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Full Length Research Paper

Studies on *Agrobacterium*-mediated genetic transformation of embryogenic suspension cultures of sweet potato

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In this study, genetic transformation of embryogenic suspension cultures of sweet potato (Ipomoea batatas) cultivar Xu55-2 was conducted utilizing the *Agrobacterium tumefaciens* strain EHA105 that contains the binary vector pBIN19/SBD2 with SBD2 (starch binding domain 2) gene and neomycin phosphotransferase (NPT II) gene. The presence of the SBD2 gene in the genomic DNA of transgenic plants was verified by PCR amplification and confirmed by Southern blot analysis. Results suggested that cefotaxime (Cefo), at the concentration of 200 mg/L, was able to effectively suppress the growth of *Agrobacterium* after co-cultivation. The optimal concentration for kanamycin (Kan) was 10 mg/L for selecting resistance calli, somatic embryo formation and plant regeneration. The highest frequency of shoot induction (30.9%) was obtained on the MS medium containing 10 mg/L Kan, 200 mg/L Cefo, 1.0 mg/L abscisic acid (ABA) and 1.0 mg/L gibberellic acid (GA_3).

Key words: *Ipomoea batatas*, *Agrobacterium*-mediated transformation, SBD2 gene, embryogenesis.

INTRODUCTION

Sweet potato (*Ipomoea batatas*) is the sixth most important crop in the world after wheat, rice, corn, white potato and barley (Vietmeyer, 1986). Sweet potato improvement through conventional breeding is a complicated process and has not been very successful. This is due to the fact that sweet potato is a hexaploid crop and genes from the cultivated sweet potato gene pool are not easily accessible by direct sexual hybridization.

A highly promising alternative to the conventional breeding is the introduction of foreign genes into plants through genetic transformation. Attempts over the last few years to produce transformed sweet potato plants utilized different gene transfer systems including the electroporation of protoplast (Nishiguchi et al., 1992) and the transformation of *Agrobacterium rhizogenes* (Dodds et al., 1991; Otani et al., 1993) and *Agrobacterium tumefaciens* (Al-Juboory and Skirvin, 1991; Carelli et al., 1991; Lowe et al., 1994). Since the *Agrobacterium*-mediated transformation system does not involve sophisticated equipment and frequently produces cleaner events (intact integrations and single copy) than particle gun bombardment (Hansen et al., 1997), it remained to be the more favorite approach. The transformation process is also especially advantageous for clonally propagated crops like sweet potato, as the primary transformation event is after thorough characterization of the final transformed variety (Luo et al., 2006).

Embryogenic calli are generally recognized to possess high proliferation and high regeneration abilities. Successful production of transgenic plants via co-cultivation of embryogenic callus with *Agrobacterium* has been previously reported for both monocotyledonous (Delbreil et al., 1993; Cheng et al., 1997) and dicotyledo-

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; ABA, abscisic acid; AS, acetosyringone; Cefo, cefotaxime; GA, gibberellic acid; Kan, kanamycin; MS, Murashige and Skoog; NPT II, neomycin phosphotransferase; PCR, polymerase chain reaction; SBD, starch-binding domain.

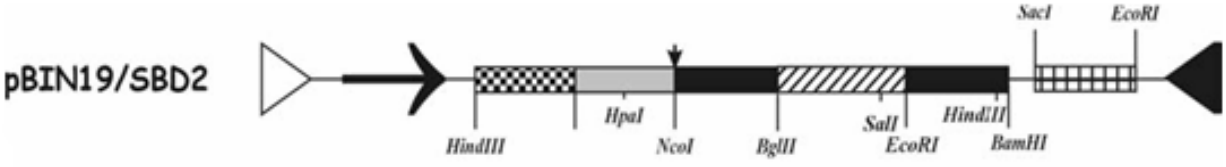

Figure 1. Schematic representation of T-DNA region of the binary vector pBIN19/SBD2.
→ : kan resistance; ▼ : cleavage site transit peptide; : potato GBSSI promoter; : potato GBSSI transit peptide; : SBD; : PT-rich linker; : NOS terminator.

nous plant species (Nakano et al., 1994), including sweet potato cultivars Jewel (Moran et al., 1998) and Li Zixiang (Zhai and Liu, 2003). However, previously we observed the formation of calli and root-forming calli when various tissues of sweet potato (cultivar Xu55-2) were used as explants for *Agrobacterium* transformation. Yet after the formation of shoot-forming calli, plant regeneration stopped.

In this study, the *A. tumefaciens* strain EHA105 harboring the pBIN19/SBD2 plasmid was used for the transformation and regeneration studies of the embryogenic suspension cells of sweet potato Xu55-2. An efficient plant transformation and regeneration system was established, which would provide guidance on the improvement of sweet potatoes through genetic engineering.

MATERIALS AND METHODS

Agrobacterium and the plasmid

A. tumefaciens strain EHA105 (rifampicine resistant) harboring the plasmid pBIN19/SBD2 was transformed into sweet potato cultivar Xu55-2. The pBIN19/SBD2 plasmid contained two SBD fusions through an artificial PT-rich linker. The SBD2 gene expression was under the control of the tuber-specific potato GBSSI promoter from the plant expression vector pBIN19. Amyloplast entry of SBD2 was mediated by the potato GBSSI transit peptide from the vector. The NPT II gene was designed for the selection of kanamycin (Kan) resistance (Figure 1) (Ji et al., 2004).

Establishment of embryogenic suspension cultures

The top buds (about 1–1.5 cm in length) were cut from sweet potato plants, washed with running water, surface sterilized in 70% ethanol for 30 s before soaked in 0.1% $HgCl_2$ solution containing one drop of Tween-20 for 3 min. The buds were then rinsed five times with sterile distilled water. The shoot apices of 1 mm in length were cut from the sterilized buds and placed on MS (Murashige and Skoog, 1962) solid medium containing 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) (CIM media, Table 1) at 28ºC in the dark for 40-60 d, refreshing medium at 25 d intervals. The embryogenic callus, derived from shoot apices of the Xu55-2, was used to initiate embryogenic suspension cultures in the CIM liquid medium with shaking at 100 rpm, at 28ºC with a 14 h photoperiod for 80 days. Fresh medium was replaced every week during the experiments.

Culture of the *Agrobacterium*

The *Agrobacterium* with the pBIN19/SBD2 plasmid was grown in 50 mL LB (pH 7.0) medium containing 100 mg/L rifampicine and 50 mg/L kanamycin with shaking at 180 rpm at 28ºC in the dark until OD_{600} reached 0.6. Cells were collected by centrifugation at 4000 rpm for 10 min and re-suspended in 50 mL MS liquid medium.

Plant transformation

After sub-culturing in the CIM liquid medium for 3 days, the embryogenic suspension cultures were treated with 0.45 mmol/L mannitol for 60 min and washed twice with 0.16 mol/L $CaCl_2$. Subsequently, the cell aggregates were incubated in *Agrobacterium* suspension for 10 min, and blotted dry on sterile filter papers. The mixed cell aggregates were then incubated in the CIM liquid medium supplemented with 0.1 mmol/L acetosyringone (AS) at 28ºC in the dark for 4 days.

Determination of the *Agrobacterium*-killing cefotaxime concentration

Cefotaxime was used to kill the rest of *Agrobacterium* after co-cultivation. The effective killing concentration of Cefo in the medium was investigated. The co-cultivated cell aggregates were transferred to CM0, CM1, CM2, CM3, CM4 and CM5 liquid media (Table 1) and incubated at 28ºC with shaking at 100 rpm and a 14 h photoperiod. Observation on the growth suppression of *Agrobacterium* was conducted for 7 days.

Kanamycin sensitivity test

To test the kanamycin sensitivity of the embryogenic callus, cell aggregates were incubated on CIM, CIM1 , CIM 2 , CIM 3 , CIM 4 and CIM 5 media containing various amounts of Kan (Table 1). Calli were refreshed on the same solid medium once. Six weeks after inoculation, the frequency of embryogenic callus livability and proliferation were examined.

Regeneration of transgenic plants

After the growth of *Agrobacterium* was suppressed with Cefo, cell aggregates (0.5 - 0.7 mm in size) from embryogenic suspension cultures were transferred to the MS medium containing 2 mg/L 2,4-D, 10 mg/L Kan and 200 mg/L Cefo (SPM medium) and incubated for two months to form the Kan-resistance embryogenic calli. The embryogenic calli were further transferred to different RM media containing either abscisic acid (ABA) alone or in combination with different concentrations of gibberellic acid (GA_3) (Table 1). Calli

Table 1. Media used for kannmycin and cefotaxime sensitivity tests and plant regeneration.

Medium	Basic medium	2,4-D (mg/L)	Kanamycin (mg/L)	Cefotaxime (mg/L)	ABA (mg/L)	GA_3 (mg/L)
CIM	MS	2				
CIM1	CIM		5			
CIM2	CIM		10			
CIM3	CIM		15			
CIM4	CIM		20			
CIM5	CIM		25			
CM0	CIM (liquid)			0		
CM1	CIM (liquid)			100		
CM2	CIM (liquid)			200		
CM3	CIM (liquid)			300		
CM4	CIM (liquid)			400		
CM5	CIM (liquid)			500		
RM 1	MS		10	200	1.0	
RM2	MS		10	200	1.0	1.0
RM3	MS		10	200	1.0	2.0

All media listed in Table 1 were autoclaved at 120ºC for 20 min. Growth regulators and antibiotics were filter-sterilized and added to the medium after autoclaving. For the solid medium, 8 g/L agar was added. The pH of all media was adjusted to 5.8.

were incubated for 4-10 weeks to allow the induction of the somatic embryo formation and shoot regeneration. Media were replaced every three weeks during this period. The formed shoots were harvested and tested for root growth on the MS medium containing 10 mg/L kanamycin (GRM medium) in 4 weeks.

Molecular characterization of transgenic plants

Total genomic DNA was isolated from fresh leaves of transformed and untransformed (control) plants according to the CTAB protocol (Ukoskit et al., 1997) with minor modifications. The DNA was then used for PCR amplification and Southern blot analysis.

The presence of SBD2 gene in genomic DNA of the transgenic sweet potato was confirmed first by PCR amplification with the primers 5'- AAGCCAAACTTCACTAGACACCAA-3' and 5'-AGATCTCTGCCAATTCACGTTAA-3'. The pBIN19/SBD2 plasmid DNA was used as a positive control. Amplification was carried out under the following conditions: 94ºC for 4 min as initial denaturetion, followed by 30 cycles of 94ºC for 1 min, 57ºC for 45 s, 72ºC for 1 min and the final extension at 72°C for 10 min. Amplified DNA fragments were separated by electrophoresis on 0.8% (w/v) agarose gel.

For Southern blot analysis, the plant genomic DNA samples from transformed and untransformed (control) plants were digested with BamHI and NcoI restriction endonuclease before separated on 0.8% (w/v) agarose gel and blotted onto Hybond-N+ nylon membrane (Amersham). The membrane was fixed by UV cross-linking and hybridized with DIG-labeled SBD2 as a probe (SBD2 gene fragment of 720 bp). Probe labeling was performed according to the manufacturer's instructions (Roche, Germany). The pBIN19/SBD2 plasmid DNA was used as a positive control.

RESULTS

Preparation of embryogenic suspension cells and transformation

The sterilized shoot apices were obtained from sweet potato plants and cultured on the CIM medium for 40-60 days. After the formation of the friable and yellow granular calli, the calli were transferred into liquid CIM medium. About 80 days later, rapidly proliferating and well dispersed embryogenic suspension cultures were established and used for the genetic transformation in this study (Figure 2A).

Determination of Cefo concentration for killing *Agrobacterium*

After the cell aggregates were co-cultured with *Agrobacterium*, cefotaxime was used for killing the rest of *Agrobacterium*. Results of growth suppression of *Agrobacterium* from different concentration of Cefo were summarized in Table 2. It was observed that infection of *Agrobacterium* around the transformed cell aggregates occurred after 3 days on CM0 and 4 days on CM1, whereas infection of *Agrobacterium* did not happened on CM2 (containing 200 mg/L Cefo) medium, indicating that the efficiency concentration of Cefo to kill the rest of *Agrobacterium* after co-cultivation was at 200 mg/L. Therefore, this concentration was used in all subsequent experiments.

Kanamycin sensitivity test

Various Kan concentrations were tested for embryogenic callus livability and proliferation. The observations were recorded 6 weeks after the induction and summarized in Table 3. Data suggested that increased Kan concentration in the medium significantly decreased the frequency of embryogenic callus liveliness and proliferation. When

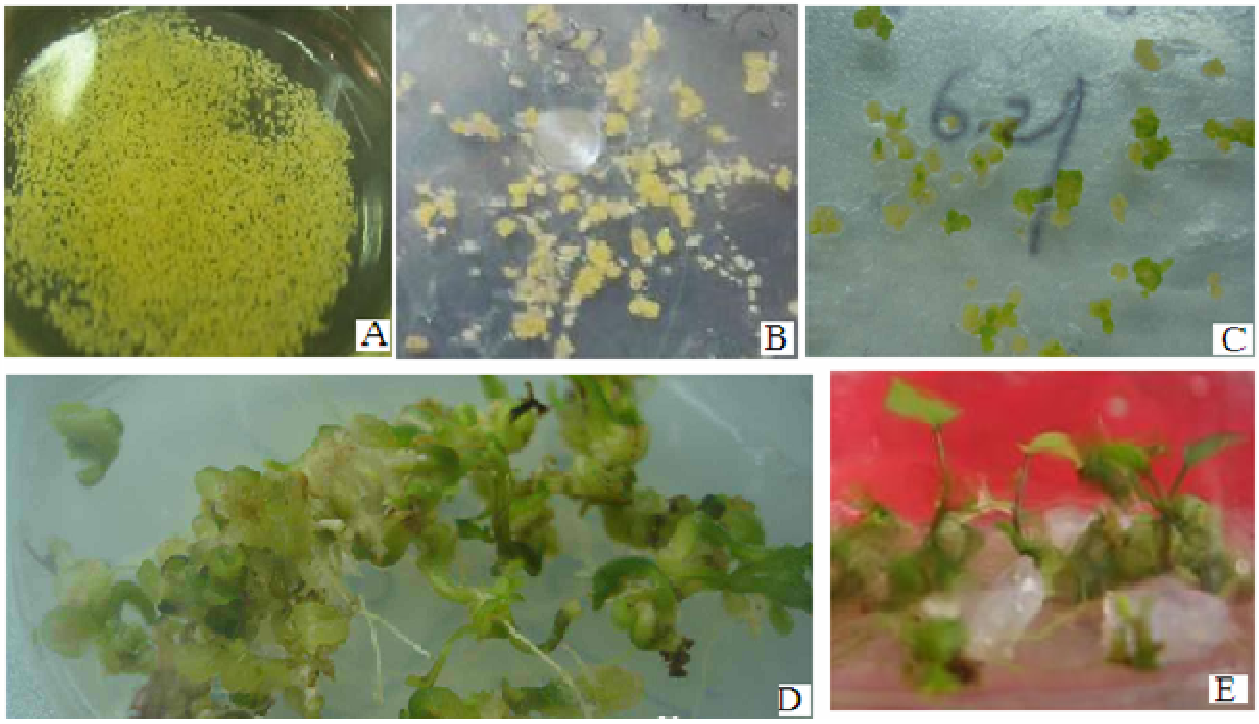

Figure 2. Growth and differentiation state of embryogenic calli co-cultured before and after with *Agrobacterium*. (A) Embryogenic suspension cells. (B) Kan-resistance calli (white calli meaned non-kan-resistance calli, yellow calli were Kan-resistance calli). (C) Starting to form somatic embrygenic calli. (D) Bigger somatic embryonic growth. (E) Shoot formation from somatic embrygenic calli.

Table 2. Suppression of *Agrobacterium* growth by cefotaxime.

Medium	Growth of *Agrobacterium*					
	2d	3d	4d	5d	6d	7d
CM0	-	+	+	+	+	+
CM1	-	-	+	+	+	+
CM2	-	-	-	-	-	-
CM3	-	-	-	-	-	-
CM4	-	-	-	-	-	-
CM5	-	-	-	-	-	-

+ = *Agrobacterium* growth; - = no *Agrobacterium* growth.

Kan was at 10 mg/L, the frequency of embryogenic callus liveliness and proliferation was 74.5 and 19.6%, respectively. However, when the concentration was increased to 15 mg/L, the frequency of embryogenic callus livability dropped to 61.5% and the proliferation was completely inhibited. Therefore, 10 mg/L of Kan was chosen as an optimal concentration for the selection of Kan-resistance callus in subsequent experiment.

Induction of Kan-resistance callus

The co-cultured cell aggregates were transferred onto the SPM medium (MS adding 2 mg/L 2,4-D, 10 mg/L Kan and 200 mg/L Cefo) for selection and proliferation of embryogenic callus. A total of 180 Kan-resistance embryogenic callus were obtained after two months (Figure 2B).

Effects of growth regulators on somatic embryogenic formation and shoot regeneration

The effects of ABA (1.0 mg/L) and GA_3 (1.0 and 2.0 mg/L) on somatic embryo formation and shoot regeneration were examined. Results presented in Table 4 sug-

Table 3. Effects of Kan concentration on embryogenic callus livability and proliferation.

Medium	Number of cell aggregates	Livability of embryogenic callus		Proliferation of embryogenic callus	
		Number	%	Number	%
CIM	35	35	100	35	100
CIM1	42	41	97.6	40	95.2
CIM2	51	38	74.5	10	19.6
CIM3	39	24	61.5	0	0
CIM4	36	19	52.8	0	0
CIM5	28	13	46.2	0	0

Table 4. Effects of growth regulators on somatic embryogenic formation and shoot regeneration.

Medium	ABA (mg/L)	GA_3 (mg/L)	Number of Callus	Frequency of somatic embryogenic formation (%)	Frequency of shoot regeneration (%)
RM1	1.0	0	78	98.7a	19.2b
RM2	1.0	1.0	55	96.4a	30.9a
RM3	1.0	2.0	47	87.2b	6.4c

a, b and c indicate values that are significantly different at the 5% level ($p \leq 0.05$) using Tukey's multiple comparison test.

gested no significant change in the rate of formed somatic embryogenic between RM1 and RM2 group ($P>0.05$). However, significant difference ($P<0.05$) between RM2 and RM3 group was observed, indicating compromised consistency in the rate of somatic embryogenic formation on the RM1 and RM2 media. Thus, 1.0 mg/L GA_3 did not have a significant effect on the somatic embryogenic formation. Furthermore, when GA_3 concentration was increased to 2.0 mg/L, the rate of somatic embryogenic formation remarkably decreased.

A notable difference in the rate of shoot regeneration was found between RM2 and RM1 or RM3 groups ($P<0.05$), indicating that the rate of shoot regeneration on RM2 was higher than that on RM1 and RM3. However, in the presence of 2.0 mg/L GA_3, the rate of shoot regeneration decreased. Data clearly demonstrated that adding of GA_3 at low concentration (1.0 mg/L) to the medium containing 1.0 mg/L ABA improved the shoot regeneration. However, higher concentration of GA_3 (2.0 mg/L) affected both the rates of somatic embryogenic formation and shoot regeneration.

Regeneration of transgenic plants

Embryogenic callus began differentiation (Figure 2C) and continued to grow (Figure 2D) on RM2 medium. After 4-10 weeks, thirty five independent shoots were harvested (Figure 2E). Root formation was then induced on the GRM medium. Four weeks after the initiation, 12 root-forming shoots were obtained.

Molecular analysis of transformants

The integration of the SBD2 gene into the genome of the transgenic plants was confirmed by PCR and Southern blot analysis. PCR was performed using SBD2 primers. PCR fragments (approximately 500 bp) corresponding to the amplified internal fragment of SBD2 gene were detected in the transgenic plant samples as well as in the positive control, but not detected in untransformed plant (Figure 3A), indicating the presence of SBD2 gene in the genome of the transgenic plants. Based on the PCR result, the transgenic plants were further investigated by Southern blot analysis. Results in Figure 3B showed the presence of the hybridization signals at 0.72 kb in all transformed plants as well as in the positive control, but absent in the untransformed negative control, confirming the integration of SBD2 gene in the genome of the transgenic plants.

DISCUSSION

In our previous study, a SBD-technology for incorporating foreign proteins into starch granules during the biosynthesis process has been developed (Ji et al., 2003). The use of microbial starch-binding domain (SBD)-encoding region of cyclodextrin glycosyltransferase (CGTase) from Bacillus circulans as an anchor is the key of this technology, in which SBDs can be fused to a large choice of effector proteins to generate starches with new or improved functionalities. Furthermore, we have shown

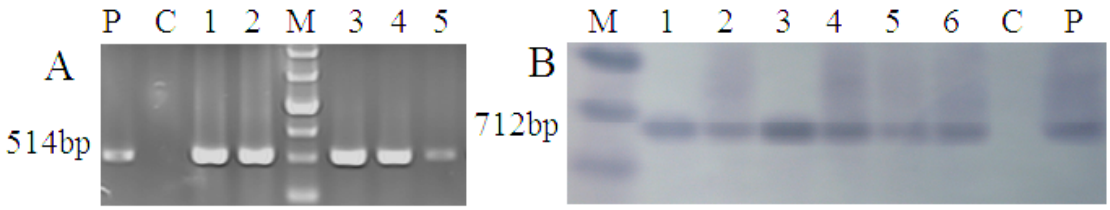

Figure 3. Confirmation of SBD2 gene integration into the sweet potato genome by PCR (A) and Southern blot analysis (B). P: positive control, pBIN19/SBD2 plasmid DNA used as template; C: negative control, untransformed plant genomic DNA used as template; 1-6: Transgenic plant; M: 4500 bp molecular marker.

that an artificial tandem repeat of SBD (SBD2) has much higher affinity for starch granules than single SBD (Ji et al., 2004), and this SBD2 can be used as an alternative for making SBD fusion proteins.

The sweet potato Xu55-2 is a major cultivar in China and it is grown mainly for human food. The development of an efficient and reproducible transformation system is needed for genetic manipulation of sweet potato via the SBD-technology to improve the crop.

In this study, genetic transformation of embryogenic suspension cultures of sweet potato cultivar Xu55-2 was conducted by using *A. tumefaciens* strain EHA105 containing the binary vectors pBIN19/SBD2 with SBD2 gene and NPT II gene. During the plant regeneration, an efficient selection concentration of Kan is required for the production of transgenic plants. It is known that Kan concentration for resistance embryogenic callus selection, proliferation and plant regeneration of various sweet potato cultivars varied from 25 mg/L to 100 mg/L (Moran et al., 1998; Zhai and Liu, 2003). Our results indicated that Xu55-2 sweet potato cells were more sensitive to Kan than other cultivars with an optimal concentration of 10 mg/L for selecting resistance calli, somatic embryos formation and plant regeneration. The difference in Kan concentration among various cultivars could attribute to the genotypic differences in sweet potato cultivars. We also observed that the process of shoot formation could be controlled by ABA and GA_3. A higher rate of shoot formation could be obtained in the medium containing 1.0 mg/L ABA and 1.0 mg/L GA_3 than in the medium containing only ABA. Data indicated that 1.0 mg/L GA_3 acted with 1.0 mg/L ABA in a synergistic manner for shoot formation. However, when the concentration of GA_3 in the medium was increased to 2.0 mg/L, not only this synergy disappeared but also the somatic embryos formation and the shoot regeneration were inhibited.

Results from this study suggested that the optimal concentration of cefotaxime for killing the rest of *Agrobacterium* after co-cultivation was 200 mg/L and the optimal concentration for kanamycin was 10 mg/L for both somatic embryo formation and plant regeneration. In addition, the highest frequency of shoot induction was obtained on the MS medium containing 10 mg/L Kan, 200 mg/L Cefo, 1.0 mg/L ABA and 1.0 mg/L GA_3. Based on the information obtained from the investigation, a genetic transformation system of embryogenic suspension cultures of sweet potato cultivar Xu55-2 using *A. tumefaciens* strain EHA105 with the pBIN19/SBD2 plasmid was established. The obtained embryogenic suspension cultures were precultured in the CIM liquid medium for 3 days. After transformation, the optimal co-culture time was 4 days. The formation and proliferation of embryogenic callus were carried out on the MS medium containing 2 mg/L 2,4-D, 10 mg/L Kan, 200 mg/L Cefo for two months. The somatic embryogenic formation and shoot regeneration were carried out on the MS medium containing Kan 10 mg/L, Cefo 200 mg/L, ABA 1.0 mg/L and GA_3 1.0 mg/L for 4-10 weeks. The formed shoots were harvested and induced for root growth on the MS medium containing 10 mg/L kanamycin for 4 weeks. The somatic embryo-derived transgenic sweet potato plants were successfully obtained. PCR and Southern blot analysis confirmed that SBD2 gene has been integrated into the sweet potato genome.

In summary, we have established a genetic transformation system in embryogenic suspension cultures of sweet potato with *A. tumefaciens* strain EHA105, which will guide the modification of sweet potato cultivar Xu55-2 for improvement via the SBD-technology.

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