

## The development of a reproducible *Agrobacterium tumefaciens* transformation system for garlic (*Allium sativum* L.) and the production of transgenic garlic resistant to beet armyworm (*Spodoptera exigua* Hübner)

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### Abstract

This paper describes the development of a reliable transformation system for garlic (*Allium sativum* L.) and its application in producing insect resistant GM garlic lines. The transformation system is based on *Agrobacterium tumefaciens* as a vector, using young callus derived from different callus sources: callus induced from both apical and non-apical root segments of *in vitro* plantlets, true garlic seeds and bulbils. Two different reporter genes were used in our garlic transformation experiments, namely the *gusA* gene coding for  $\beta$ -glucuronidase and the *gfp* gene coding for green fluorescent protein. A total of seven independent transformed callus lines derived from different callus sources were obtained. The advantage of the system developed is the short time period needed for completion of the protocol (about 6 months) and the year-round availability of high quality callus from *in vitro* roots. The highest transformation frequency in a single experiment (1.47%), was obtained using garlic cv. 'Printanor'. Differences existed between cultivars in transformation frequency but were not significant. The same was found for the plasmids used in transforming garlic. Via PCR the presence of the *gusA*, *hpt* (hygromycin phosphotransferase) and *gfp* genes could be demonstrated in putative transformed *in vitro* plants. Southern hybridization showed that the reporter gene *gusA* and the selective gene *hpt* were stably integrated into the garlic genome. After transfer to the greenhouse of *in vitro* regenerants, transgenic garlic harbouring the *gusA* gene survived and grew well, whereas the *gfp* transgenic garlic gradually died under these conditions.

Using this protocol transgenic garlic resistant to beet armyworm using the *cryICa* and *H04* resistance genes from *Bacillus thuringiensis* were developed. Via Southern hybridization it was shown that the *cryICa* sequence was stably integrated into the garlic genome. After transfer of the transgenic *in vitro* garlic plants to the greenhouse, the *cryICa* plants developed normally and grew well to maturity with normal bulbs. However, all transgenic *in vitro* *H04* garlic plants did not survive after transfer to the greenhouse. Transgenic *cryICa* garlic plants proved completely resistant to beet armyworm in a number of *in vitro* bio-assays. This finding will facilitate the development of new garlic cultivars resistant to beet armyworm.

**Abbreviations:** *cryICa* – a synthetic gene from *Bacillus thuringiensis*; GFP – green fluorescent protein; *gfp* – green fluorescent protein gene; GUS –  $\beta$ -glucuronidase; *H04* – a hybrid gene from *Bacillus thuringiensis*; *hpt* –

– hygromycin phosphotransferase gene; LB – T-DNA left border; RB – T-DNA right border; sGFP – synthetic GFP with a threonine instead of a serine at position 65; *gusA* –  $\beta$ -glucuronidase gene.

## Introduction

Garlic (*Allium sativum* L.) is a very important and widely cultivated crop, which is known for its culinary and medicinal use. The overriding majority of the garlic germplasm analyzed proved to be sexually sterile, consequently all commercially available garlic cultivars have been developed using clonal selection of landraces or spontaneous mutants. One of the most promising ways to improve these asexual garlic cultivars is via the use of genetic transformation. Despite the recent progress made in the development of garlic cultivars via sexual hybridization (for review see Kik 2002), the availability of a reliable genetic transformation system remains highly valuable even for sexually reproducing garlic cultivars as in general not all agronomically important traits can be transferred via sexual hybridization. This can be due, amongst others, to the lack of genetic variation in the germplasm available or due to insurmountable species barriers.

The common vectors used for genetic modification of plants are *Agrobacterium tumefaciens* and particle bombardment (Songstad et al. 1995). Many protocols for the production of transgenic crops were developed in the last decade, however, for *Allium* species the development of these protocols proved to be difficult. Only recently, reports were published showing that genetic transformation has become possible in onion and shallot (*A. cepa*) and garlic (*A. sativum*) (for review see Eady 2002). Particle bombardment was primarily used in garlic transformation studies (Barandiaran et al. 1998; Ferrer et al. 2000). Kondo et al. (2000) were the first who developed stable transgenic garlic plants using highly regenerative calli derived from shoot primordial-like tissues of mature cloves via a complicated suspension culture procedure and *Agrobacterium*-mediated gene transfer. Via particle bombardment, Park et al. (2002) and Sawahel (2002) reported that transgenic garlic plants were generated using callus induced on apical meristems of (im)mature cloves. However, the *Agrobacterium*-mediated transfer system developed by Kondo et al. (2000) and the particle bombardment protocols developed by Park et al. (2002) and Sawahel (2002) can be improved with respect to the duration of the transformation protocol, the number of different tissues to be

used and the transformation frequency. Therefore, in this paper we report the development of an efficient, simple and reliable *Agrobacterium tumefaciens*-mediated transformation system for garlic based on young callus derived from different sources: callus induced from both apical and non-apical root segments of *in vitro* plantlets, true garlic seeds and bulbils. From these sources, root segments are the most easily available.

The beet armyworm (*Spodoptera exigua* Hübner) is an extremely polyphagous insect (Goh et al. 1991), which threatens garlic cultivation in the dry season especially in (sub) tropical countries. To introduce resistance in garlic to the beet armyworm, we adopted a transformation approach using genes encoding for insecticidal proteins originating from *Bacillus thuringiensis*. This approach has been very successful in the past for a number of crops (De Maagd et al. 1999; Naimov et al. 2003; Perlak et al. 2001; Schuler et al. 1998; Tu et al. 2000). The *cry* gene family from *Bacillus thuringiensis* is a large, still growing family of homologous genes, with each gene encoding a protein active on insect larvae of a subset of species usually belonging to the same order (De Maagd et al. 2001; Schnepf et al. 1998). Cry1 proteins are generally active against lepidopterans (larvae of moths and butterflies). For example, a synthetic *cry1Ca* gene, encoding a *Bacillus thuringiensis* delta-endotoxin, confers resistance to *S. exigua* in alfalfa and tobacco (Strizhov et al. 1996) and overexpression of *Bt cry2Aa2* in chloroplasts resulted in 100% killing of beet armyworm after consuming transgenic leaves (De Cosa et al. 2001). In this paper we report the development of transgenic garlic resistant to beet armyworm using the *cry1Ca* resistance gene.

## Materials and methods

### *Callus induction*

Callus was induced on both apical and non-apical root segments derived from *in vitro* plantlets. In this context the most widely cultivated European garlic cultivars, namely cvs. ‘Messidrome’, ‘Morasol’ and ‘Printanor’ were used. Furthermore, true mature zygotic embryos and immature bulbils, from garlic ac-

cessions collected in Central Asia, were used for the induction of callus. The procedure of isolation of garlic mature zygotic embryos is the same as described earlier for onion and shallot (Zheng et al. 1998, 1999). Immature bulbils, developing after flowering, were surface-sterilized by immersion in 70% ethanol for 30 s, and subsequently in 1% (w/v) Na-hypochlorite (containing two drops of Tween-20 per 100 ml) for 12 min under continuous agitation. Immature bulbils were rinsed three times in sterile water prior to placing these on callus induction medium. Callus induction and callus propagation from root segments, mature embryos and immature bulbils were carried out on solidified MS medium with 1 mg l<sup>-1</sup> 2,4-D (2,4-dichlorophenoxyacetic acid), 0.1 mg l<sup>-1</sup> 2ip (6-( $\gamma$ - $\gamma$ -dimethylallylamino) purine) and 30 g l<sup>-1</sup> sucrose in the dark at 25 °C. The procedure of callus induction and callus propagation followed the procedure as described earlier by Zheng et al. (2003).

#### *Agrobacterium strains and DNA constructs for garlic transformation*

*Agrobacterium* strain AGL0 was used in this study. AGL0 is similar to EHA105 which we had used in onion and shallot transformation previously (Zheng et al. 2001a). It is an L, L-succinamopine strain with a C58 chromosome background (Lazo et al. 1991). It contains as virulence helper plasmid pEHA101, originally derived from supervirulent pTiBo542 (Hood et al. 1986, 1993). The plasmid pCAMBIA1301 is a normal binary vector from the Center for Application of Molecular Biology to International Agriculture, Canberra, Australia (CAMBIA) having *hpt* and intron-*gusA* genes in the T-DNA region (Figure 1A). pCAMBIA1301 has an intron-interrupted *gusA* gene, the expression of *gusA* only occurs in transformed calli and plants rather than in *A. tumefaciens* itself (Ohta et al. 1990).

Plasmids pPB34 and pPB36 were constructed as described below. The *H04* (Carozzi et al. 2002) and *cryICa* (Strizhov et al. 1996) genes were inserted at the *NcoI* and *BglII* sites in plasmid pRBC respectively. They were under the control of a chrysanthemum ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (Rubisco SSU) promoter and terminator (Outchkourov et al. 2003). The *H04* gene is a hybrid gene which encodes domains I and II of Cry1Ab and domain III of Cry1Ca (De Maagd et al. 1996) and which was optimized for expression in plants (Carozzi et al. 2002). In the pCAMBIA1301

*HindIII* site, we inserted a *H04* or *cryICa* truncated gene cassette under the control of a chrysanthemum Rubisco promoter and terminator (Figure 1B, 1C). pC1300intA and pC1300intA-sGFP were a kind gift from Dr. Pieter Ouwkerk from Leiden University, the Netherlands. A *HindIII-EcoRI* fragment of 1.4 kb from the pTH2 plasmid containing the sGFP (S65T) gene with the CaMV 35S promoter and the terminator of the NOS gene described by Chiu et al. (1996) was inserted into *HindIII-EcoRI*-cut pC1300intA, vector to make pC1300intA-sGFP (Figure 1D). Plasmid pC1300intA originates from pCAMBIA1300 modified with a catalase-1 intron in the *hpt* gene (Wang et al., 1998). Plasmids pCAMBIA1301, pPB34, pPB36, pC1300intA and pC1300intA-sGFP were introduced into *Agrobacterium* strain AGL0 by electroporation (Mattanovich et al. 1989).

#### *Transformation procedure*

AGL0(pCAMBIA1301), AGL0(pPB34), AGL0(pPB36), AGL0(pC1300intA) and AGL0(pC1300intA-sGFP) were streaked out on solidified LB medium with 50 mg l<sup>-1</sup> rifampicin, 50 mg l<sup>-1</sup> kanamycin and grown at 28 °C for three days for colonies to appear. A single bacterial colony was collected from a plate and suspended for further culture in liquid LB medium for two days. Suspensions were centrifuged at 3000 rpm for 10 min and the *Agrobacterium* pellet was resuspended in liquid callus induction medium with 100  $\mu$ M acetosyringone at an optical density of 0.5 to 1 (OD<sub>600</sub>).

One- or two-month old callus from root segments, true garlic seeds and immature bulbils were used for transformation. The calli were chopped directly after retrieving them from the *Agrobacterium* suspension or calli were chopped in advance and suspended in liquid culture one week before co-cultivation. The solidified co-cultivation medium contained callus induction medium with 10 g l<sup>-1</sup> glucose and 100  $\mu$ M acetosyringone. About 20 calli were examined immediately after the co-cultivation period of 4 days for GUS or GFP transient expression. The remaining calli were put on selective medium, i.e. callus induction medium supplemented with 400 mg l<sup>-1</sup> cefotaxime and 100 mg l<sup>-1</sup> vancomycin and 25 mg l<sup>-1</sup> hygromycin. After continuous selection for one or two months and subculturing the calli every two weeks, putative transformed calli were transferred to regeneration medium. The regeneration medium contained MS salts with 30 g l<sup>-1</sup> sucrose, 1 mg l<sup>-1</sup> kinetin and 25

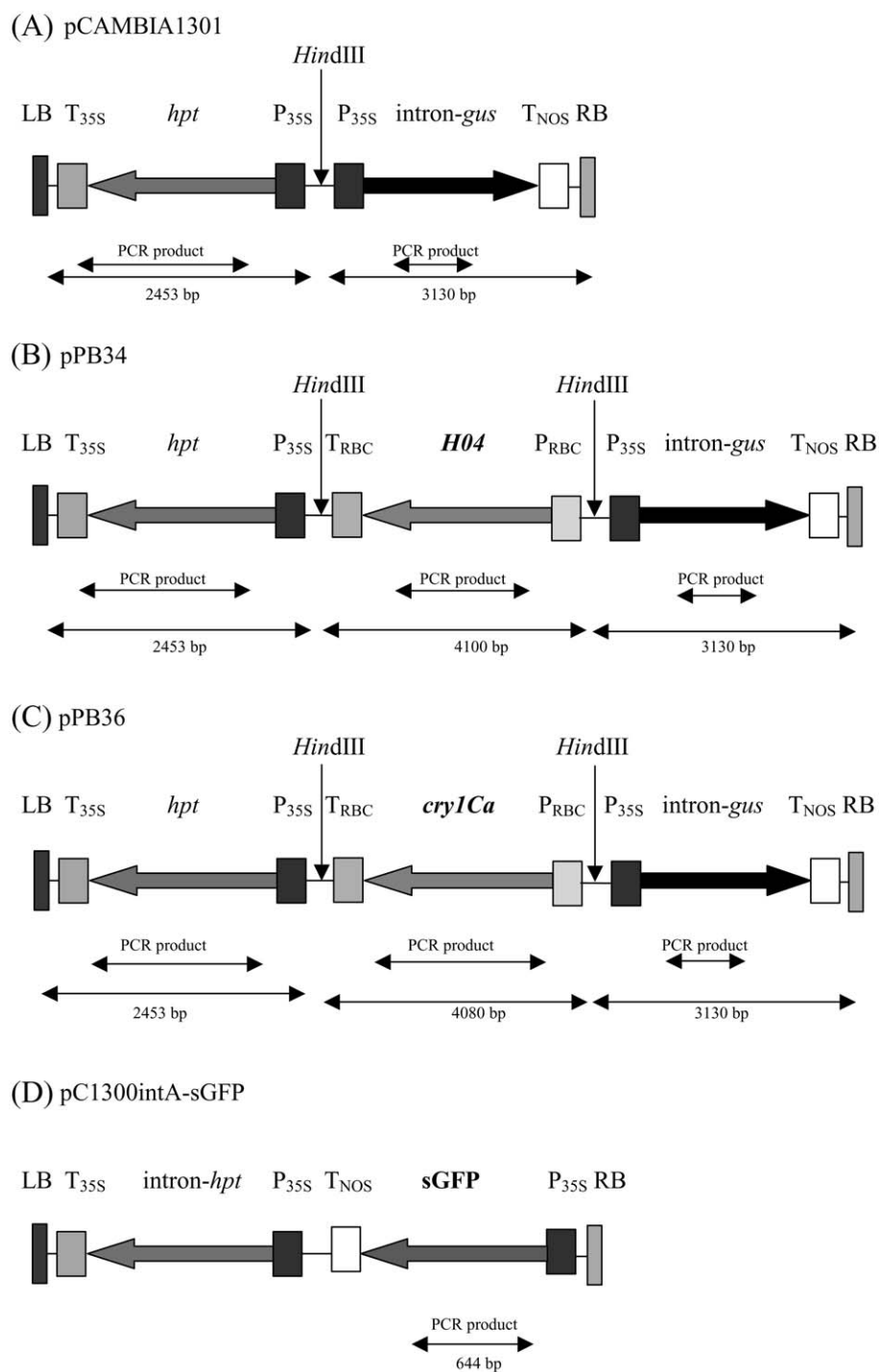


Figure 1. T-DNA region of (A) pCAMBIA1301, (B) binary vector pPB34, (C) binary vector pPB36 and (D) binary vector pC1300intA-sGFP, showing the size of T-DNA, the region for PCR amplification, PCR products used as a probe and *Hind*III restriction sites used for Southern hybridization. Abbreviations: RB: right border; LB: left border; P<sub>35S</sub> and T<sub>35S</sub>: CaMV 35S promoter and terminator; P<sub>NOS</sub> and T<sub>NOS</sub>: nopaline synthase promoter and terminator; P<sub>RBC</sub> and T<sub>RBC</sub>: ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) promoter and terminator; *hpt*: hygromycin phosphotransferase; *intron-hpt*: intron interrupted hygromycin phosphotransferase; *intron-gus*: intron interrupted  $\beta$ -glucuronidase; sGFP: synthetic GFP with a threonine instead of a serine at position 65; *H04*: a hybrid *Bt* gene; *cry1Ca*: synthetic *Bt* gene.

mg l<sup>-1</sup> hygromycin. Regeneration was carried out at an ambient temperature of 25 °C with a 16 h photoperiod (ca. 60 μE m<sup>-2</sup> s<sup>-1</sup>; lamps used: Philips, TLD 50W/840HF, and Electronic NG). After one month, all material was subcultured. Shoot regeneration was observed after two months.

Transient GUS expression detection, resistant callus selection and transformed callus regeneration and rooting conditions were similar as described for onion and shallot (Zheng et al. 2001a). Transgenic plantlets were obtained after resistant calli were transferred to regeneration medium for at least two months. These plantlets were rooted and transplanted into pots (8 × 8 × 8 cm<sup>3</sup>) in the greenhouse. The plantlets were first covered with a plastic cap for one week; then they were grown in the greenhouse at 16/20 °C (night/day) with additional light for 16 hours (approx. 100 μE s<sup>-1</sup>m<sup>-2</sup>; lamps used: SON-T 400 watt).

#### *Histochemical GUS-assay and GFP detection*

Expression of GUS in garlic callus, and transformed leaves and roots were assayed as described by Jefferson et al. (1987) with some modifications. As a substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) was used. Calli, leaf, root material were stained overnight at 37 °C with 1 mM X-Gluc in 50 mM phosphate buffer (pH 7.5), supplemented with 10 mM EDTA and 0.1% Triton X-100. The GUS staining was stopped by washing with 70% ethanol until destaining was complete and the ethanol remained colourless.

Green fluorescent protein transient and stable expression was observed using a Leica MZ FLIII fluorescent binocular microscope. This microscope was equipped with GFP2 (plus) excitation filter (480/40 nm), dichromatic beam splitter (505 nm LP), and barrier filter (510 nm LP), GFP3 (plant) excitation filter (470/40 nm), dichromatic beam splitter (495 nm), and barrier filter (525/50 nm). The GFP3 filter is used to block the red autofluorescence from chlorophyll. A 100 W high-pressure mercury bulb provided the light source. Fluorescent photographs were taken using a Nikon Coolpix 990 camera.

#### *PCR analysis*

Approximately 0.25 g fresh leaf tissue from putative transgenic greenhouse-grown plants was collected. DNA was isolated from these plants via a miniprep

protocol (van Heusden et al. 2000). DNA concentration was estimated using an Eppendorf Biophotometer. Optimal conditions, such as temperature, DNA concentration and suitable primers for PCR were checked in primary experiments. Successful PCR was performed using specific primers for *gusA* (forward: 5'GGAATTGATCAGCGTTGGTG3' and reverse: 5'TAGATATCAC ACTCTGTCTG3'), for *hpt* (forward: 5'ATGAAAAAGCCTGAACTCA3', and reverse: 5'ACTGGATTTTGGTTTTAGG3'), for *gfp* (forward: 5'ATGGTGAGCAAGGGCGAGGAGCTGTTTC3', and reverse: 5'CTTCTCGTTGGGGTCTTTGCTCAGGGC3'), for *cry1Ca* (forward: 5' TGATCACATAACAACCGACT 3', and reverse: 5' CTGTGAATCCTGGTCCTGTA 3') and for *H04* (forward: 5'ACAGCATCACCATCTACACC3', and reverse: 5'CAGGATGATCTCGATCTTGT3'). The PCR cycle was 94 °C for 2 min (1 cycle); 94 °C for 1 min, 56 °C for 1 min, 72 °C for 2 min (35 cycles); 72 °C annealing extension for 10 min. The reactions were carried out in PTC-200 (DNA Engine, MJ Research Inc., USA). Primers for *gusA* amplify a sequence of 710 bp, while *hpt*, *gfp*, *cry1Ca* and *H04* primers amplify four sequences of 1.2 kb, 644 bp, 802 bp and 953 bp, respectively.

#### *Southern DNA blot analysis*

Putative transgenic garlic genomic DNA was isolated from 1–1.5 g frozen leaf tissue collected from greenhouse-grown plants with a midiprep DNA-isolation method as described by van Heusden et al. (2000). The DNA concentration was estimated using an Eppendorf Biophotometer. From each sample 25 μg DNA was digested overnight with 100 units of *Hind*III. After digestion, the DNA was loaded on a 1.0% agarose gel with TAE at 25 V for electrophoresis (running time about 17 h). DNA was transferred to a nylon Hybond<sup>TM</sup>-N+ membrane (Amersham Life-science, UK) by vacuum blotting. 100 ng DNA of PCR products from *gusA*, *hpt*, *cry1Ca* and *H04* were used for random prime labeling, respectively. The RadPrime DNA labeling system (GibcoBRL<sup>®</sup>, Life Technologies) was used. For pre-hybridisation, hybridisation and blot washing standard procedures were followed. Signals were detected using a Storm 860 Phospho Imager (Amersham Biosciences).

### Bio-assay for resistance to beet armyworm

The protocol was based on the toxic compound bio-assay method described earlier Zheng et al. (2000). In brief, four replications from transgenic garlic and control plants were used, with each 3–4 g fresh leaves on 0.8% sterile water agar in a Petri dish. Five 3-day old larvae were present in each replication. Four days after the start of the experiment the percentage of surviving larvae per Petri dish was determined.

### Statistical analyses

The data of the garlic transformation experiments were analysed using a generalized linear model (McCullagh and Nelder 1990) based on a binomial distribution using a logit as a link function.

## Results

### Garlic transformation protocol

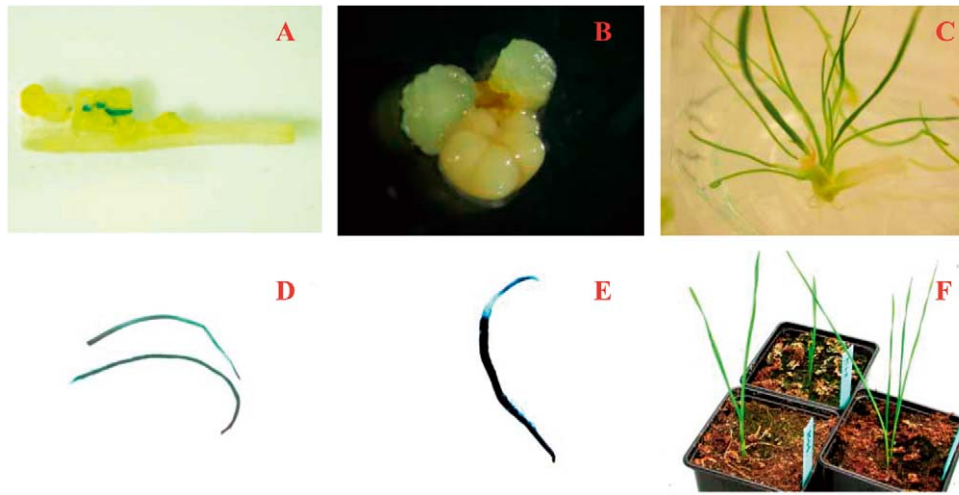
In seven of the nine experiments plasmids pCAM-BIA1301, pC1300intA, pPB34 and pPB36 were used for garlic transformation (Table 1). Plasmids pCAM-BIA1301, pC1300intA, pPB34 and pPB36 have an intron-*gusA* gene as a reporter. Transient expression of GUS was observed in callus derived from root segments, true seeds and bulbils immediately after co-cultivation for four days with *Agrobacterium*. GUS assays showed that in most cases blue spots were located around the surface or the middle part of the callus (Figure 2A). The root segment part did not show any GUS expression. No blue spots were observed in untransformed calli. After co-cultivation for four days with *Agrobacterium*, calli were transferred to the selective medium and subcultured every four weeks. Resistant calli gradually appeared and were selected for further propagation after two months (Figure 2B). After resistant callus lines had grown homogeneously on selective medium for another one-two months, they were transferred to regeneration medium, which contained 25 mg l<sup>-1</sup> hygromycin, for another one-two months. After putative transgenic shoots had fully developed, they were isolated and transferred to rooting solidified MS medium that contained 25 mg l<sup>-1</sup> hygromycin and 30 g l<sup>-1</sup> sucrose without any phytohormones (Figure 2C). The maximum transformation frequency was 1.47% (Table 1). Transgenic plants were identified by the GUS assay

on leaf and root tissues while the plantlets grew on the regeneration medium containing hygromycin. Before the transgenic plants were transferred to the greenhouse, leaf and root tissues were collected for an additional histochemical GUS assay. Transgenic garlic plants had visible GUS activity in their entire tissues (Figure 2D, Figure 2E). These individual plants were transferred to the greenhouse (Figure 2F). The transgenic plants harbouring the *cry1Ca* gene grew well, had a normal phenotype and produced bulbs. However, the transgenic plants harbouring the *H04* gene died after transfer to the greenhouse (Table 1).

In three of the nine experiments that were carried out, plasmid pC1300intA-sGFP harbouring a synthetic GFP gene, was used for garlic transformation as a reporter gene (Table 1). After transformation with pC1300intA-sGFP, transient expression in calli derived from root segments of cvs. 'Messidrome', 'Morasol' and 'Printanor' or true garlic seeds (bulbils not tested) was visible as early as two days after *Agrobacterium* infection and reached maximum levels after four days (Figure 3AA, 3BB and 3CC). GFP expression showed that in most cases green spots were located around the surface of the callus (Figure 3AA and 3CC). The number of green spots varied from a few isolated spots (1–2) to a larger area (Figure 3BB). No green fluorescence was observed when an *Agrobacterium* sample was checked under the fluorescence microscope (not shown). Although the most dramatic and the earliest transient GFP activity was seen after four days co-cultivation with *Agrobacterium*, the expression of GFP decreased quickly in the majority of calli after they were transferred to the selective medium containing hygromycin. At the beginning, non-transformed callus without GFP expression was surrounded by transformed callus. After approximately two-three weeks of growth, the GFP regions were excised and cultured separately. This procedure was repeated until resistant calli appeared with homogeneous green fluorescence expression (Figure 3DD). After transferring these homogeneous calli onto regeneration medium containing hygromycin for one-two months, transgenic shoots with GFP expression were produced (Figure 3EE and 3FF). Later they were isolated and put on rooting solidified MS medium containing 25 mg l<sup>-1</sup> hygromycin (Figure 3F). Unfortunately, the transgenic rooted plants harbouring the *gfp* gene gradually died after transfer to the greenhouse (Table 1).

Table 1. Summary of garlic transformation experiments

Exp	Cultivar or source	Explant	Plasmid	Number of callus lines on selective medium (A)	Number of callus lines regenerating transgenic plants (B)	Transformation efficiency (B/A, %)	Number of transgenic in vitro plants to greenhouse	Number of transgenic plants surviving after 4 months in greenhouse
1	'Printanor'	root	pPB34	68	1	1.47	42	0
1	'Printanor'	root	pPB36	81	1	1.23	55	46 (bulbs for storage)
1	'Morasol'	root	pPB34	44	0	0	0	0
1	'Morasol'	root	pPB36	91	0	0	0	0
2	'Printanor'	root	pPB34	56	0	0	0	0
2	'Printanor'	root	pPB36	68	0	0	0	0
3	'Printanor'	root	pPB36	91	0	0	0	0
3	'Messidrome'	root	pPB34	17	0	0	0	0
3	'Messidrome'	root	pPB36	72	0	0	0	0
4	True seed	embryo	pPB36	313	1	0.32	3	0
4	True seed	embryo	pCI300intA	320	0	0	0	0
4	True seed	embryo	pCI300intA-sGFP	341	0	0	0	0
4	'Printanor'	root	pPB36	134	0	0	0	0
4	'Printanor'	root	pCI300intA-sGFP	123	0	0	0	0
4	'Messidrome'	root	pCI300intA-sGFP	52	0	0	0	0
5	'Printanor'	root	pCAMBIA1301	209	0	0	0	0
5	'Messidrome'	root	pCAMBIA1301	28	0	0	0	0
5	'Morasol'	root	pCAMBIA1301	64	0	0	0	0
5	Bulbil	bulbil	pCAMBIA1301	116	1	0.86	5	4
6	'Printanor'	root	pCI300intA-sGFP	406	0	0	0	0
6	'Morasol'	root	pCI300intA-sGFP	231	0	0	0	0
7	'Printanor'	root	pCI300intA-sGFP	268	1	0.37	11	0
7	'Messidrome'	root	pCI300intA-sGFP	149	0	0	0	0
7	'Morasol'	root	pCI300intA-sGFP	182	0	0	0	0
8	'Printanor'	root	pCAMBIA1301	85	1	1.18	2	0
8	'Messidrome'	root	pCAMBIA1301	176	0	0	0	0
8	'Morasol'	root	pCAMBIA1301	135	0	0	0	0
9	'Printanor'	root	pPB36	246	0	0	0	0
9	'Messidrome'	root	pPB36	161	1	0.62	5	0
9	'Morasol'	root	pPB36	104	0	0	0	0



*Figure 2.* Overview of the garlic transformation procedure. A. Transient expression of GUS in two-month old callus derived from root segment of cv. 'Printanor'; infection with AGL0 (pPB36) after four days of co-cultivation. B. Hygromycin-resistant callus of cv. 'Printanor' after two months growth on selective medium. C. Plant regeneration of cv. 'Printanor' transformed with AGL0 (pPB34). The photograph was taken four weeks after the hygromycin-resistant shoot had been transferred to rooting medium with hygromycin. D. Expression of GUS in the leaves of a transformant. E. Expression of GUS in the root of a transformant. F. Transgenic garlic plants in the greenhouse.

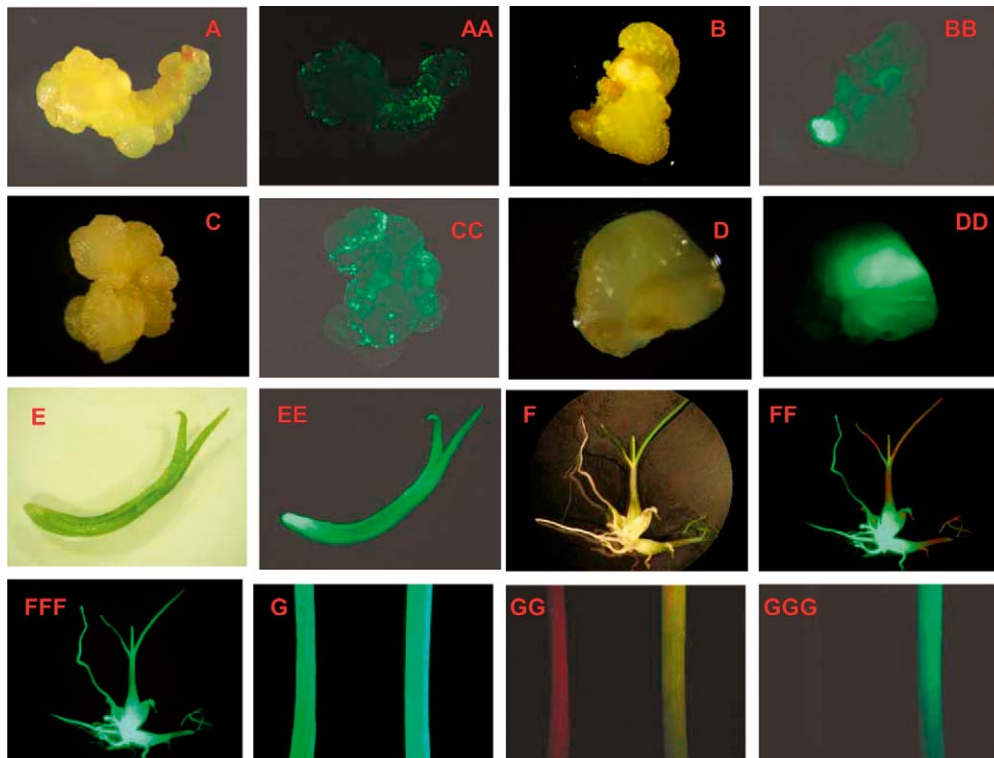


Table 2. Overview of the results obtained in nine successive garlic transformation experiments studying the effects of garlic cultivars, callus source and plasmids on transformation efficiency. \* X/Y;X: number of calli lines regenerated *in vitro* plants. Y: number of calli lines on selective medium.

Plasmid	Root			True seed	Bulbil	Total
	'Printanor'	'Morasol'	'Messidrome'			
pPB34	1/68*	0/44	0/17			1/185
	0/56					
pPB36	1/81	0/91	0/72	1/313		3/1361
	0/68	0/104	1/161			
	0/91					
	0/134					
	0/246					
pC1300intA-sGFP	0/123	0/231	0/52	0/341		1/1752
	0/406	0/182	0/149			
	1/268					
pCAMBIA1301	0/209	0/64	0/28		1/116	2/813
	1/85	0/135	0/176			
pC1300intA				0/320		0/320
Total	4/1835	0/851	1/655	1/974	1/116	7/4431
	5/3341					

Figure 3. Overview of garlic transformation procedure using the GFP monitoring system. A and AA. Light and fluorescence images (GFP2 filter), respectively, showing transient GFP expression in the cells at the surface of two-month old callus derived from root segment of 'Printanor'; infection with AGL0 (pC1300intA-sGFP) after four days of co-cultivation. B and BB. Light and fluorescence images (GFP2 filter), respectively, showing transient GFP expression in the cells at one large area of two-month old callus derived from root segment of cv. 'Printanor'; infection with AGL0 (pC1300intA-sGFP) after four days of co-cultivation. C and CC. Light and fluorescence images (GFP2 filter), respectively, showing transient GFP expression in the cells at the surface of two-month old callus derived from root segment of cv. 'Messidrome'; infection with AGL0 (pC1300intA-sGFP) after four days of co-cultivation. D and DD. Light and fluorescence images (GFP2 filter), respectively, stable and uniform expression of GFP in hygromycin-resistant callus of cv. 'Printanor' after two months growth on selective medium. E and EE. Light and fluorescence images (GFP3 filter), respectively, regenerated shoot of 'Printanor'. F, FF and FFF. Light and fluorescence images (GFP2 and GFP3 filters), respectively; note that FF is a fluorescence image without the chlorophyll fluorescence blocked in young garlic plants. G, GG and GGG. Light and fluorescence images (GFP2 and GFP3 filters) of non-transgenic (left) and transgenic garlic leaf (right) from the greenhouse, respectively. Note that GG is a fluorescence image without the chlorophyll fluorescence blocked which shows red fluorescence in non-transgenic cv. 'Printanor' leaf (left) and greenish yellow fluorescence in transgenic cv. 'Printanor' leaf (right). Only green fluorescence in transgenic cv. 'Printanor' leaf (right) is observed under GFP3 filter.

#### Analysis of cultivar, plasmid and tissue effects on transformation frequency

In total 4431 callus lines were subcultured and seven callus lines produced transgenic plants (Table 2). Of these seven lines only two lines produced plants which were able to develop into normal looking plants in the greenhouse. Differences in transformation frequency (number of callus lines regenerated *in vitro* plants / number of callus lines on selective medium) were observed among the three French garlic cultivars used (Table 2). 'Printanor' had the highest transformation frequency (overall 0.22%) followed by cv. 'Messidrome' (0.15%), whereas cv. 'Morasol' did not produce any plants. However these differences proved to be statistically not significant (Table 3). Also, it was observed that cv. 'Printanor' could be

transformed with all four plasmids yielding plants, whereas this was only the case with pPB36 using cv. 'Messidrome'. With respect to the effect of the different plasmids on mean transformation frequency, it was observed that pPB34 yielded the highest transformation frequency (0.54%), followed by pCAMBIA1300 (0.25%) and pPB36 (0.22%), and pC1300intA-sGFP (0.06%). However no statistically significant differences were found (Table 3). Comparisons among source tissues for genetic transformation (root, bulbil and true seed) were not possible due to the unbalanced nature of the data. However, in our hands all three source tissues could be genetically transformed to yield *in vitro* plants. The multiplication rate of *in vitro* plantlets differed greatly between the primary *in vitro* regenerants (2–55 plantlets / regenerant; Table 1).

Table 3. Analysis of deviance of the effect of cultivar, plasmid and tissue source on garlic transformation efficiency. Ratios are based on a dispersion parameter with value 1. d.f.: degree of freedom, M.D.: mean deviance. NS: not significant.

Source of variation	d.f.	M.D.	Deviance ratio	Approximately F prediction
Cultivar	2	1.5255	2.00	0.173 <sup>NS</sup>
Plasmid	3	0.5797	0.76	0.536 <sup>NS</sup>
Cultivar × Plasmid	6	0.3559	0.47	0.823 <sup>NS</sup>
Residual	14	0.7646		
Total	25	0.7052		



Figure 4. PCR amplification of genomic DNA from garlic transformants. DNA amplified with *gusA* primers resulting in a 710 bp fragment. Lane P: plasmid pPB34 as positive control; Lane N: untransformed garlic as negative control; Lane M: 1kb DNA ladder marker; Lanes 1–3 in 4A: individual transgenic garlic plants with AGL0 (pPB34). Lanes 1–17 in 4B: individual transgenic garlic plants with AGL0 (pPB36).

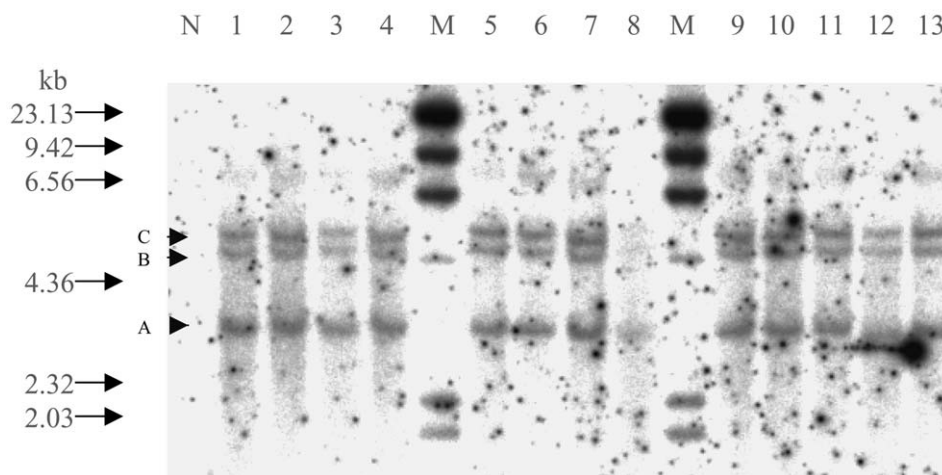
#### Characterization of transgenic garlic

All transgenic garlic plants transformed with pP34 and pP36 were analysed by PCR to check whether the *gusA*, *hpt* and the *cry1Ca* or *H04* gene sequences were present. The presence of *cry1Ca*, *gusA* and *hpt* sequences was analysed in greenhouse plants, whereas the presence of *gfp* and *H04* was analysed in *in vitro* plantlets. To this end genomic DNA from the putative transformants was amplified with the *gusA*, *hpt* and the *cry1Ca* or *H04* gene primers. All genes were present in all transformed plants and PCR products were generated with the expected fragment size of 710 bp (Figure 4A, Figure 4B), 1.2 kb, 802 bp and 953 bp, respectively (data not shown).

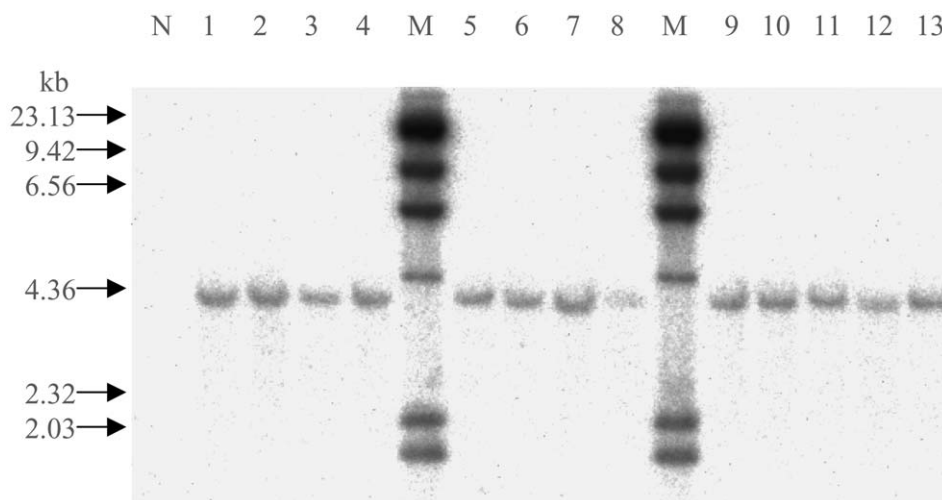
In order to determine whether T-DNA integration had taken place and how many copies of the T-DNA were present in the plant's genome, Southern hybridization was carried out. DNA was extracted from young leaf tissue of individual transgenic plants, digested with *Hind*III and hybridized with probes from the PCR products of the *gusA*, *hpt* and *cry1Ca* genes, respectively. DNA from non-transformed plants was used as a negative control and showed no hybridization to *gusA*, *hpt* and *cry1Ca* probes, respectively. Because the T-DNA of pCAMBIA1301 has only one *Hind*III site, which is located between the *hpt* and *gusA* gene (Figure 1A), the second *Hind*III site must be located on the plant genome. Hence, probing with

either the *gusA* or *hpt* gene sequence gives an indication of the number of T-DNA copies integrated. Because the T-DNA of pPB36 has two *Hind*III sites, probing with *cry1Ca* gene sequence will give an internal fragment harbouring the *cry1Ca* gene when the *cry1Ca* gene PCR product is used as a probe (Figure 1B). Probing with either the *gusA* or *hpt* gene sequence will give an indication of the number of T-DNA copies integrated. Thirteen plants were used for the analysis, these 13 plants were randomly taken from the 46 *in vivo* plants produced in experiment 1 (Table 1). The thirteen plants analysed had one band (A) with a fragment size of approximately 3 kb and two other bands (B and C) with sizes of approximately 4.6 and 5 kb (Figure 5), when probing with the PCR product of the *hpt* fragment. This suggests three copies. However, these 13 plants had only one band with a fragment size of 6 kb when probing with the PCR product of *gusA* fragment (data not shown). These findings suggest that the 13 plants originated from one transformed callus cell and were genetically identical. The 13 transgenic plants had one band with the expected size of 4080 bp (Figure 6), when probing with the PCR product of the *cry1Ca* gene. This indicates that no truncated integration of the *cry1Ca* gene into the garlic genome took place.

In transgenic garlic plants, which were transformed with GFP, transgenic tissue could be easily detected via the expression of fluorescent protein under a UV



**Figure 5.** Southern blot analysis of transgenic garlic plants. DNA from hygromycin-resistant transformants was digested with *Hind*III, fractionated by electrophoresis, transferred to a nylon membrane, and allowed to hybridise to a probe. DNA from a PCR amplification using *hpt* primers generated a 1.2 kb fragment. This 1.2 kb fragment was used as a probe. Lane M:  $\lambda$ DNA digested with *Hind*III; Lane N: untransformed garlic plant as negative control. Lanes 1–13: clonal plants originating from one line transformed with AGL0 (pPB36).



**Figure 6.** Southern blot analysis of transgenic garlic plants. DNA from hygromycin-resistant transformants was digested with *Hind*III, fractionated by electrophoresis, transferred to a nylon membrane, and allowed to hybridise to a probe. DNA from a PCR amplification using *cry1Ca* primers generated a 802 bp fragment. This 802 bp fragment was used as a probe. Lane M:  $\lambda$ DNA digested with *Hind*III; Lane N: untransformed garlic plant as negative control. Lanes 1–13: clonal plants originating from one line transformed with AGL0 (pPB36).

microscope. As a comparison, a non-transgenic garlic leaf was red using a GFP2 filter because the chlorophyll fluorescence was not blocked in the young leaf part while a transgenic garlic leaf was pale yellow (Figure 3FF and 3GG). Using a GFP3 filter, a transgenic garlic leaf was homogenous green while a non-transgenic leaf was not visible (Figure 3GGG). All transgenic garlic plants were analysed by PCR to confirm whether the GFP gene sequences were

present in the garlic genome. In all transformed plants a 644 bp fragment of PCR product was generated indicating the presence of the GFP gene (Figure 7).

#### *Beet armyworm bio-assay*

To establish whether garlic plants transformed with pPB36 were resistant to beet armyworm, five 3-day old larvae of beet armyworm (*Spodoptera exigua*

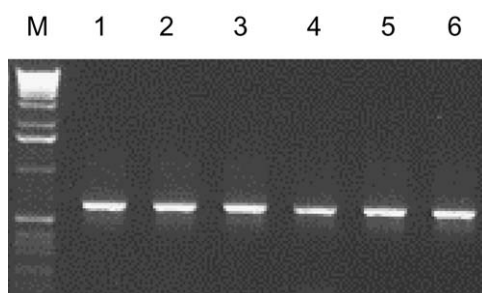


Figure 7. PCR amplification of genomic DNA from garlic transformants. DNA amplified with *gfp* primers resulting in a 644 bp fragment. Lane M: 1kb DNA ladder marker; Lane 1–6: individual transgenic garlic plants with AGL0 (pC1300intA-sGFP).

Hübner) were fed on transgenic and non-transgenic leaves. Four days after the start of the experiment all five larvae survived on non-transgenic garlic leaves and had developed normally, while all five larvae were killed on transgenic garlic leaves (Figure 8). The same results were obtained in two successive replicate experiments.

## Discussion

We have developed a simple and reliable transformation system for garlic. The transformation procedure is based on *Agrobacterium tumefaciens* using as target tissue young callus derived from both apical and non-apical root segments, true garlic seeds and immature bulbils. This is an improvement to the previous protocols for garlic transformation as these only used callus induced on apical meristems of (im)mature cloves for successful transformation (Kondo et al. 2000; Park et al. 2002 and Sawahel 2002). However, cloves age during storage and, therefore, may be less suitable for callus induction.

The possibility to use both apical and non-apical root segments from *in vitro* plantlets in our system appears to be a significant advance, because it is now feasible to carry out garlic transformation experiments year-round. We demonstrated that shoot regeneration from callus induced on non-apical segments was higher, although not significant, compared to callus induction from apical root segments (Zheng et al. 2003). In this context, Shuto et al. (1993) showed that as much as 20% shoot regeneration took place in garlic via root-tip derived callus and Haque et al. (1997) obtained high frequency shoot regeneration and plantlet formation from garlic root-tips. Other impor-

tant issues for a successful transformation system are transformation frequency and time required to obtain transgenic plants. Our transformation protocol takes about six months from callus induction to the production of *in vitro* transgenic plants. We induced very young and small calli (Figure 2A) on root segments of *in vitro* garlic plantlets and these behave similarly to the 3-week old calli derived from onion and shallot seeds in a previous study (Zheng et al. 2001a, b). The highest transformation frequency obtained was 1.47% using garlic cv. 'Printanor'. This frequency was comparable to our previous developed onion and shallot transformation protocol (Zheng et al. 2001a). We obtained seven lines with transgenic garlic plants in six different independent experiments by using different constructs (Table 2). Kondo et al. (2000) obtained 20 shoots, which were induced on about 1000 calli using one construct. Fifteen of the 20 plants expressed  $\beta$ -glucuronidase activity upon staining with X-Gluc. Their transformation frequency was approximately 1.5%, which is similar to our transformation frequency. The transformation procedure of Kondo and co-workers took at least eight–ten months from starting material to the production of transgenic garlic. Biolistic transformation via the protocol of Park et al. (2002) took about 14 months and the transformation frequency reported was 0.63%. On the basis of the results obtained from all garlic transformation studies carried out until now, it seems that our protocol is straightforward having an acceptable transformation frequency. Evidence is also accumulating that in garlic, the transformation frequency is higher in *Agrobacterium tumefaciens*-mediated protocols compared to particle gun protocols (1.5 and 1.47% vs 0.63%). Amongst others this might be due to the presence of a strong endogenous nuclease activity (Barandiaran et al. 1998) interfering to a large extent with the latter transformation system. Only via blocking of DNase activity using aurintricarboxylic acid Sawahel (2002) was able to produce transgenic plants. In a previous study on onion and shallot transformation (Zheng et al. 2001a, b) it was shown that the use of different cultivars is an important factor influencing transformation frequency. Also in the present study we found indications for this effect as cvs. 'Printanor' and 'Messidrome' produced transgenic *in vitro* plants and cv. 'Morasol' did not (Table 2). Furthermore, we investigated if the presence of a catalase-1 intron in the *hpt* gene might improve our protocol as it was observed in rice (Wang et al. 1998). They found that a catalase-1 intron in the

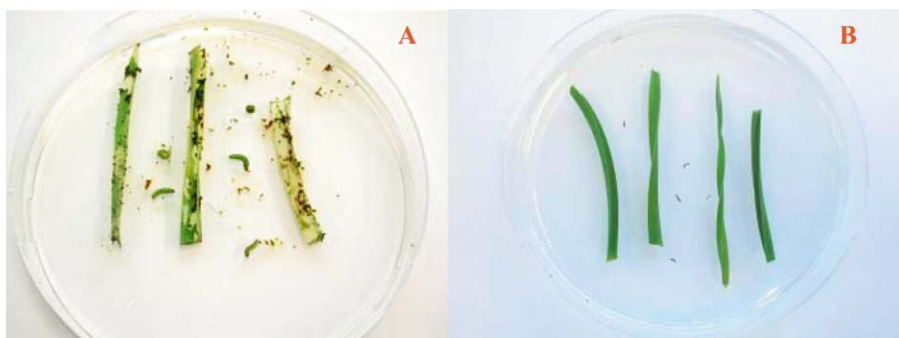


Figure 8. Transgenic cv. 'Printanor' transformed with pPB36 resistant to beet armyworm, right image B; left image A: non-transgenic cv. 'Printanor'.

*hpt* gene resulted in a higher resistance towards hygromycin, which allowed an easier killing of *Agrobacterium* during the regeneration of transgenic plants. The easier killing of *Agrobacterium* resulted in a higher transformation frequency. However, the use of intron-*hpt* plasmids pC1300intA-sGFP and pC1300intA in our hands did not improve garlic transformation efficiency compared to the other plasmids without intron-*hpt* (Table 3). Concerning the molecular analysis of the 13 transgenic plants from cv. 'Printanor', we found that the number of bands hybridizing to the *gusA* (RB) and the *hpt* (LB) probes were different. There were three bands hybridizing to the *hpt* probe, while only one band hybridized to the *gusA* probe. This finding could be interpreted as a result of a head-to-head tandem repeat, which occurred during the integration of T-DNA into the garlic genome. Using the same basic plasmid pCAMBIA1301 for rice transformation, Sallaud et al. (2003) also found that the number of bands hybridizing to the *gusA* (RB) and the *hpt* (LB) probes were different.

In this study, we produced transgenic garlic plants harbouring the *cry1Ca* gene. Using an *in vitro* assay we showed that the *cry1Ca* gene functioned satisfactorily as transgenic garlic was not affected by beet armyworm (Figure 8). Furthermore, the *cry1Ca* transgenic plants grew well in the greenhouse, had a normal phenotype and produced bulbs. Therefore, these transgenic plants show great promise in the fight against beet armyworm in (sub-) tropical *Allium* cultivations. Remarkably, transgenic plants harbouring the *H04* gene died after transferring to the greenhouse. However, in our shallot transformation experiments (unpubl. data) we observed that *in vivo* transgenic shallot plants could be produced harbouring either *cry1Ca* or *H04* gene albeit looking at overall frequencies *H04* plants generally perform worse

than *Cry1Ca* plants. At present it is unclear what caused this discrepancy. A similar phenomenon occurred when using GFP in garlic transformation experiments. Also with this gene transgenic garlic plants died after transfer to the greenhouse. However, Eady et al. (2000, 2003) showed that in onion transformation plants could be produced harbouring the *gfp* gene. This difference might be due to the fact that they used a different construct (*m-gfp5-ER*). However, further research is clearly needed to solve this problem. The use of GFP in our garlic transformation research was very helpful because it allowed us to monitor the transformation process in detail without having to remove the tissue from the culture plates and without adversely affecting tissue growth.

Our study showed that genetic transformation of garlic via *Agrobacterium tumefaciens* can be achieved using callus induced from different source tissues (preferably from *in vitro* roots) and that the procedure from start to end can be reduced to six months. Furthermore, using this new transformation protocol we were able to develop garlic resistant to beet armyworm which is a major step forward in combating this serious pest in (sub-) tropical *Allium*.

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## References

- Barandiaran X., Pietro A.D. and Martin J. 1998. Biolistic transfer and expression of a *uidA* reporter gene in different tissues of *Allium sativum* L. *Plant Cell. Rep.* 17: 737–741.
- Carozzi N.B., Rabe S.M., Miles P.J., Warren G.W. and De Haan P. T. 2002. Novel insecticidal toxins derived from *Bacillus thuringiensis* crystal proteins. International Application Published Under The Patent Cooperation Treaty WO 02/15701.
- Chiu W.L., Niwa Y., Zeng W., Hirano T., Kobayashi H. and Sheen J. 1996. Engineered GFP as a vital reporter in plants. *Current Biol.* 6: 325–330.
- De Cosa B., Moar W., Lee S.B., Miller M. and Daniell H. 2001. Overexpression of *Bt cry2Aa2* operon in chloroplasts leads to formation of insecticidal crystals. *Nature Biotechnology* 19: 71–74.
- De Maagd R.A., Kwa M.S.G., van der Klei H., Yamamoto T., Schipper B., Vlak J.M., Stiekema W. and Bosch D. 1996. Domain III substitution in *Bacillus thuringiensis* delta-endotoxin CryIA(b) results in superior toxicity for *Spodoptera exigua* and altered membrane protein recognition. *Appl. Environ. Microbiol.* 62: 1537–1543.
- De Maagd R.A., Bosch D. and Stiekema W. 1999. *Bacillus thuringiensis* toxin-mediated insect resistance in plants. *Trends in Plant Sci.* 4: 9–13.
- De Maagd R.A., Bravo A. and Crickmore N. 2001. How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *Trends in Genet.* 17: 193–199.
- Eady C.C., Weld R.J. and Lister C.E. 2000. *Agrobacterium tumefaciens*-mediated transformation and transgenic-plant regeneration of onion (*Allium cepa* L.). *Plant Cell Rep.* 19: 376–381.
- Eady C.C. 2002. Genetic transformation of onions. In: Rabinowitch H.D. and Currah L. (eds), *Allium Crop Science: Recent Advances*. CAB International, Wallingford, UK, pp. 119–144.
- Eady C.C., Davis S., Farrant J., Reader J. and Kenel F. 2003. *Agrobacterium tumefaciens*-mediated transformation and regeneration of herbicide resistant onion (*Allium cepa*) plants. *Ann. Appl. Biol.* 142: 213–217.
- Ferrer E., Linares C. and Gonzalez J. M. 2000. Efficient transient expression of the beta-glucuronidase reporter gene in garlic (*Allium sativum* L.). *Agronomie* 20(8): 869–874.
- Goh H.G., Park J.D., Choi Y.M., Choi K.M. and Park I.S. 1991. The host plants of beet armyworm, *Spodoptera exigua* (Hübner), (Lepidoptera: Noctuidae) and its occurrence. *Korean J. Appl. Entomol.* 30: 111–116.
- Haque M.S., Wada T. and Hattori K. 1997. High frequency shoot regeneration and plantlet formation from root tip of garlic. *Plant Cell, Tissue and Organ Cult.* 50: 83–97.
- Hood E.E., Helmer G.L., Fraley R.T. and Chilton M. D. 1986. The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. *J. Bact.* 168: 1291–1301.
- Hood E.E., Gelvin S.B., Melchers L.S. and Hoekema A. 1993. New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgenic Res.* 2: 208–218.
- Jefferson R.A. 1987. Assaying chimeric genes in plants; the GUS gene fusion system. *Plant Mol. Biol. Rep.* 5: 387–405.
- Kik C. 2002. Exploitation of wild relatives for the breeding of *cultivated Allium* species. In: Rabinowitch H.D. and Currah L. (eds), *Allium Crop Science: Recent advances*. CAB International, Wallingford, UK, pp 81–100.
- Kondo T., Hasegawa H. and Suzuki M. 2000. Transformation and regeneration of garlic (*Allium sativum* L.) by *Agrobacterium*-mediated gene transfer. *Plant Cell Rep.* 19: 989–993.
- Lazo G.R., Stein P.A. and Ludwig R.A. 1991. A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. *Bio/Technology* 9: 963–967.
- Mattanovich D., Rüker F., Machado A.D.C., Laimer M., Regner F., Steinkellner H., Himmler G. and Katinger H. 1989. Efficient transformation of *Agrobacterium* spp. by electroporation. *Nucl. Acids Res.* 17: 6747.
- McCullagh P. and Nelder J.A. 1990. *Generalized Linear Models*. Chapman and Hall, London and New York.
- Naimov S., Dukiandjiev S. and De Maagd R.A. 2003. A hybrid *Bacillus thuringiensis* delta-endotoxin gives resistance against a coleopteran and a lepidopteran pest in transgenic potato. *Plant Biotechnology J.* 1: 51–57.
- Ohta S., Mita S., Hattori T. and Nakamura K. 1990. Construction and expression in tobacco of a beta-glucuronidase (GUS) reporter gene containing an intron within the coding sequence. *Plant and Cell Physiol.* 31: 805–813.
- Outchkourov N.S., Peters J., De Jong J., Rademakers W. and Jongsma M.A. 2003. The promoter-terminator of chrysanthemum *rbcS1* directs very high expression levels in plants. *Planta* 216: 1003–1012.
- Park M.Y., Yi N.R., Lee H.Y., Kim S.T., Kim M., Park J.H., Kim J.K., Lee J.S., Cheong J.J. and Choi Y.D. 2002. Generation of chlorosulfuron-resistant transgenic garlic plants (*Allium sativum* L.) by particle bombardment. *Mol. Breed.* 9: 171–181.
- Perlak F.J., Oppenhuizen M., Gustafson K., Voth R., Sivasubramanian S., Heering D., Carey B., Ihrig R.A. and Roberts J.K. 2001. Development and commercial use of Bollgard<sup>®</sup> cotton in the USA: Early promises versus today's reality. *Plant J.* 27: 489–501.
- Sallaud C., Meynard D., van Boxtel J., Gay C., Bès M., Brizard J.P., Larmande P., Ortega D., Raynal M., Portefaix M., Ouwerkerk P.B., Rueb S., Delseny M. and Guiderdoni E. 2003. Highly efficient production and characterization of T-DNA plants for rice (*Oryza sativa* L.) functional genomics. *Theor. Appl. Genet.* 106: 1396–1408.
- Sawahel W.A. 2002. Stable genetic transformation of garlic plants using particle bombardment. *Cell. Mol. Biol. Lett.* 7: 49–59.
- Schnepf E., Crickmore N., van Rie J., Lereclus D., Baum J., Feitelson J., Zeigler D. R. and Dean D.H. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* 62: 775–806.
- Schuler T.H., Poppy G.M., Kerry B.R. and Denholm I. 1998. Insect-resistant transgenic plants. *Trends in Biotechnology* 16: 168–175.

- Shuto H., Abe T. and Sasahara T. 1993. *In vitro* propagation of plants from root apex-derived calli in Chinese chive (*Allium tuberosum* ROTTLER) and garlic (*Allium sativum* L.). Japan. J. Breed. 43: 349–354.
- Songstad D.D., Somers D.A. and Griesbach R.J. 1995. Advances in alternative DNA delivery techniques. Plant Cell Tissue Organ Cult. 40: 1–15.
- Strizhov N., Keller M., Mathur J., Koncz K.Z., Bosch D., Prudovsky E., Schell J., Sneh B., Koncz C. and Zilberstein A. 1996. A synthetic cryIC gene, encoding a *Bacillus thuringiensis* delta-endotoxin, confers *Spodoptera* resistance in alfalfa and tobacco. PNAS (USA) 26: 15012–15017.
- Tu J., Zhang G., Datta K., Xu C., He Y., Zhang Q., Khush G.S. and Datta S.K. 2000. Field performance of transgenic elite commercial hybrid rice expressing *Bacillus thuringiensis* delta-endotoxin. Nature Biotechnology 18: 1101–1104.
- van Heusden A.W., van Ooijen J.W., Vrielink van Ginkel R., Verbeek W.H.J., Wietsma W.A. and Kik C. 2000. A genetic map of an interspecific cross in *Allium* based on amplified fragment length polymorphism (AFLP<sup>TM</sup>) markers. Theor. App. Genet. 100: 118–126.
- Wang M.B., Li Z.Y. and Matthews P. R. 1998. Improved vectors for *Agrobacterium tumefaciens*-mediated transformation of monocot plants. Acta Horticulturae 463: 401–407.
- Zheng S.J., Henken B., Sofiari E., Jacobsen E., Krens F.A. and Kik C. 1998. Factors influencing induction, propagation and regeneration of mature zygotic embryo-derived callus from *Allium cepa* L.. Plant Cell Tissue Organ Cult. 53: 99–105.
- Zheng S.J., Henken B., Sofiari E., Keizer P., Jacobsen E., Kik C. and Krens F. A. 1999. The effect of cytokinins and lines on plant regeneration from long-term callus and suspension cultures of *Allium cepa* L.. Euphytica 108: 83–90.
- Zheng S.J., Henken B., Sofiari E., Jacobsen E., Krens F.A. and Kik C. 2000. Development of bio-assays and screening for resistance to beet armyworm (*Spodoptera exigua* Hübner) in *Allium cepa* L. and its wild relatives. Euphytica 114: 77–85.
- Zheng S.J., Khrustaleva L., Henken B., Sofiari E., Jacobsen E., Kik C. and Krens F.A. 2001a. *Agrobacterium tumefaciens*-mediated transformation of *Allium cepa* L.: the production of transgenic onions and shallots. Mol. Breed. 7: 101–115.
- Zheng S.J., Henken B., Sofiari E., Jacobsen E., Krens F.A. and Kik C. 2001b. Molecular characterization of transgenic shallots (*Allium cepa* L.) by adaptor ligation PCR (AL-PCR) and sequencing of genomic DNA flanking T-DNA borders. Transgenic Res. 10: 237–245.
- Zheng S.J., Henken B., Krens F.A. and Kik C. 2003. The development of an efficient cultivar independent plant regeneration system from callus derived from both apical and non-apical root segments of garlic (*Allium sativum* L.). *In Vitro* Cell. Dev. Biol. Plant 39: 288–292.