Agrobacterium tumefaciens-mediated transformation in shallot (Allium cepa L.)

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Abstract

This paper describes the use a reliable protocol for producing transgenic plants of 2 cultivars of shallot from mature zygotic embryos. Embryos were isolated and cultured in MS medium containing 1 mg/l 2,4-D for producing embryogenic callus. Three weeks old calli were inoculated *with Agrobacterium tumefaciens* AGL0 pCAMBIA 1301-cry1Ca, and AGLOpCAMBIA 1301-ho4. Calli were co-cultivated in MS medium supplemented with 100 µM acetosyringone and examined their transient expression of after 4 days culture. The infected calli were then transferred to selection medium MS containing 1 mg/l 2,4-D, 400 mg/l cefotaxime, 100 mg/l vancomycin and 50 mg/l hygromycin for 2 months which they were subculture in the same medium every 2 weeks. The resistant calli were transferred to regeneration medium MS with 1 mg/l kinetin and 50 mg/l hygromycin. Characterizations of transgenic plants were done by PCR analysis by amplifying *hpt* gene and genomic DNA flanking T-DNA by means of adaptor ligation PCR (AL-PCR). GUS transient expression after 4 days co-cultivation were up to 75 percent in shallot. From total of 2 836 embryos of shallot, 2 244 callus lines were transferred to selection medium and 291 of them were able to survive and transferred to regeneration medium. Plants were formed either by organogenesis or formation of somatic embryos after one-week culture. In shallot cv. 'Tropix' (pCAMBIA 1301-cry1Ca), 34 shoots were produced from individual callus of 18 lines from a total of 36 lines cultured in regeneration medium. In the case with pCAMBIA 1301-ho4 26 plants were produced from individual callus of 16 lines from a total of 41 lines cultured in regeneration medium. Amplifying the *hpt* gene by specific primers produced single band of 1.2 kb indicating that all examined plant samples were transgenic. The AL-PCR confirmed the result.

INTRODUCTION

Shallot (*Allium cepa* L.) is important culinary plants as well as a medicinal herbs due to their ability to improve the taste of food. Shallot is vulnerable to a number of diseases and pests (Rabinowith, 1977). In tropical region purple blotch (*Alternaria porri*), anthracnose (*Colletotricum gleoesporiodes*) and beet armyworm (*Spedoptera exigua*) (Zheng et al., 2001a). The Armyworm can be very

devastating pest for shallot cultivation especially in tropical regions. It is very difficult to control with chemicals, because the young larvae weave leaves together and feed under the webbing that protect them from insecticides, while older larvae are more tolerant to pesticides. Introduction of resistant genes are very important. The gene can be from *Lycopersicon hirsutum* that has to types of resistance, namely trichome-based and a lamellar factor-based (Zheng, 2000), but introgression of such traits to shallot has never been performed. The other alternatives are introgressing genes from other genera, insects or bacteria by means of transformation. As example the powerful alien gene that is able of controlling such a disease there is Bt gene (Cry-gene) from *Bacillus thuringienses.* This gene encodes a crystal protein and the crystals are toxic to different insects such as lepidoptera, diptera and coleoptera. For instance cry1Ba is active against larvae of moths, fires and beetles (de Maagd et al., 2001).

Published protocols for transformation and regeneration of shallot and garlic are very rare. As monocotyledonous plant, both crops do not to be susceptible to *Agrobacterium*-mediated transformation. The development of direct gene transfer techniques soon led to particle bombardment as the favoured method of monocotyledon transformation. However such techniques have low transformation frequency and a high frequency of unusual integration patterns (Eady et al., 1996). Recently, *Agrobacterium* mediated transformation of monocotyledons proved to be possible, and many monocotyledonous species like rice, wheat, barley, maize, sugarcane including shallot have now been transformed using this method (Ishada et al., 1996; Zheng, 2000; Cheng et al., 1998, Eady et al., 2000; Arencibia et al., 1998). The key component in the success of these systems has been the use of highly embryogenic tissue types and precise post-transformation selection protocols.

This paper reports repeatable protocols for production of transgenic *Alliums* plants as described by Zheng et al. (2001). We have introduced cry1Ca, Ho4 and intA-GFP genes to mature embryo-derived calli of the Dutch open pollinated cultivar "Tropix", the Indonesian open pollinated cultivars "Bawang Bali"and "Kuning"and to mature embryo-derived calli of garlic.

MATERIAL AND METHOD

Plant material and bacterial strain

True seed of shallot cv. "Tropix" and cv. "Bawang Bali" were used as a source of mature embryos. *Agrobactrium tumefaciens* strain AGL0 containing the binary vector pCAMBIA 1301-cry1Ca, and AGLO containing pCAMBIA1301-ho4 ware used.

Embryo isolation and callus induction

Sterilisation of seed was conducted by immersion in 70% ethanol for 30 minutes and subsequently in 10 % (v/v) sodium hypoclorite containing two drops of Tween-200 per 100 ml for 1 hour under continuous agitation. The seed were then washed with sterile MQ water for 10 times over a 2-hour period. After storing in sterile MQ water at 4 °C over night, the second disinfection was performed by

immersing the seed in 5 % (v/v) sodium hypoclorite for 10 minutes and subsequently washed in sterile water 10 times over a 2 hour period.

The procedure of isolating mature zygotic embryo has been described by Zheng et al (1998). The embryos were aseptically excised using stereo microscope. The parts of the embryo containing the radicle, the shoot apex and the lower part of cotyledon were separated from the main part of the cotyledon and cultured in a 6 cm petridish containing 10 ml callus induction medium. The callus which are derived from a single embryo were considered as a line. The callus induction medium was MS supplemented with 1 mg/l 2,4-D, 0.2 g/l casein hydrolisate, 30 g/l sucrose and solidified with 4 g/l gelrite. The cultures were then incubated in the dark at 25 °C for six weeks.

Co-cultivation, selection and regeneration

Agrobactrium tumefaciens from stock solution (-80 °C) containing the plasmids were suspended for further culture on 2 ml LB liquid containing 25 mg/l rifampicin and 50 mg/l kanamycin in shaker (300 rmp) at 28 °C for 1 day then subsequently added to 20 ml LB liquid medium containing 25 mg/l rifampicin, 50 mg/l kanamycin and 100 µM acetosyringone and shaking (300 rpm) at 28 °C and grow for another 1 day. Suspensions were then centrifuged at 3000 rpm for 10 minutes and the *Agrobacterium* pellet was resuspended in MS liquid medium supplemented with 1 mg/l 2,4-D, 0.2 mg/l casein hydrolisate, 30 g/l sucrose, and 100 µM acetosyringone at an optical density of 0.5 to 1 at OD_{600}. The resuspended *Agrobacterium* strains were then transferred to small jars.

The 3-6 weeks old embryo-derived callus was immersed in the bacterial suspension for at least 10 minutes. The immersed callus were then wounded by chopping callus with forceps in order to give an easy acces for *Agrobacterium*. Subsequently the chopped calli were tranferred on solidified co-cultivation medium. The co-cultivation medium was MS supplemented with 1 mg/l 2,4-D, 30 g/l sucrose, 10 g/l glucose and 100 µM acetosiringone. About 25 of calli were examined immediately after the co-cultivation period of 4 days for GUS transient expression of shallot or GFP transient expression of garlic. The remaining calli were put on selection medium MS containing 1 mg/l 2,4-D, 30 g/l sucrose, 400 mg/l cefotaxime, 100mg/l vancomycine

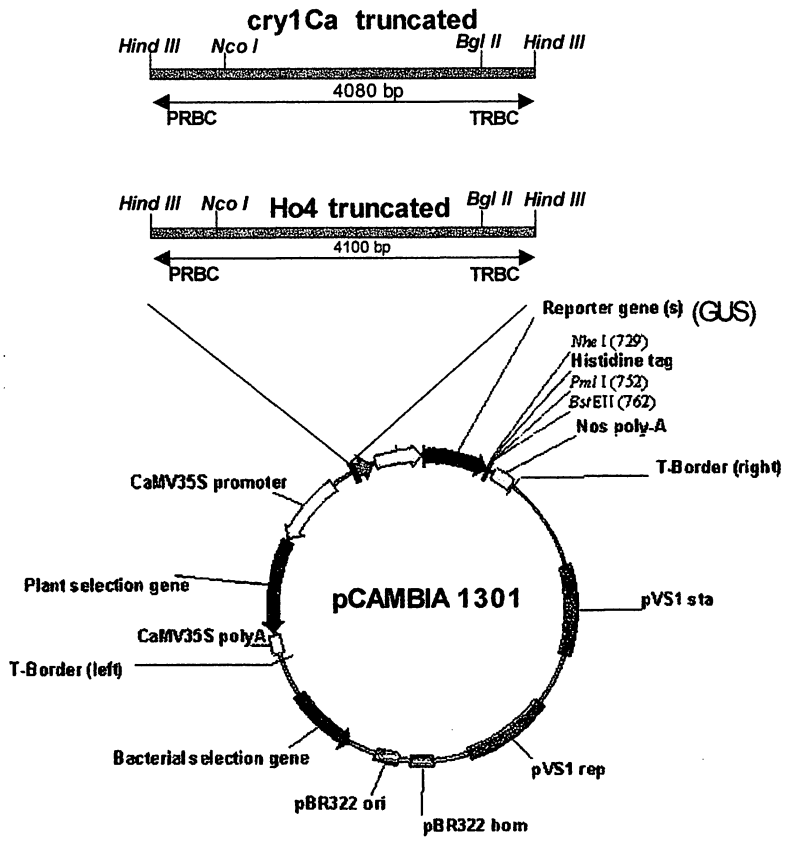

Figure 1. Plasmid map pCAMBIA 1301 inserted with gene ho4 and cry1Ca

and 50 mg/l hygromycine solidified with 4 g/l gelrite. After continuos selection for at least two months and subculturing the calli every two weeks, putative transformed calli were put on regeneration medium MS containing 30 g/l sucrose, 50 mg/l hygromycin and 1 mg/l kinetin. Regeneration was carried out at an ambient temperature of 25 °C with a 16 h photoperiod. (approx. 100 $\mu Es^{-1}m^2$, lamps used: SON-5 400 watt., Phillip).

Histological GUS-assay

Expression of GUS in shallot callus, and transformed leaf was assayed as described by Jefferson et al (1987) with some modifications. 5-bromo-4-chloro-3-indole glucoronide (x-gluc) was used as a substrate. Calli and leaf were stained over night at 37 °C with 1 mM x-gluc in 50 mM phosphate buffer (pH7.5), supplemented with 10 mM EDTA and 0.1% Triton x-1000. The GUS staining was stopped by washing with 70% ethanol until distaining was complete.

Plant genomic DNA isolation

Fresh leaves of putative transgenic plants were collected from greenhouse. Plant genomic DNA was isolated with mini prep DNA isolation methods. Approximately 1-1.5 g fresh leaf material was collected in Eppendorf tube, frozen

and grind. A 750µl aliquot of DNA isolation buffer (IB) with $Na_2S_2O_5$ (3.5 g/l) was added to leaf materials. This mixture was incubated for 60 minutes at 65^0C with occasional inverting of tube. Isolation buffer was lysis buffer: extraction buffer:5% (w/v) sarkosyl (2.5:2.5:1). Lysis buffer : 0.2 M tris-HCl pH 7.5, 0.05 M EDTA, 2 M NaCl, 2% (w/v) CTAB. Extraction buffer: 0.35 M sorbitol, 0.1 M tris-HCl pH 7.5, 5 mM EDTA. The DNA was further purified by adding 750 µl chloroform:Iso-amylalcohol (24:1), mix well and centrifuge for 5 minutes at 15 000 rpm at room temperature. After transfer of the supernatant to a new Eppendorf tube 1.5 ml, the DNA was precipitated by the addition of 400 µl isopropanol and mix carefully. The DNA could be pelleted by centrifuge for 6 min at 15 000 rpm at room temperature. The DNA sample were then washed once with 500 µl of 70% ethanol and resuspended in 50-100 µl TE and dissolved the pellet. DNA concentration was determined by DNA sample machine.

PCR analysis

Two different PCR analysis have been performed. The first was targeted to amplify a fragment containing the *hpt* gene in the T-DNA of putative transgenic plants, while the second analysis was targeted to amplify flanking fragment of the restriction side. This technique is called adaptor ligation PCR (AL-PCR) (Zheng et al., 2001).

The *hpt* gene of putative transgenic plants was amplified using the specific primer 5'ATGAAAAAGCCTGAACTCA3' as forward and 5'ACTGGATTTTG GTTTTAGG3' as reverse. The PCR cycle was 94 ^{0}C for 2 minutes (1 cycle), 94^0C for 1 minutes, 56 ^{0}C for 1 minutes, 72 ^{0}C for 2 minutes (35 cycle); 72 ^{0}C annealing extension for 10 minutes. The PCR reaction volume was 25 µl containing 2.5 µl super Taq buffer, 0.1 µl super Taq enzyme (5 U/µl),, 1.0 µl dNTP 10 mM, 3.75 µl *hpt*-R 50 ng/µl, 3.75 µl *hpt*-F 50 ng/µl, and 10 µl DNA 10 ng/µl. The reaction were done in a PCR machine PTC-200 (DNA Engine, MJ Research, Inc, USA). The *hpt* primer amplified a sequence of 1.2 kb (Figure 2).

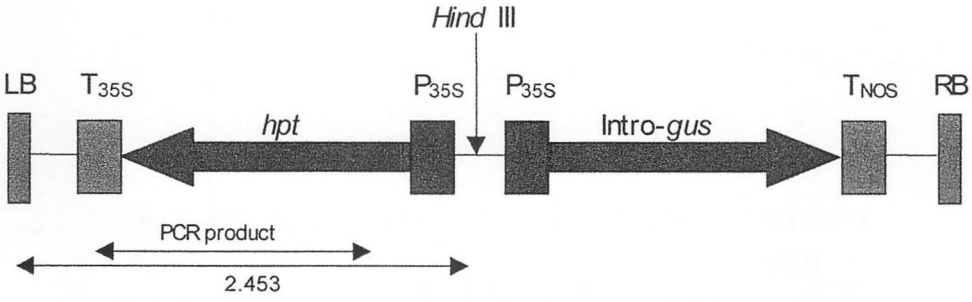

Figure 2. T-DNA region of binary vector pCAMBIA 1301 showing the size of T-DNA, the region for PCR amplification. Abbreviation: RB: righ border; LB: left border, P35S and T35S: CaMV 35S promotor and terminator; PNOS and TNOS: nopaline synthase promotor and terminator; hpt: hygromycin phosphotransferase; intron-gus: intron interupted β-glucuronidase

The second PCR analysis performed after digestion with restriction enzymes and ligation of specific adaptors. The enzyme used (*Ssp*I or *Alu*I) were selected because they generated blunt-end fragment which are easily ligated to adaptors (Zheng et al., 2001). Digestion was performed at final concentration 40 µl containing 4 µl OPA (One-Phor-All^{TM}) buffer 10x, 1 µg DNA sample and 1 µl (5 U) *Alu*I or 1 µl (10 U) *Ssp*I. The reaction were then incubated at 37 C for 1.5 hours. Ligation of the adaptors (upperstrand (48 nucleotides), 5'GTAATACGACTCA-CTATAGGGCACGCGTGGTCGACGGCCC-GGGCAGGT3'. and lower strand, 5'PO_4-ACCTGCCCNH_2-3') to the restriction site of genomic DNA was performed in final concentration 50 µl. This reaction was done by adding the digestion mixture with 2 µl CT adaptor, 5 µl ATP 10 mM, 1 µl OPA buffer 10 x, and 1 µl T4 DNA ligase 5 U/µl. This ligation mixture was then incubated overnight at room temperature.

PCR amplification was targeted to the adaptor sequence specific restriction side with adaptor and to specific sequence in the left border (LB) or right border (RB) sequences of the T-DNA. Specific primers were design, which homologous to sequences close to RB or LB of the T-DNA (Fig. 3).

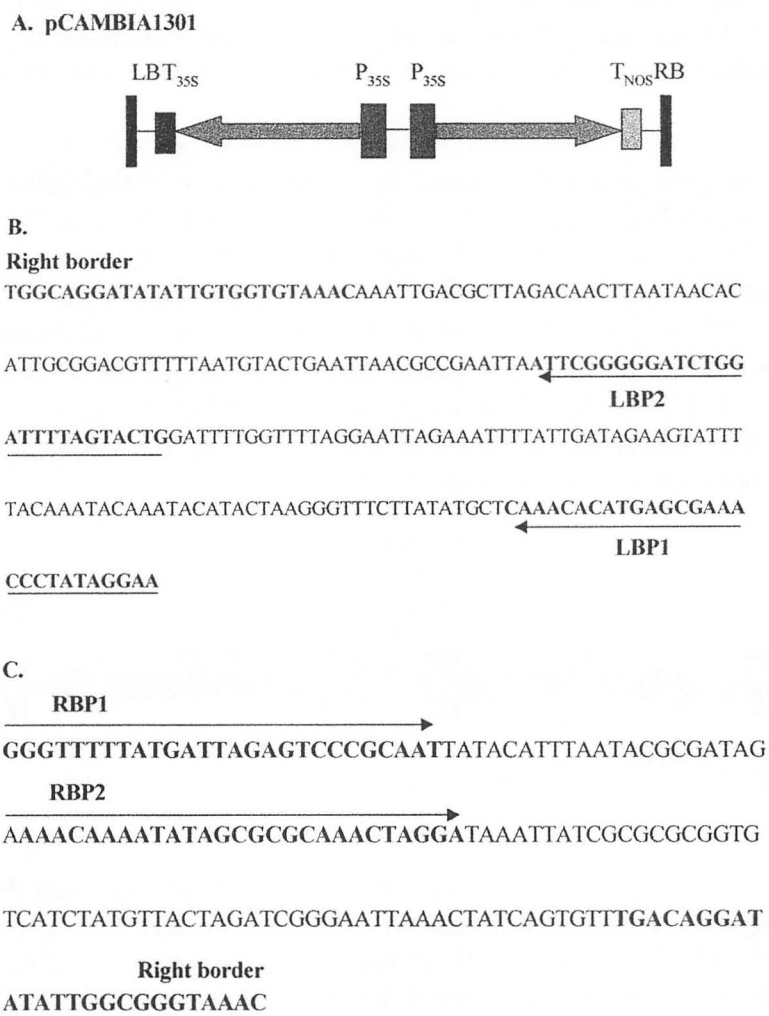

Figure 3. Left and right border sequences of binary vector pCAMBIA1301. A. General overview of the T-DNA region of pCAMBIA 1301, B. T-DNA sequences

close to left border. Solid arrows indicate the positions used for primer design, C. T-DNA-sequence close to right border

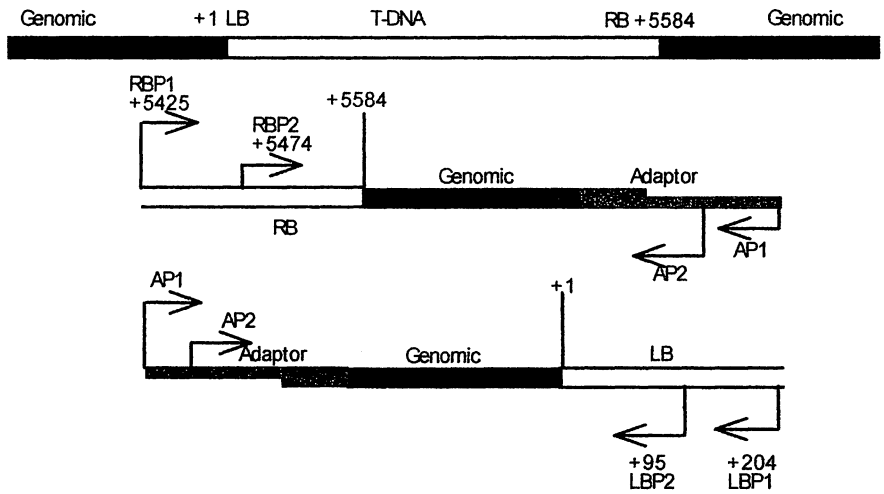

Figure 4. Schematic representation of the T-DNA border region. A. T-DNA region of the pCAMBIA 1301 insert in the Allium genome. Open box: full region of the T-DNA insert. B. After digestion with specific enzyme restriction, adaptor were ligated to the genomic DNA AL-PCR was performed on the right border using AP1 and AP2 adaptor primers and RB1 and RB2 (right border specific primers). C. After digestion with specific enzyme restriction, adaptor were ligated to the genomic DNA AL-PCR was performed on the left border using AP1 and AP2 adaptor primers and LB1 and LB2 (right border specific primers)

Firstly PCR analysis were done in 25 µl volume containing 5 µl of 10 diluted DNA from ligation mixture, 2.5 µl PCR buffer no MgCl 10xi, 0.5 µl dNTP 10 mM, 1.5 µl MgCl 10 mM, 1 µl adaptor AP1 primer 10 mM, 1 µl RBP1 primer 10 mM and 1 U of Taq DNA polymerase (Promega, Wisconsin, USA). AP1 primer was 5'GTAATACGACTCACTATAGGGC3' and right-border (RB)-specific primer RBP1 was 5'GGGTTTTTATGATTAGAGTCCCGCAAT3'. PCR was done after a hot start at 94 ^{0}C for 3 min. Three step cycling condition were used: the denaturating step at 94 ^{0}C for 30 s was followed by annealing and extension step. The first five cycle were done with annealing temperature starting at 65 ^{0}C for 30 s with a decline at rate of 1 ^{0}C every cycle. The extension step was done at 72 ^{0}C for 1 min at each cycle. Additional 40 cycles were done with a denaturating step at 94 ^{0}C for 30 s, an annealing step at 60 ^{0}C for 30 s and an extension step at 72 ^{0}C for 1 min. PCR was terminated with a 15 min extension step at 72 ^{0}C.

The further PCR amplification were done with 5 µl of 50 x dilution of first PCR using nested adaptor primer AP2 (5'-ACTATAGGGCACGCGTGGT-3') and the right border specific primer RBP2 (5'-AAACAAATATAGCGCG-3'). The same PCR mixture conditions were used and 25 additional cycle were performed.

The same amplification were also done with a set of primer specific to the left border LBP1 (5'-CAACACATGAGCGAAACCCTATAGGAA-3') and LBP2 (5'-GTCATGATTTTAGGTCTGGGGGCTT-3'). Ten microliters of second PCR product were analyzed via electrophoresis in 1.5% agarose gel.

RESULT

Callus production

A total of 2 836 embryo's from 3 cultivars of shallot (cv. "Tropix", and "Bawang Bali") were isolated for producing embryogenic calli. Callus formation from embryo could be observed after four days of culture. The morphology of calli was the friable type with no apparent structure. This friable callus was abundantly produce by cotyledon parts. During callus initiation and growth, mucilaginous substances were sometime produced. After 3 weeks of culture the size of calli were about 0.5 mm in diameter. A large enough size callus was achieved after 6 weeks of cultures. There were no differences in morphology of callus within the cultivars of shallot. From 2836 isolated embryo of shallot cultured, there were 2 244 of them could grow and inoculated with *Agrobacterium* (Tabel 1).

Tabel 1. The number of selected calli after 3 subsequent subculture

Cultivar	Plasmid	Number of callus line on selection medium	Number of surviving callus line (weeks)			Number of callus lines put on the regeneration medium
			2	4	6	
Tropix	PCAMBIA 1301-cry1Ca	936	168	146	145	145
	PCAMBIA 1301-ho4	1050	129	128	125	125
Bawang Bali	PCAMBIA 1301-cry1Ca	128	10	9	9	8
	PCAMBIA 1301-ho4	130	29	29	26	13
Total		2 244	336	312	305	291

Inoculation of *Agrobacterium* was done by chopping the callus into small pieces. After a co-cultivation period of four days, GUS assay showed that in most cases blue spots located around the shoot apex area. The percentage of calli showing transient expression of *uid*A gene could amount was observed up to 75 % (Tabel 2). No effect on transient GUS expression between callus inoculated with *Agrobacterium tumefacient* AGL0 pCAMBIA 1301-cryICa and AGLO pCAMBIA 1301-ho4.

Tabel 2. Percentage of GUS and GFP transient expression after 4 days inoculation of *Agrobacterium.*

Cultivar	Number GUS^+	
	AGLO (pCAMBIA 1301-cr1Ca)	AGLO (pCAMBIA 1301-ho4)
Tropix	58	77.1
Kuning	43.2	66.0

Selection and plant regeneration

From a total 2 244 callus lines that were inoculated with *Agrobacterium*, 336 callus lines were able to survive after two months culture in selection medium and finally 291 callus lines put on the regeneration medium (Tabel 1). In order to reduce the number of escapes (untranformed calli), 3 subsequent subcultures in the same medium were done. The selected calli were cut into smaller in order to make better contact to the selection medium. The resistant calli were selected and kept separated during subculture.

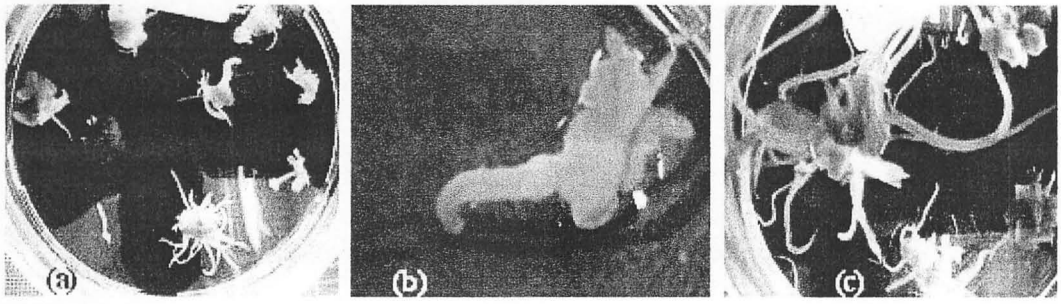

Figure 5. Formation of shoots in regeneration medium, a: organogenesis, b: somatic embryo and c: development of shoots after 2 months

Putative transgenic calli were regenerated in the regeneration medium where the 50 mg/l hygromycin was present. The putative transgenic plants regenerated after 1 week to 2 months depending on the cultivars used. There was also variation among lines within the cultivars. Some lines had a higher regeneration capacity. The type of regeneration was either by organogenesis or by forming of somatic embryos (Fig. 5). Some calli only produced root and/or green leaves-like structures. Some of this structures still produced shoots after another 2 months culturing and subculturing. In shallot cv. Tropix in experiment 1 (pCAMBIA 1301 cry1Ca), 34 plants were produced from individual callus of 18 lines from a total of 36 lines cultured in regeneration medium. For pCAMBIA 1301 ho4, 26 plants were produced from individual callus of 16 lines from 41 lines cultured in regeneration medium. The putative transgenic shoots were then subcultured in the MS medium without supplemented hormone and with 50 mg/l hygromycin. As soon as the size was sufficient, the shoots were transplanted to the greenhouse for further molecular characterisation.

Characterisation of transgenic plants

The transgenic plants were identified by GUS assay on leaf tissue. Most of plants originating from the greenhouse had visible GUS activity in their entire tissue (Fig. 6), but dot-like expression was also observed. GUS activity was also different from leaf to leaf in the same putative plant. Although the putative transgenic plants showed GUS expression and could grow in medium containing hygromycin, the molecular characterisation is necessary to confirm the transgenic nature of the plants.

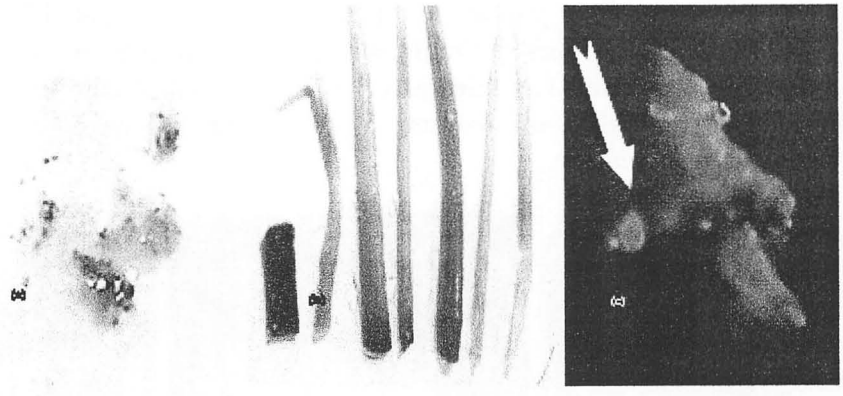

Figure 6. a: GUS positive in shallot 4 days after inoculation, b: GUS positive leaves of transgenic plants and

Several putative transgenic plants from the greenhouse were examined by amplifying *hpt* gene. The result showed that the genes were present in all transformed plant and a PCR product was generated of 1.2 kb (Fig. 7). With this analysis, it is not possible to determine real integration of T-DNA and also not how many copies of the T-DNA were present in the plant's genome. There are several alternatives can be done such as Southern hybridisation or AL-PCR. The use of Southern hybridisation labour-intensive when many samples have to be assayed, and it requires

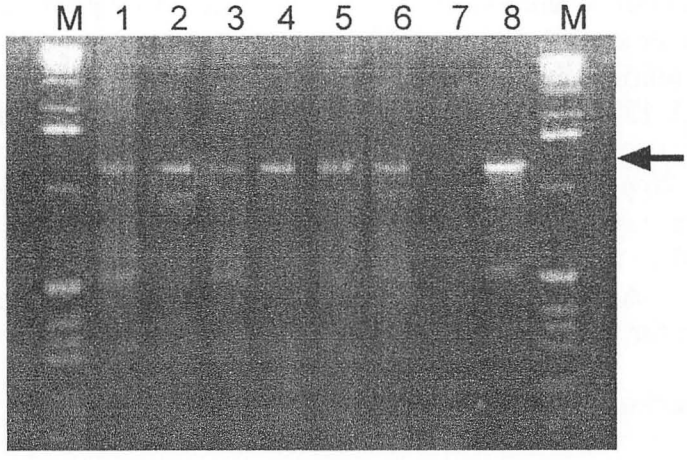

Figure 7. a: normal PCR to amplify hpt gene, lane M: 1kb DNA ladder marker, lane 1,2,3: putative transgenic with pTOK233, lane 4,5,6 putative transgenic with pCAMBIA1301, 7: negative control, 8: positive control (plasmid pCambia1301).

relatively large amounts of genomic DNA, especially in plants with a large genome. The AL-PCR is therefore one of the alternatives. The main procedures of amplification of T-DNA flanking genomic DNA sequences consist of three steps,

namely a) construction of DNA library by digestion of genomic DNA with several restriction enzyme, (b) ligation of adaptors to the fragments in all libraries and (c) two successive PCR amplification primer using primary and nested primer. By doing this, PCR product will only be obtained when T-DNA is integrated into the genomic DNA and the maximum number of amplified fragments identified in the individual libraries represents a minimum estimation of the number of integrated copies. The blunt-end cleavage restriction enzyme could be chosen because they yield were different to which blunt-end adaptors could be easily ligated. Using *Alu*I and *Ssp*I followed by the adaptor ligation PCR of three transgenic lines gave a good result. Figure 8 showed PCR amplification of left and right border of the three transgenic lines. All lines tested gave clear PCR product indicating T-DNA insertion in shallot genome. There were three transgenic plants (1,2,3 = 4,5,6 = 7,8,9 = 10,11,12). Lane 1-6 represent PCR product of genomic DNA flanking T-DNA right border (1-3 digested by *Alu*I, 4-6 digested by *Ssp*I). Lane 7-12 represent genomic DNA flanking T-DNA left border (lane 7-9 digested by *Alu*I, and lane 10-12 digested by *Ssp*I). Using right

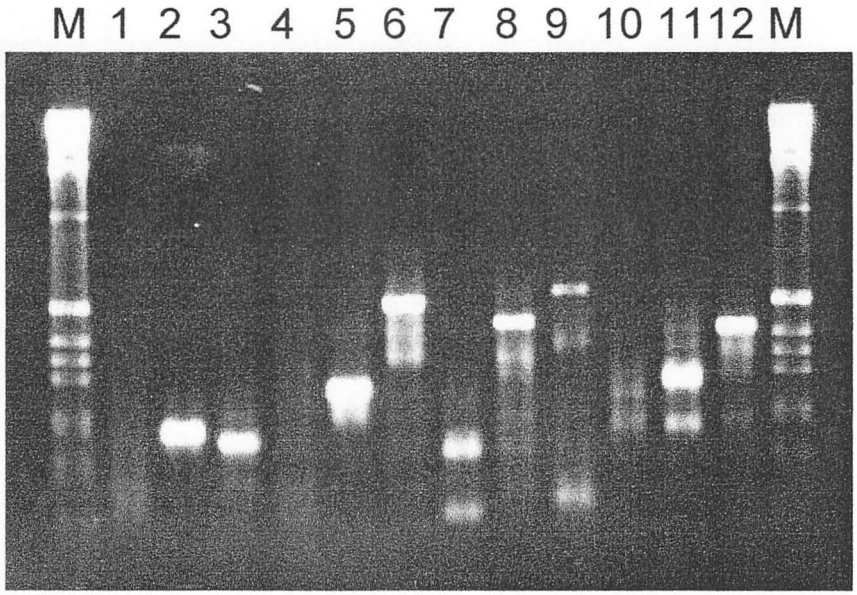

Figure 8. AL-PCR resulted from putative transgenic plants with pCAMBIA 1301. M: 1 kb DNA ladder marker, lane 1-6: AL-PCR was performed on the right border using AP1 and AP2 (adaptor primers) and RBP1 and RBP2 (right border specific PCR primers). Lane 7-12: AL-PCR was performed on the left border using AP1 and AP2, LBP1 and LBP2 (left border specific PCR primer). Lane 1-3 and lane 7-9:digested with Alu1. Lane 4-6 and lane 10-12 digested with Ssp1

border specific primer (lane 1-6), the PCR product showed that lane 2, 3, 5 and 6 have clear bands, while lane 1 and 4 (transgenic plant line 1) do not amplified fragments. Amplification left border specific primer (lane 7-12) had a similar results. Lane 7-12 had 1 fragments.

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