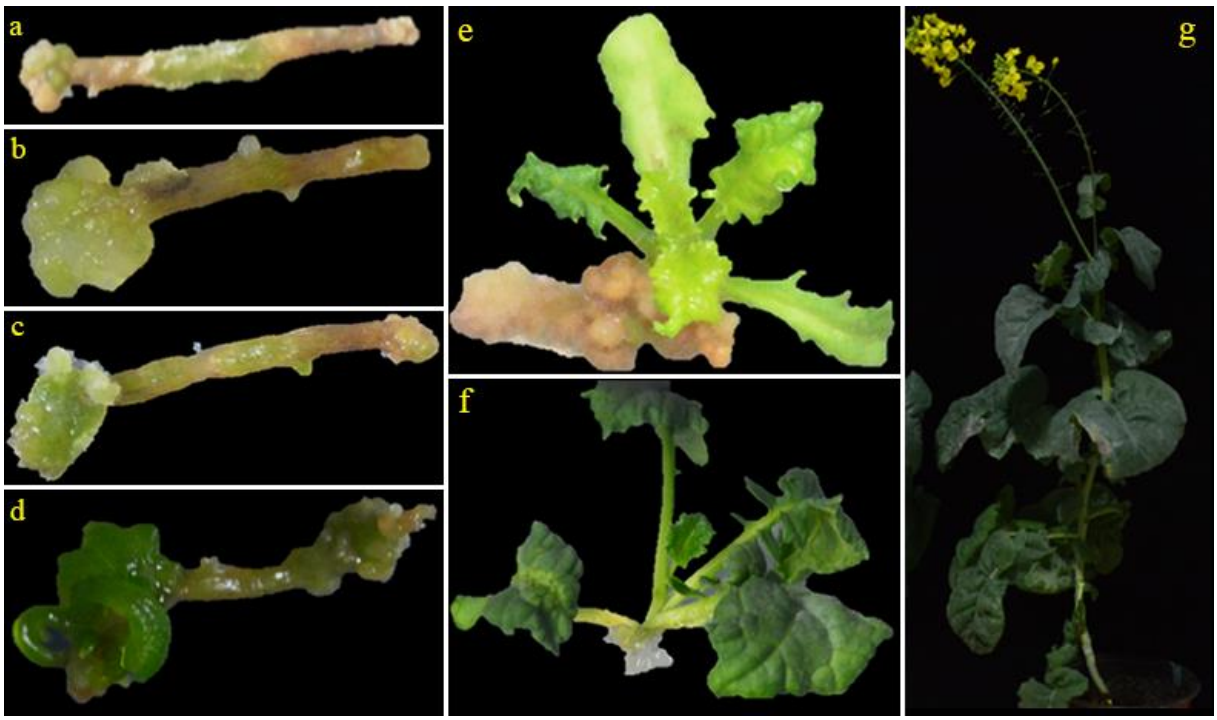


Figure S1. The formation process of hypocotyls from calli to a regenerated plantlet. (a and b) represented calli were induced from hypocotyls, (c) represented calli were prepared to swollen to shoots, (d and e) represented shoots formation and cotyledon development companied with the appearance of growth point.

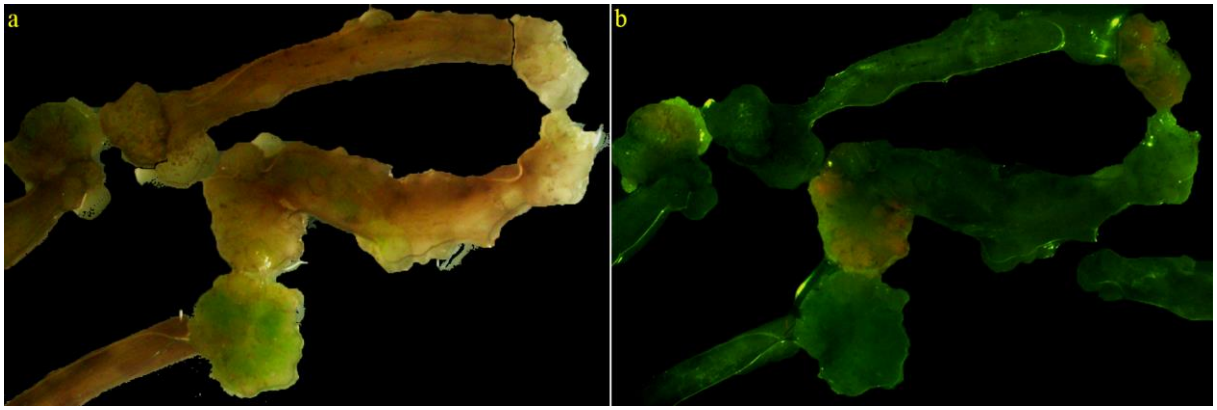
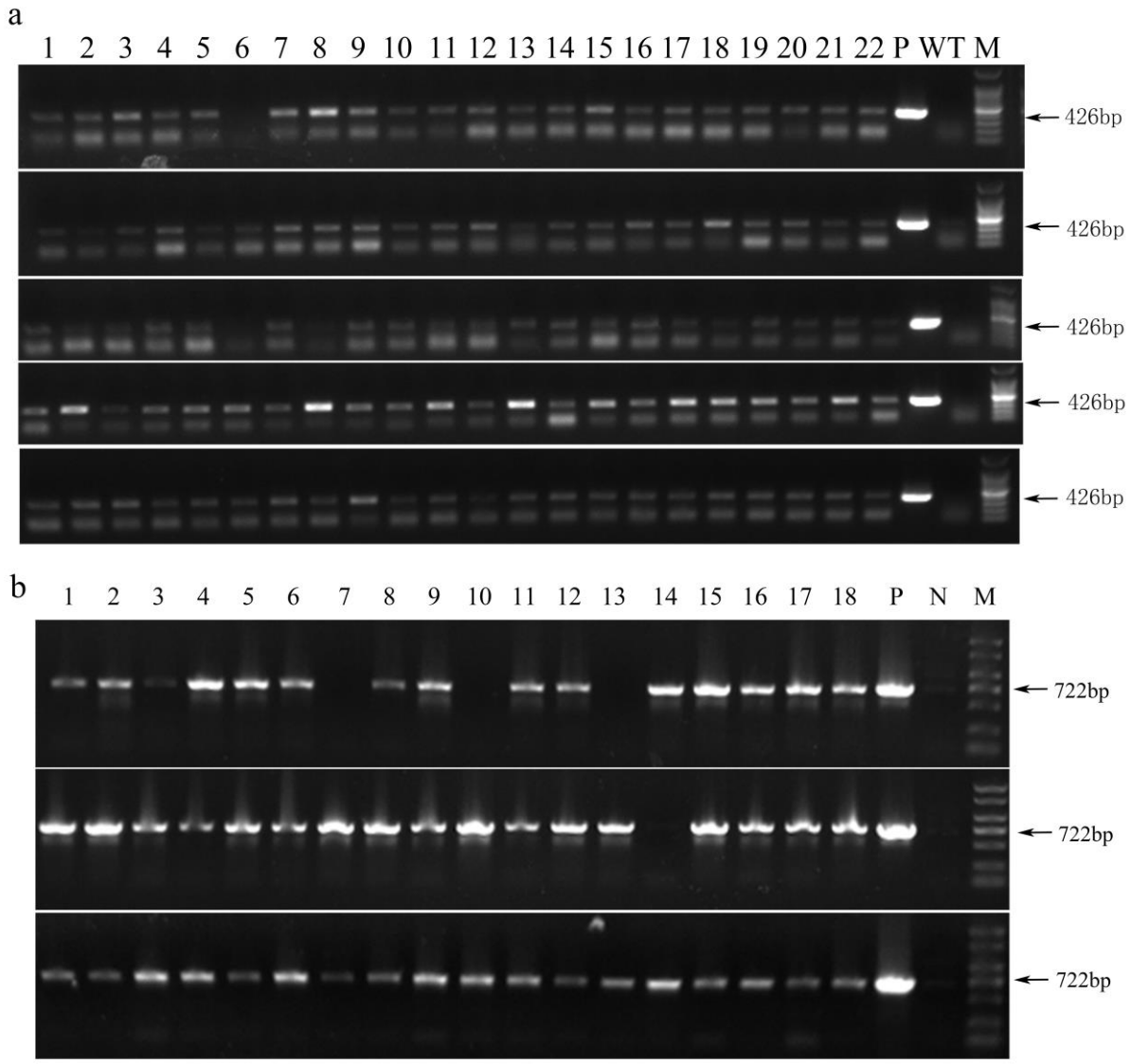


Figure S2. Visual screening for transgenic-positive and/or -negative tissues under fluorescence stereomicroscope. Bright field images for tissues in the medium (a), and dark-field images for tissues in the medium using hand-held green fluorescent flashlight in (b).



730

731 **Figure S3.** PCR amplification for transgenic-positive identification in calli. (a) Amplification
732 of *DsRed* from calli of Jia9709, Jia2016, Zhong shuang 8, Zhong shuang 11 and Zhong you
733 821, respectively. P, plasmid. WT, wild type plant. Marker, DL 100bp ladder. (b)
734 Amplification of *Bna*A07g17400D from calli in 7633, B351 and Shan3B, respectively. P,
735 plasmid. N, wild type plant. Marker, DL 2000bp.

736

737

Table S1. Primers Used in This Study

R1-S-F	5’ CCGGTACCAGGCCTGAAATTTCTCCCAGTCATA	RNAi vector construction
R1-S-R	5’ ACGTAGGGGCGATAGAGTGCATCCTTAGTCA 3’	
R1-A-F	5’ TGGAAGACGCGTTACAGTGCATCCTTAGTCA 3’	
R1-A-R	5’ GGATCCGTCGACTACAAATTTCTCCCAGTCATA 3’	
R2-S-F	5’ CCGGTACCAGGCCTGTGCCTGTCCCTCGAAA 3’	
R2-S-R	5’ ACGTAGGGGCGATAGAAGAGATTTGACCAGTGT 3’	
R2-A-F	5’ TGGAAGACGCGTTACTTACTAACAGCTGACACA 3’	
R2-A-R	5’ GGATCCGTCGACTACCATGCTCTTGTCGTTT 3’	
R3-S-F	5’ CCGGTACCAGGCCTGTGGATGTACTTCTGGGATC 3’	
R3-S-R	5’ ACGTAGGGGCGATAGTGATTCGACGGATGTGG 3'	
R3-A-F	5’ TGGAAGACGCGTTACTGATTCGACGGATGTGG 3’	
R3-A-R	5’ GGATCCGTCGACTACTGGATGTACTTCTGGGATC 3’	
DsRed-F	5'CCGGCGCGCCAAGCTCTAGTAGAAGGTAATTATCCA3'	
DsRed-R	5'GTAGGGAGCTAAGCTCCCGATCTAGTAACATAGAT3'	
BnaA07g17400D	5'CCGCGGCCGCGAATTCATGGCGATGGCAGCAGCA3'	over-expression vector construction
	5'CCTTGTAATCGAATTCCTTCTGCTTCTCCTCCAC3'	
BnaC05g34170D	5'CCGCGGCCGCGAATTCATGGAAAAGAAACTTACAAACT3'	
	5'CCTTGTAATCGAATTCAAAGTTCATTTTTTTACTAATTTG3'	
Actin	F: AGAGTCATGCCAAGTTCATGGTT	RT-PCR
	R: CCTCATAAGCACACCATCAACTCTAA	
BnaA07g17400D-RT	F: 5' AGAGGCAAAACTTAAAGCAGCACA 3'	
	R: 5' TTTGGTGCGAGGAATCAACACATT 3'	
DsRed-RT	F: 5' CCCGCCGACATCCCCGACT 3'	
	R: 5' CGAAGTTCATCACGCGCTCCC 3'	
BnaA07g17400D-OE	F: 5' TAAAACACTTACAACACCGGAT 3'	PCR identification
	R: 5' GAGGACATAGCAAACTGCC 3'	
DsRed-OE	F: 5' GCCAAGCTCTAGTAGAAGGT 3'	
	R : 5' TATATAGGAAAACTCAAGGGCAAA 3'	
BnaC05g34170D-OE	F: 5' CCATAGCCATGCATACTGA 3'	
	R: 5' TTTCGCCTCTGTGTAATCTG 3'	

738

Figures

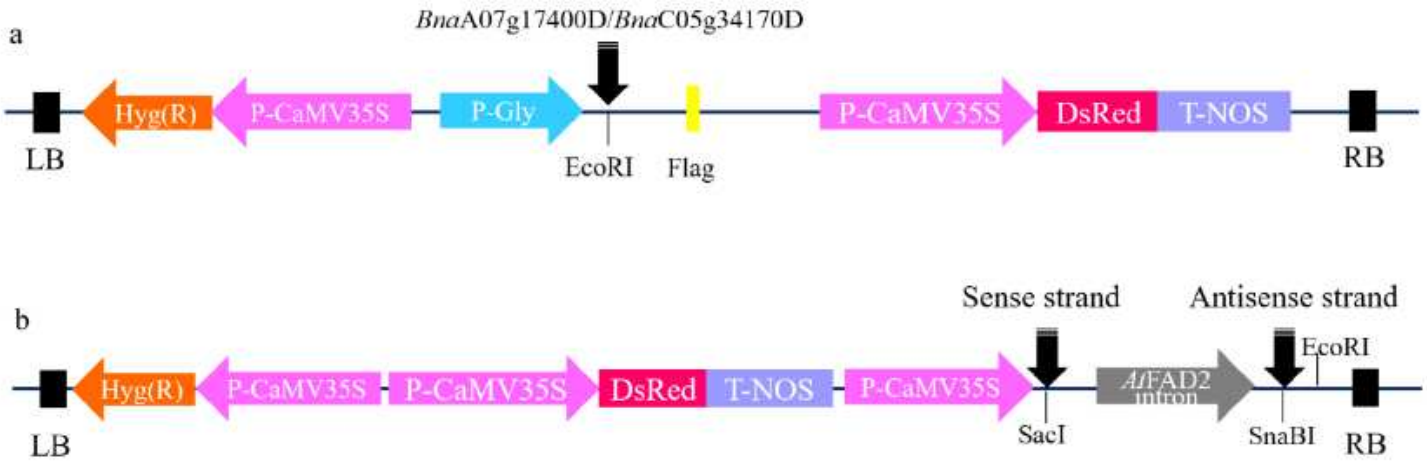


Figure 1

Vectors construction for gene over-expression and knock-down. (a) Diagram of the construction for over-expression vector. The expression of BnaA07g17400D and BnaC05g34170D is driving by glycinin promoter. The N terminus of BnaA07g17400D and BnaC05g34170D is companied with a flag. (b) Diagram of the construction for RNAi knock-down vector. The expression of sense strands and antisense strands is driven by the 2x35S promoter and with a 2x nuclear localization signal (NLS) at the N terminus. DsRed in both vectors was also under the control of the CaMV35S promoter.

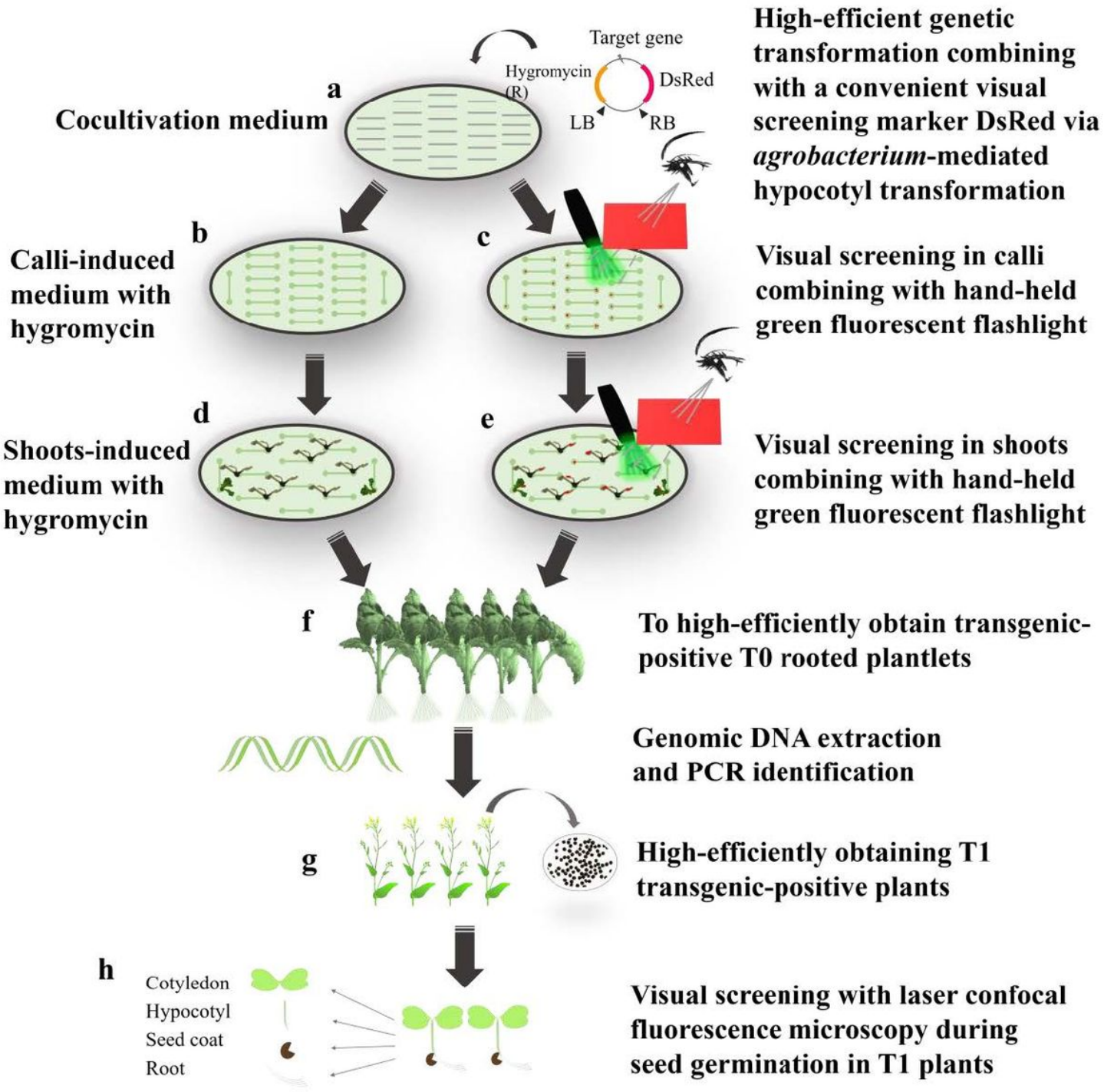


Figure 3

Overview of the novel method for high-efficiently obtaining the transgenic lines combing with a visual screening method compared with antibiotics screening in B. napus. (a) High-efficient genetic transformation combining with a convenient visual screening marker DsRed via agrobacterium-mediated hypocotyl transformation in B. napus. Hypocotyls were transformed with a vector companied with DsRed and hygromycin via agrobacterium- mediated transformation system. The light grey lines inside the oval represented hypocotyls from B. napus. (b and c) Calli formation and screening during calli-induced stage with or without visual screening method. The calli would be survived under antibiotics screening with

hygromycin in b, and further picked by visual screening using a hand-held green fluorescent flashlight in c. The endpoints in the line represented the formed callus, and the red spots in the endpoints represented the red fluorescence observed in the calli. (d and e) Shoots formation in shoots-induced medium with or without visual screening method. The calli would be survived under antibiotics screening with hygromycin in d, and further picked by visual screening using a hand-held green fluorescent flashlight in e. The red spots in the shoots represented the red fluorescence observed in the cotyledons. (f and g) To high-efficiently screen the transgenic-positive rooted T0 plantlets and T1 transgenic-positive plants. Transgenic-positive rooted T0 plantlets and T1 transgenic-positive plants could be rapidly and high efficiently obtained when combining with the convenient visual screening method. (h) Visual screening in cotyledons, hypocotyls, seed coats and roots during seed germination. The seedlings could be screened with visual marker DsRed using laser confocal fluorescence microscopy.

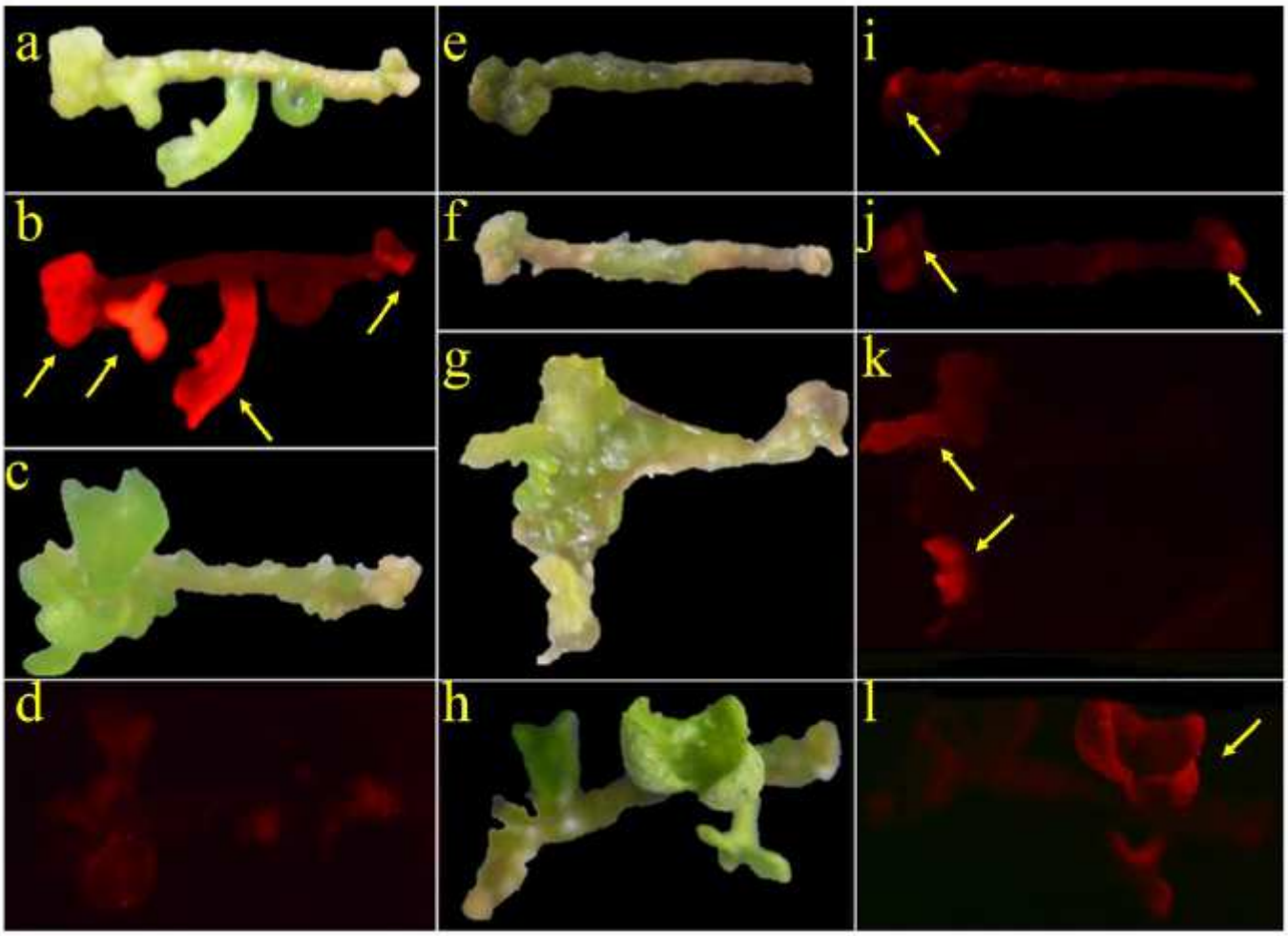


Figure 5

Efficient and convenient screening in the primary stage of tissue culture. (a-d) n showed that the transgenic-positive and/or -negative tissues could be distinguished clearly using the convenient hand-held green fluorescent flashlight by visual screening. The middle and the right column showed that the red fluorescence could be observed clearly the calli and shoot by visual screening using the same method through the stage of tissue culture. The yellow arrows indicated red fluorescence.

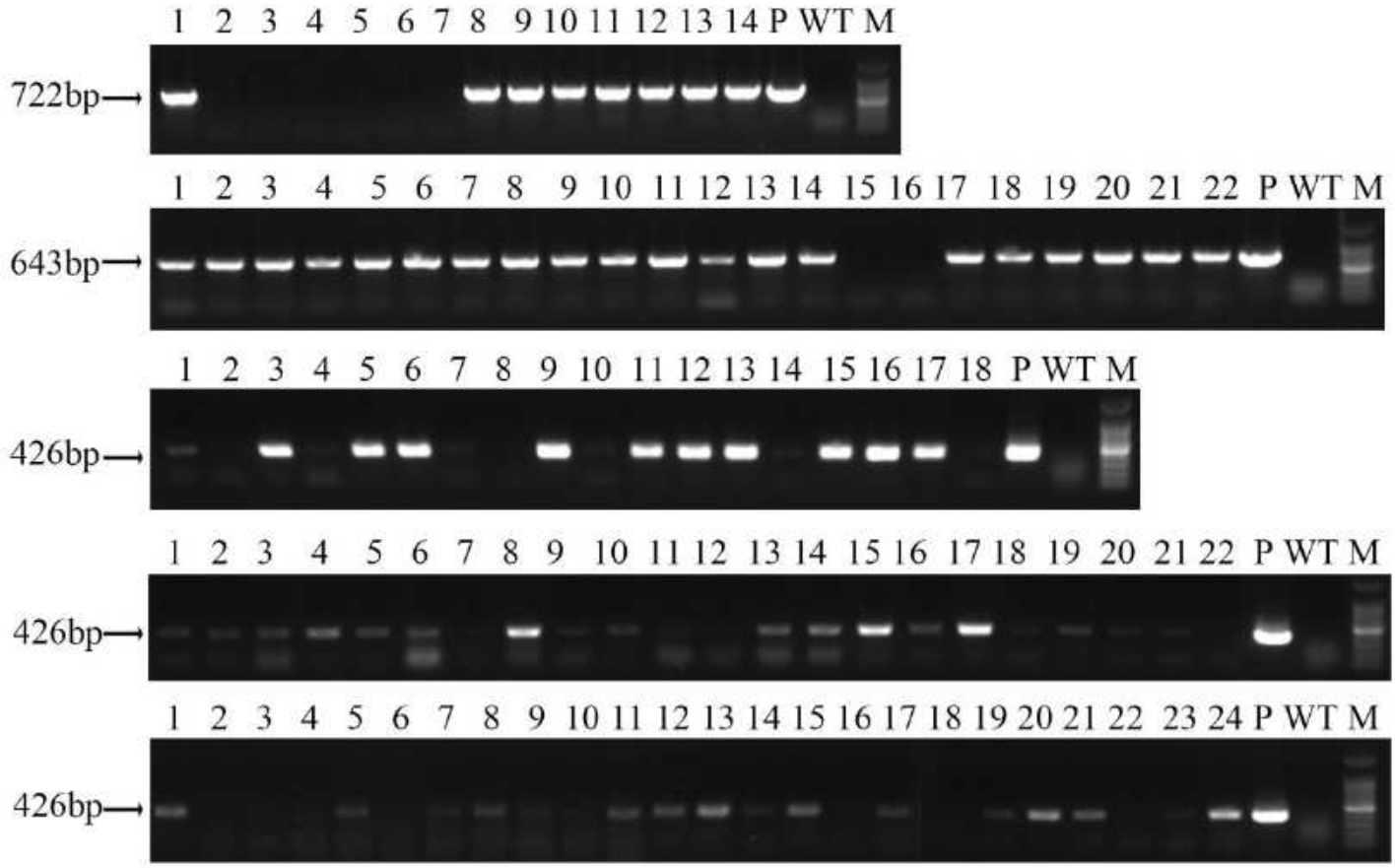


Figure 7

Transgenic-positive plants identification in T0 plants. Amplification of BnaA07g17400D gene in E1 transformed lines, and BnaC05g34170D gene in E2 transformed lines, as well as DsRed gene in R1, R2 and R3 transformed lines by designed primers, respectively. P, plasmid. WT, wild type plant. Marker, DL 100bp ladder.

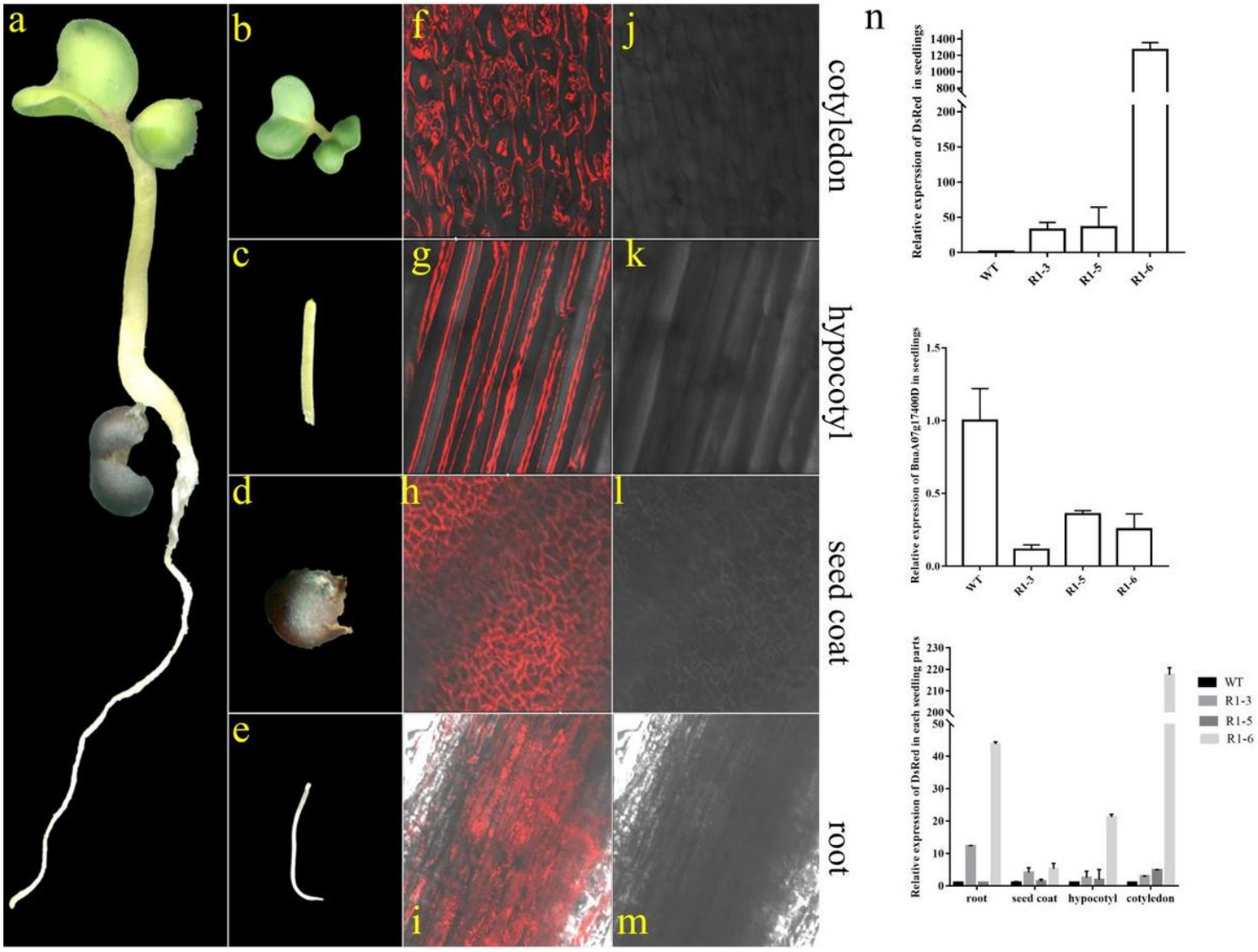


Figure 10

Convenient screening during seed germination. (a-m) Observation of the red fluorescence through the primary stage of seedling. The red fluorescence could be observed at the cotyledon (b, f and j), hypocotyl (c, g and k), seed coat (d, h and l) and root (e, i and m) for rapeseed seedling under laser confocal fluorescence microscopy FV1000, respectively. (n) Relative expression of BnaA07g17400D and DsRed in the seedlings with red fluorescence. The upper one showed that the relative expression of DsRed gene in the seedlings of root, seed coat, hypocotyl and cotyledon. The middle one showed the relative expression of DsRed gene in seedlings of different plants. The lower one showed that the relative expression of BnaA07g17400D gene in seedlings of different plants.

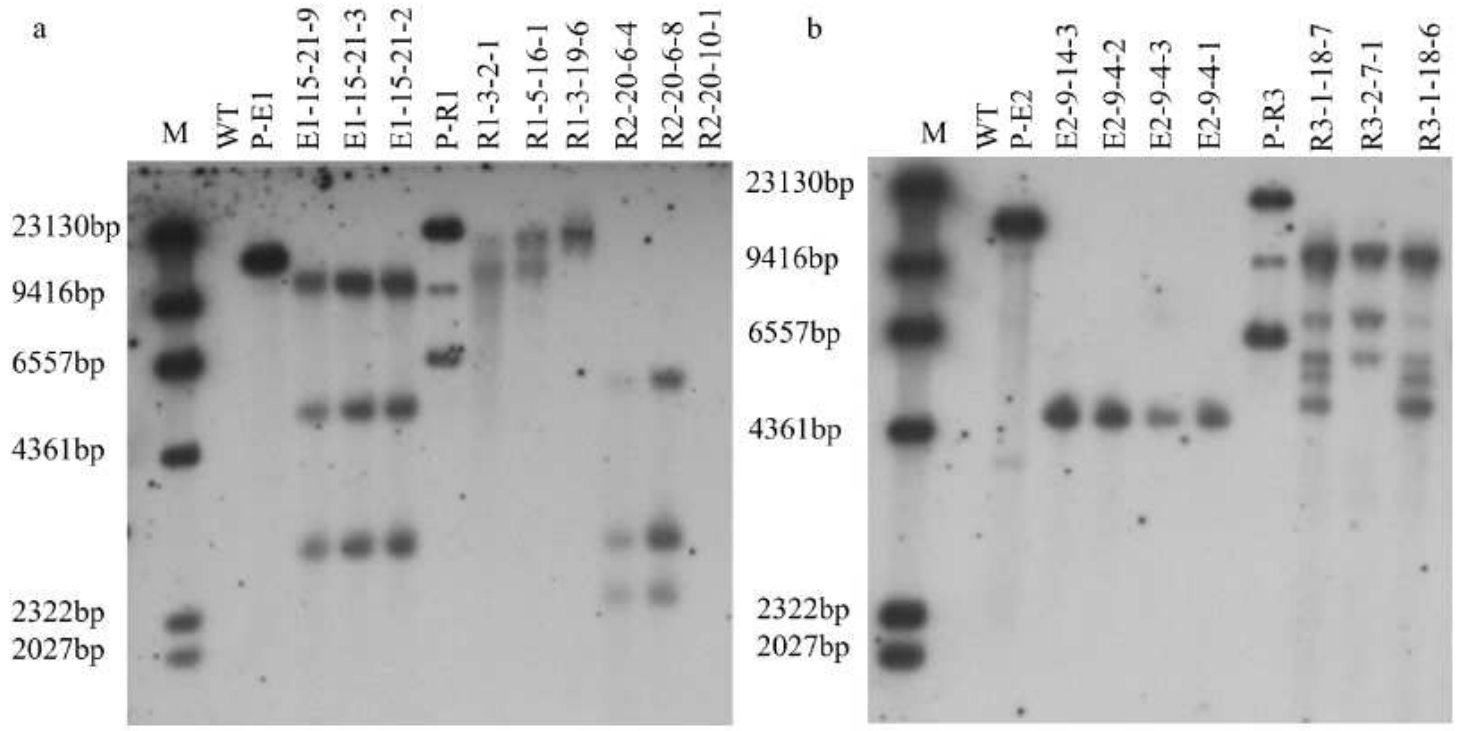


Figure 12

Southern blot analysis of DsRed genes for copy number identification in T3 transgenic rapeseed plants. (a) Southern blot analysis for BnaA07g17400D over-expression and knock down. (b) Southern blot analysis for BnaC05g34170D over-expression and knock down. WT, wild type; P-E1/E2, over-expression plasmid pcmabia-1303; P-R1/R2/R3, knock down plasmid p35S-1390; M: λ DNA/Hind III- Plus DNA marker.