

Construction of Potato DM1-3-516-R44 (DM) Transgenic System Based on Agrobacterium Transformation

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Research

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Abstract

Background: The sequencing potato DM1-3-516-R44 played an irreplaceable role in the study of gene function. So far, no one research the transformation system about DM. Therefore, our experiment was studied from three aspects: plant regeneration system, optimization of agrobacterium infection conditions and the effect of hygromycin on DM. Results: A relatively suitable method for genetic transformation of DM was obtained: 1) The stem callus induction medium was MS + IAA 0.5 mg/L + 6-BA 2.0 mg/L, the leaf callus induction medium was MS + NAA 1.0mg/L + 6-BA 0.5mg/L and the shoot differentiation medium was MS + 6-BA 3.0 mg/L + ZT 0.5 mg/L. 2) The specific transformation condition was the agrobacterium concentration kept the OD600 = 0.3, and co-culture time consisted 3 days in the dark. The hygromycin concentration chose 8 mg/L to screen the transgenic plants. 3) Using hygromycin to screen about 100 transgenic shoots, 75 shoots were obtained and 53 strains were identified had target stripe by PCR technology. Conclusion: The efficiency of the transformation system we created was over 50%. It provided a good basis for the study of potato gene function.

Background

Potato had become the world's third largest food crop and played a vital role in food security in pre-columbian times [1, 2]. Potatoes had played an important role in the period of food security, human nutritional demand and large population growth for the past 8 centuries [3]. The United Nations Department of Agriculture had even awarded the potato the 'hidden treasure'. It believed that the potato was the most important reliance on the human diet in the future. [13]

Over the past 15 years, the area planted with transgenic crops had gradually increased. One hundred and seventy million hectares were planted around the world until 2012 and genetically modified crops of corn, soybean and cotton which contained insect and herbicide resistant genes were mainly genetically modified crop in currently. Moreover, amylose-free line transgenic plants had also been approved for the commercialization [4].

Through gene transformation technology could possible obtain some characters that were not available in traditional breeding and enrich their genetic information. The advantage of gene transformation technology was that it did not influence the unique genetic integrity in a particular plant and many traits that were difficult to acquire from wild species could be incorporated into the genome [5, 6]. There were a variety of methods to improve crops according to genetic transformation. The two commonly methods were particle bombardment and Agrobacterium-mediated. Comparing these two methods, Agrobacterium-mediated methods had higher transformation frequency and efficiency and particle bombardment were beneficial to transform multiple genes. Although each method had its own advantages and disadvantages, the Agrobacterium-mediated method was currently the most widely used and preferred potato transformation method.

Dale was researched that only a half of 34 potatoes could be transformed with tubers and some varieties could neither be transformed with tubers nor leaves [7]. Han and their team showed that expect potato Jowon had high transformation efficiency and other species (Namseo, Chuback, Jopoong and Jasim) had lower efficiency with stems and leaves [8]. Some researchers from China [9–12] were identified some potato species but no one researched the regeneration system of sequenced species and these species had clearly genetic information to support the study of potato gene function.

Thus, in this study, a double haploid potato DM1-3-516-R44 (DM) [13] that was potato sequencing variety was used as the research object, and potato stems and leaves were used to establish an efficient regeneration system. On the other hand, optimizing agrobacterium transformation to improve transformation efficiency and provided support for research on revealing potato gene function.

Results And Discussion

Selection of callus induction medium for stems and leaves

Dedifferentiation and redifferentiation of explants was the main point of plant transformation. However, this point was based on the plant hormone ratio. Referenced some researchers results[14-18], we selected 54 combinations of 4 phytohormones to determine the ratio of hormones which were most suitable for dedifferentiation and form callus of DM (Supplement table 1-3).

The stems could induce the calluses under the MS with 6-BA and IAA cultured for about 20 days. But different concentration could lead to different types which were the callus took root and could not differentiate when the 6-BA concentration was lower than the IAA concentration (Figure 1A), and it was easy to produce white villi-like dense callus for a long time when the difference between 6-BA and IAA concentration was too large (Figure 1B).

Comparing with callus types under the MS with 6-BA and NAA showed that the leaves would be brown even dead under higher 6-BA concentration (Figure 1C) and callus took root under higher NAA concentration (Figure 1D). Only 6-BA and NAA had equally concentration could induce healthy callus (Figure 1E). And the combination of this hormone was not suitable for callus formation of stem.

Most of the concentration ratios could induce the loose and transparent callus or take root in MS medium containing 2,4-D of stems and leaves. Therefore, it was concluded that 2,4-D was not suitable for induce callus of DM (Figure 1F and G).

The results showed that the leaves could not induce the healthy calluses even turn into brown and death, but the stem could form better callus and green swollen appeared at both ends under the 6-BA and IAA hormone ratio (Figure 1H). On the contrary, under 6-BA and NAA hormone ratio, only leaves could induce better callus (Figure 1E). Even though stems and leaves could form some calluses, but the calluses were turned brown and dead under 6-BA and 2,4-D hormone ratio. Due to these ratios could induce some stems and leaves to form calluses, thus, so we selected some hormone ratio repeated were still unsatisfactory.

Finally, we could only select 2 different hormone ratios to induce stem and leaf to form callus, respectively. We chose 2.5 mg/L 6-BA and 0.5 mg/L IAA to induce stem to form callus and 1 mg/L 6-BA and 1 mg/L NAA to induce leaf.

Selection of shoots induction medium for callus

From previous studies, there were many studies on the ratio of hormones required for the differentiation of potato callus. Qiu [19] showed the optimum shoot induction medium of *Shapody* and *Fovorita* were MS+ ZT 2.0 mg/L+NAA 0.1 mg/L+GA₃ 5 mg/L and MS+6-BA 2.0 mg/L+ZT 2.0 mg/L+GA₃ 5 mg/L, respectively. The research of Zhang [20] indicated that the concentration of 6-BA with the highest shoot induction rate were *Atlantic*, *Fovorita*, *Shapody* were 2 mg/L and *Zi-Huabai* and *Desiree* were 1 mg/L. and the GA₃ also had different concentration in different species. The results of others research also indicated that the hormone ratio required for different genotypes of potato to differentiate into shoots was different, and the differentiation efficiency was similarly different [21-25].

Similar to callus induction medium selection, hormone selection also played a vital role in shoots induction. We also selected 4 hormones to determine the ratio of hormones (Supplement table 4-6) [23, 26-28]. Large difference in induction rate led to different concentration ratios. The higher the cytokinin concentration, the better the callus differentiation rate, but when the 6-BA concentration was higher than 4 mg/L, it would affect the time required for differentiation. And the NAA-added differentiation medium was not suitable for the differentiation of leaf callus. With the ratio of 6-BA and ZT, not only the differentiation time was reduced, but also the callus of leaves and stems could be affected (Figure 2A and B). Similarly, The ratio of 6-BA to GA₃ was favorable for the differentiation (Figure 2C).

The results showed the shoot induce ratio was highest under 3 mg/L 6-BA with the combination of different hormone types. Almost all of callus could be induced under the hormone ZT and 6-BA (3 mg/L 6-BA and 0.5 mg/L ZT, 3 mg/L 6-BA and 1 mg/L ZT) and GA₃ and 6-BA (2 mg/L 6-BA and 0.1 mg/L GA₃, 3 mg/L 6-BA and 0.1 mg/L GA₃). Finally, we selected 3 mg/L 6-BA and 0.5 mg/L ZT as the shoot induction because the higher inductivity and cost-effective.

Optimization of agrobacterium infection conditions

Infection concentration and co-cultivation time were the transformation of the factors influence based on agrobacterium infection. Some researchers also analyzed and adjusted the agrobacterium concentration, co-culture time and infection method, and finally obtained a better way [18, 27-30]. But everyone had their own method which might be related to the type of agrobacterium and potato varieties. And a lot of studies used OD₆₀₀ from 0.3 to 0.8 and Formation of callus and inhibition of agrobacterium was the key indicator of selecting. We used about 30 explants per treatment to observe there growth (Table 3).

The results showed co-culture 2 days was not efficient for agrobacterium infection under the OD₆₀₀ from 0.3 to 0.5. About half of explants were turned to tawny and others did not have obvious callus formation after 2 weeks later cultured in callus induction medium (Figure 3C and D). Until the co-culture was

extended to 3 days, a majority of explants were induced into callus (Figure 3E and F). But the higher concentration was hard to inhibit and led the explants were dead. This phenomenon was appeared when the OD600 was 0.5 (Figure 3A and B). To avoid this situation, we finally selected OD600 = 0.3 as infection concentration and 3 days for co-cultivation (Figure 3 E and F).

At present, the genetic transformation method using agrobacterium as a vector had been applied to a large number of crops, especially in dicotyledons [109]. However, because infection involved the regulation of multiple factors, the transformation efficiency was not high. This experiment combined the characteristics of potato material and the optimization of the agrobacterium transformation system by Shi Hu [98], and performed preliminary optimization from the aspects of agrobacterium concentration and co-cultivation time, and antibiotic screening. It was found that when potato explants were infected with a high concentration of agrobacterium, it was extremely difficult to inhibit the growth of bacteria, resulting in death of potato explants. Therefore, the concentration of agrobacterium used in this experiment was similar or slightly lower than that used by other researchers [110,111]. The screening results of co-cultivation time of callus in the dark showed that at a suitable concentration of agrobacterium, co-culture for more than 3 days would produce more agrobacterium, which was not easy to inhibit and not conducive to callus survival.

The influence of hygromycin concentration on plant

Hygromycin could prevent protein production in plant cells in the translation process [31]. The tolerance of transgenic plant cells to hygromycin depends on the copy number of the hygromycin resistance gene and its position in the nuclear genome. Plant cell death by hygromycin could produce phenolic compounds and their vacuolar constituents could negatively influence other cells. The cell death was not only because of the hygromycin concentration, but also the poisonous components produced in dead cells [32]. The suitable concentration of hygromycin was studied in potato stem, leaf and callus in our research.

The studies showed the leaves and stems were cultured in MS with Hyg for 20 days, the higher explants death and the higher hygromycin concentration (Table 4). The stems and leaves had same tendency. The higher hygromycin could led the explants all dead in about 20 days, the leaves and stems changed from green to tan, and the lower concentration could not play a role in selecting and could not even restrict explants growth, effectively.

Thus, the results indicated that 4 mg/L hygromycin could not restrict the explants growth and 12 mg/L hygromycin led the explants were all dead in a few days. And a half of explants were dead and others were turned brown till died a few days later under the hygromycin was 8 mg/L. These concentrations not only partially limited explants survival, but also certainly influenced plant growth.

We also used callus to select the hygromycin concentration (Table 5) and had same result as stem and leaf. Almost a half of callus cultured in 8 mg/L hygromycin were poorly growth and the higher concentration were led callus dead more and faster, about 15 days could be observed the dead callus

(Figure 4C). And the lower concentration could not screen the positive transgenic callus and hardly being the purpose concentration (Figure 4A). It could play a screening role and not affect the growth of transformed callus under the hygromycin concentration was 8 mg/L (Figure 4B).

Combined with the results we finally selected the 8 mg/L as a selection of transgenic plants. Apparently, the numbers of survival calluses were significant reduction when hygromycin concentration was 10 mg/L, and the other concentration were no obvious difference in the screening of transformed callus. Kashani and their team showed the suitable hygromycin concentration were 7.5, 12.5 and 10 mg/L for *Desiree*, *Agria* and *Marfona* cultivars, respectively [33] and their results were closely similar to mine. Simultaneously, it also verified that there were some differences in the tolerance to hygromycin between different varieties.

Identification of transgenic plants

We selected pFX-E24.2-15R vector to build the enhancer trap vector. Figure 5A showed the callus formation, differentiation and strike root. We selected some plants which had normally rooting and good growth potential for identification from a molecular way.

In order to obtain the real transgenic plants, the PCR tested the DNA could distinguish the positive plants (Fig 5B). Due to pFX-E24.2-15R vector contained hygromycin resistance gene sequence which the plants could not have these gene in nature. We used HYG primer to amplify the transgenic plants and wild DM as negative control and pFX-E24.2-15R vector as positive control to confirm the transgenic plants. The results of PCR showed the positive and transgenic plants had same strip, about 335 bp and the negative had nothing. The agarose gel electrophoresis showed that there was another stripe except the target one which might be the primer dimer. We finally identified 30 transgenic plants which all had brighter target stripe.

Conclusion

There had been many reports on methods for transforming potatoes based on agrobacterium infection. But the differences among varieties could had different responses of plant hormones. Combining the importance of sequenced potato DM in scientific research, we constructed a more efficient potato transformation system.

- 1) The stem callus induction medium was MS + IAA 0.5 mg/L + 6-BA 2.0 mg/L, the leaf callus induction medium was MS + NAA 1.0mg/L + 6-BA 0.5mg/L and the shoot differentiation medium was MS + 6-BA 3.0 mg/L + ZT 0.5 mg/L.
- 2) The specific transformation condition was the agrobacterium concentration kept the OD600 = 0.3, and co-culture time consisted 3 days in the dark. The hygromycin concentration chose 8 mg/L to screen the transgenic plants.

3) Using hygromycin to screen about 100 transgenic shoots, 75 shoots were obtained and 53 strains were identified had target stripe by PCR technology. Thus, the efficiency of the transformation system we created was over 50%.

Materials And Methods

Plant material growth conditions and reagents

The potato material, DM1-3-516-R44 (DM) which was sequenced double haploid potato, was grown with 20 g/L sugar on solid MS medium with vitamins (MS, USA) as culture medium for about four weeks in a plant incubator at $25\pm 2^{\circ}\text{C}$ under 10 000 Lx in light for 16 h and $20\pm 1^{\circ}\text{C}$ under 0 Lx for 8h.

The phytohormones were used to induce callus and differentiation including Indole-3-acetic acid (IAA), Naphthylacetic acid (NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D), 6-Benzylaminopurine (6-BA), Gibberellin (GA3) from Sangon Biotech (China) and trans-Zeatin (ZT) from Sigma (USA).

The antibiotics, Kanamycin (Kan), Rifampicin (Rif), Cefotaxime (Cef) and Hygromycin (Hyg) were used to cultivate agrobacterium and Screen of transgenic callus and plants. And these antibiotics were purchased from Solarbin (China).

Callus induction from stems and leaves

Referenced the results of potato regeneration system from some researchers showed the concentration and ratios of cytokinin and auxin were the main influence in callus formation process. Thus, we set the concentration of 6-BA as 0 mg/L, 1 mg/L, 1.5 mg/L, 2.5 mg/L, 3.5 mg/L and 4.5 mg/L and combined with IAA, NAA and 2,4-D at concentrations of 0.5 mg/L, 1 mg/L and 2 mg/L (Table 1) to find the best hormone ratio in callus induction medium (CIM). Statistics of callus formation cultured about 15 days after.

Induction of callus differentiation shoots

Combining with others' research, 6-BA, NAA, GA3 and ZT were used to induce the buds formation. Thus, different ratios of 6-BA and ZT, 6-BA and NAA, and 6-BA and GA3 were selected to determine shoot induction media (SIM). We finally set the phytohormone combination were list in table 2. The callus were cultured in the medium (MS) with different concentrations of hormones for 20 days to statistic the shoots induction.

Optimization of Agrobacterium infection conditions

We selected pFX-E24.2-15R (Enhancer trap) transformed Agrobacterium EHA105 to infect the potato stems and leaves. We set 4 different concentration of Agrobacterium solution, OD_{600} were 0.3, 0.4 and 0.5, and co-cultivation with explants for 2 or 3 days. And the specific steps for Agrobacterium transformation were detailed below.

1. Set an overnight culture of *Agrobacterium* in YEB medium with the correct antibiotics (Rif + Kan) and let grow the culture of *Agrobacteria* until $OD_{600} = 0.3, 0.4$ and 0.5 . Resuspended in the same volume YEB medium without antibiotics to an $OD_{600} = 0.3, 0.4$ and 0.5 .
2. Cut potato stems without growing point and leaves through the middle-rip one or two-fold without petiole pre-cultured in callus induction medium (pre-CIM) for about 3 days, and place them in a dish containing *Agrobacterium* and shake culture for 10 min.
3. Filter paper was blotted dry, and the stems and leaves was incubated in callus induction medium (CIM) without antibiotics for 2 or 3 days in the dark.
4. Rinsed the cultured callus with sterile water containing 500 mg/L Cef and sterile water for 4 times. Blotted dry sterile water with filter paper and transferred into CIM with 300 mg/L Cef and Hyg.
5. After 15 days transferred the callus onto shoot induction media (SIM) and changed the media every 10 days until shoots were formed.
6. The shoots reached a length of about 1-2 cm and transferred them into MS2 (MS with 20 g/L sugars) media with 250 mg/L Cef and Hyg.

Screening of hygromycin concentration

To avoid potato explant was all dead under the higher antibiotic concentration or the low concentration could not preliminary screening of transformed callus. Thus, we set 6 gradients, 0 mg/L, 4 mg/L, 8 mg/L, 12 mg/L, 16 mg/L and 20 mg/L to find out an optimal concentration before potato genetic transformation.

We cultivated potato stems and leaves in a plate containing MS medium with hygromycin for about 2 weeks to statistic the statement of these tissues. Moreover, we transferred the co-cultured callus into a differentiation medium containing 5 hygromycin concentration gradients, 2 mg/L, 4 mg/L, 6 mg/L, 8 mg/L and 10 mg/L. statistic of callus survival about 2 weeks later.

Identification of transgenic plants

The shoots were cut into MS medium with 300 mg/L Cef and Hyg, and cultured four weeks in a plant incubator at $25 \pm 1^\circ\text{C}$ under 10 000 Lx in light for 16 h and $20 \pm 1^\circ\text{C}$ under 0 Lx for 8h. Selected the transgenic plants which were normal root induction and growing to DNA extraction. Through the premier 5 software to design a suitably hygromycin-specific primer (Hyg-F: AGGCTCTCGATGAGCTGATGCTTT; Hyg-R: AGCTGCATCATCGAAATTGCCGTC) to identify the transgenic plants by PCR reaction.

Declarations

Author's contributions

C.Z., Y.Y., Q.C. conceived the experiments. C.Z., D.W. performed and analyzed the experiments. C.Z., Y.Y. wrote the paper.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Conflict of Interest

None of the authors have any actual or potential conflicts of interest.

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Tables

Table 1 Hormonal composition of callus induction media

6-BA	Hormones (mg/L)								
	IAA			NAA			2,4-D		
0.0	0.5	1.0	2.0	0.5	1.0	2.0	0.5	1.0	2.0
1.0	0.5	1.0	2.0	0.5	1.0	2.0	0.5	1.0	2.0
1.5	0.5	1.0	2.0	0.5	1.0	2.0	0.5	1.0	2.0
2.5	0.5	1.0	2.0	0.5	1.0	2.0	0.5	1.0	2.0
3.5	0.5	1.0	2.0	0.5	1.0	2.0	0.5	1.0	2.0
4.5	0.5	1.0	2.0	0.5	1.0	2.0	0.5	1.0	2.0

Table 2 Hormonal composition of shoot induction media

6-BA	Hormone concentrations (mg/L)									
	NAA				ZT			GA3		
1.0	0.0	0.1	0.5	1.0						
2.0	0.0	0.1	0.5	1.0	0.1	0.5	1.0	0.1	0.5	1.0
3.0	0.0	0.1	0.5	1.0	0.1	0.5	1.0	0.1	0.5	1.0
4.0	0.0	0.1	0.5	1.0	0.1	0.5	1.0			

Table 3 Infection of agrobacterium concentration and co-culture time on the conversion

OD ₆₀₀ value	Co-cultivation time (Days)	Callus survival	Agrobacterium inhibit
0.3	2	part of explants dead	Basic inhibit
0.3	3	formation calluses	Basic inhibit
0.4	2	part of explants dead	Basic inhibit
0.4	3	formation calluses	Basic inhibit
0.5	2	part of explants dead	hard to inhibit
0.5	3	a lot of explants dead	hard to inhibit

Table 4 potato stems and leaves of hygromycin sensitivity test

Object		Hygromycin concentration (mg/L)					
		0	4	8	12	16	20
Number of cultured explants	stems	45	45	45	45	45	45
	leaves	30	30	30	30	30	30
Number of surviving explants	stems	45	33	18	6	0	0
	leaves	30	23	10	1	0	0
Explant survival rate	stems	100%	73%	40%	13%	-	-
	leaves	100%	77%	33%	3%	-	-

Table 5 Potato callus to hygromycin sensitivity test

Object	Hygromycin concentration (mg/L)				
	2	4	6	8	10
Number of cultured callus	30	30	30	30	30
Number of surviving callus	28	24	20	17	3
Callus survival rate	93%	80%	67%	57%	10%

Additional File Legend

Additional file 1. Different proportions of hormones induced calluses and shoots.

Figures



Figure 1

Leaf and stem induced callus under different hormone concentrations. A, B and H showed the typical callus condition under the 6-BA and IAA. C, D and E were the typical callus condition under the 6-BA and NAA. F and G were the typical callus condition the 6-BA and NAA. These calluses were cultured about 20 days and changed the media every 10 days.

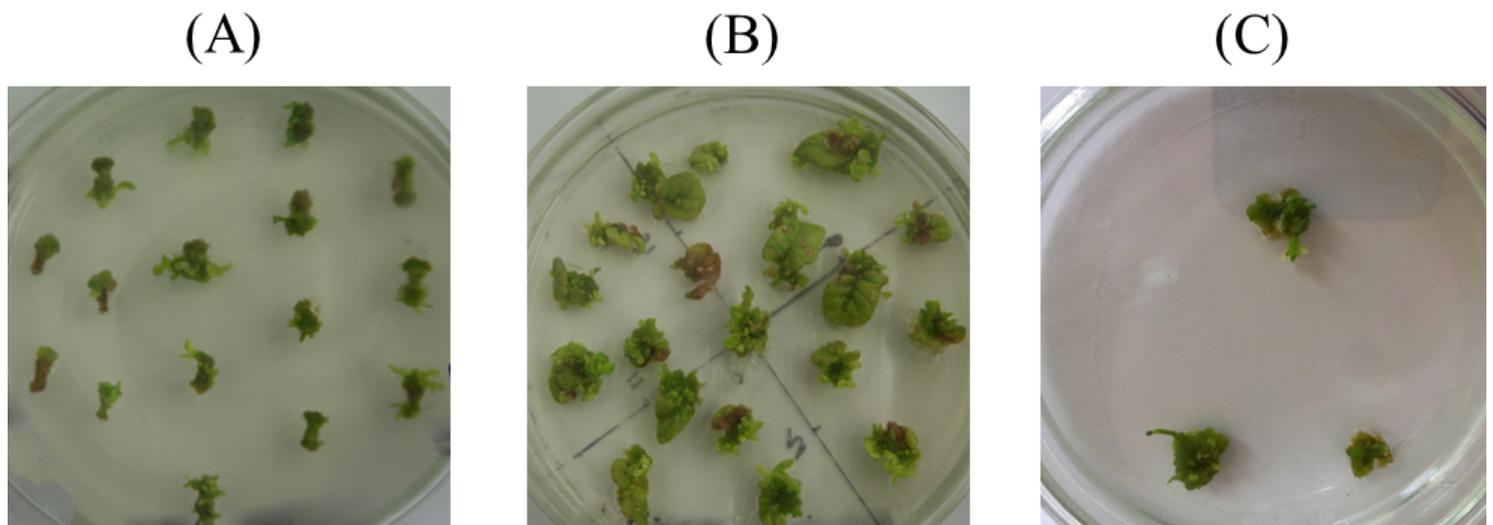


Figure 2

Calluses induced shoots under different hormone concentrations. A and B showed the induction of shoots under the 6-BA and ZT. C was the induction of shoots under the 6-BA and GA3.



Figure 3

The statuses of explants under the different co-cultivation periods and agrobacterium concentration. A and B showed the concentration of agrobacterium was hard to inhibit under the OD600 was 0.5. C and D showed the explants were turning to dead under the 2 days co-cultivation. Only E and F induced healthy calluses and could inhibit the bacteria growth under 3 days co-cultivation and the OD600 was 0.3-0.4 which was better conditions to suitable agrobacterium transformation.

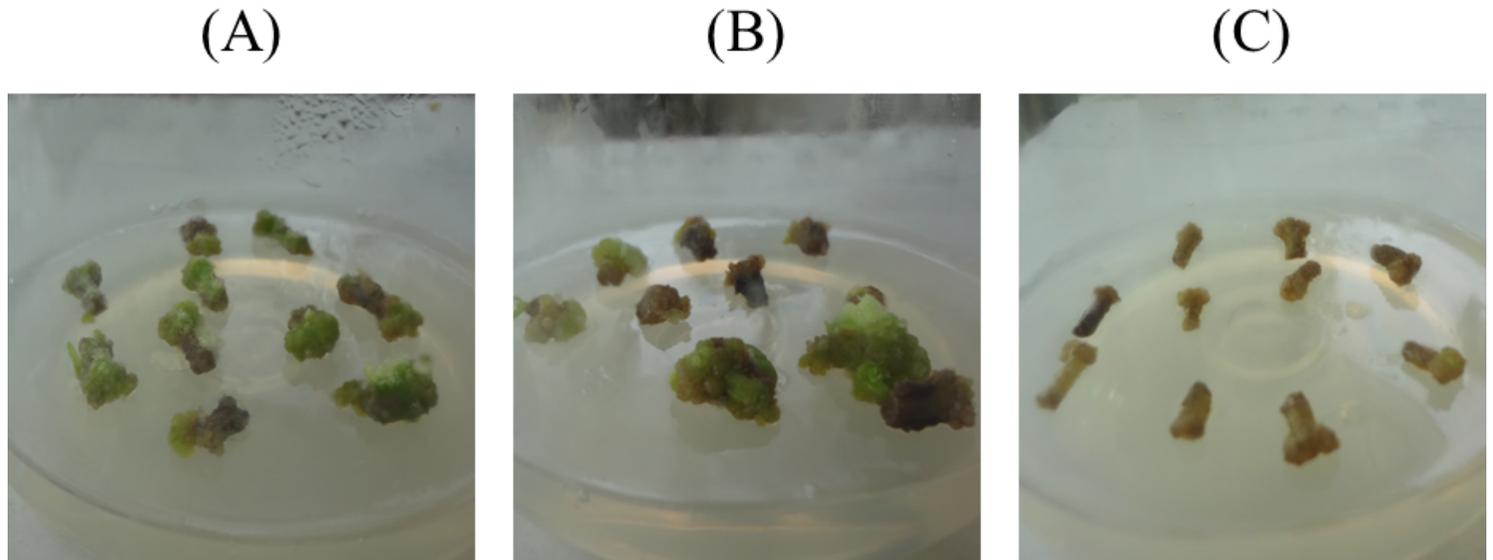
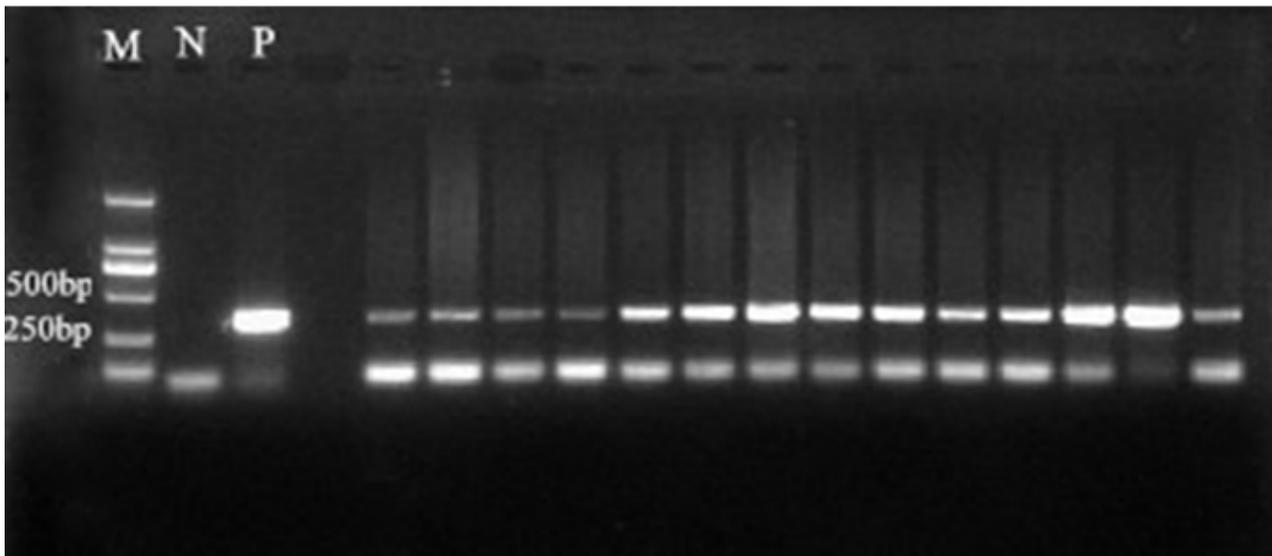


Figure 4

The calluses were cultured for 15 days after agrobacterium infection under hygromycin concentration. A was the statue of callus under 4 mg/L, almost every stem induced callus. B showed a half of stems were dead and the others were induced calluses under 8mg/L. And C showed the stems almost all dead under 10 mg/L.



A



B

Figure 5

The identification of transgenic plants. A indicated the growth of transgenic potato which was the state of normal growing transgenic plants and roots. B showed PCR detection of transgenic potato. M: DL2000 DNA Maker. N: Negative control (wild-type). P: Positive control (the plasmid of pFX-E24.2-15R). Others were transgenic plants. The Target electrophoretic band size was 335 bp. All bands have target bands except for the negative control and it could be proved the regenerated plants were transgenic, preliminarily. And these tested transgenic plants were well grown singles.

Supplementary Files

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