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ORIGINAL PAPER

Efficient *Agrobacterium tumefaciens*-mediated transformation and regeneration of garlic (*Allium sativum*) immature leaf tissue

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Abstract Transgenic garlic (*Allium sativum*) plants have been recovered directly from immature leaf material by selective culture following *Agrobacterium*-mediated transformation. This method involved the use of a binary vector containing the *mgfp-ER* reporter gene and *hpt* selectable marker, and followed a similar protocol developed previously for the transformation of immature onion embryos. The choice of tissue and post-transformation selection procedure resulted in a large increase in recovery of transgenic plants compared with previously confirmed allium transformation protocols. The presence of transgenes in the genome of the plants was confirmed using Southern analysis. This improvement in frequency and the use of clonal commercial "Printanor" germplasm now makes possible the integration of useful agronomic and quality traits into this crop.

Keywords *Allium* · Garlic · Transformation · *Agrobacterium*

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Introduction

The agronomic benefits and commercial uptake of transgenic first tier crops is now well established (www.isaaa.org) and much research into the establishment of transformation systems and applications for second tier crops has also been undertaken (Kole and Hall 2008). However, for some major second tier crops, e.g. commercial allium species, efficient transformation systems that are practical as an applied research tool have yet to be developed. Historically, allium cultivars arose from locally adapted genotypes owned by small local seed suppliers that were individually unable to fund expensive biotechnology research. Global seed acquisitions over the last 15 years have consolidated many of these companies, and since about year 2000 privately funded large allium biotechnology research projects have become a feasible proposition. Unfortunately, allium transformation systems have not progressed since the first publication on *Allium cepa* by Eady et al. (2000) and the extension to garlic and leek (Eady et al. 2005). The inefficiency of allium transformation systems is thus a major hurdle for applied research in these crops. For garlic, this is a particular problem as most published allium transformation systems have used seed-derived tissue as their primary source of transformation tissue. Commercial garlic is of clonal origin and does not form true seed; therefore, an alternative transformation system that does not use seed-derived tissue is required if application is to be realized.

Three reports of garlic transformation using somatic-derived tissue exist. Kondo et al. (2000) used *Agrobacterium* to transfer the *uidA* reporter gene and the hygromycin (*hpt*) selective gene to shoot meristem-derived calli; although plants were recovered, acknowledgement was given to the difficult job of maintaining the callus material. Park et al. (2002) used biolistics to transfer herbicide

resistance to a similar cell culture system with only limited success. Most recently, Zheng et al. (2004) introduced insect resistance traits into five lines of garlic using Bt constructs. Although this produced more transgenic events than the Kondo et al. (2000) method, it still required lengthy culture of callus, an inefficiency in itself, and prolonged callus culture runs, an increased risk of introducing somatic mutations. This is evidenced by the decreasing ability of cultures to regenerate over time compared with fresh cultures (Tanikawa et al. 1996). The objective of the work described here was to adapt the direct immature embryo transformation protocol described by Eady et al. (2000) to immature garlic leaf tissue to produce stable transgenic plants via direct regeneration from somatic tissue, thus minimizing the culture period and the potential for somatic mutation.

Materials and methods

Transformation followed the protocol of Eady et al. (2000) with the following modifications.

Plant material

Field-grown cloves of *A. sativum* cv 'Printanor' were used as a source material. These were stored in net bags under shelter in the field until ready to break dormancy and then used in transformation experiments.

Clove sterilization

Garlic bulbs were broken up into individual cloves from which the outer skins were removed. From each clove, the underside of the basal plate from where the roots emerge was removed back to clean the tissue using a scalpel blade. The cloves were then placed in glass jars and briefly rinsed in sterile distilled H_2O. The H_2O was discarded and the cloves were then covered in 70% ethanol and washed for ~30 s, and the ethanol then discarded. A 30% domestic bleach solution with two drops Tween-20 per 100 ml was then used to immerse the cloves. This was then gently agitated for 30 min. Finally, the cloves were rinsed four times in sterile distilled H_2O and placed in a flow bench ready for tissue isolation.

Preparation of *Agrobacterium* containing *pArt27-mgfp-ER*

The day prior to transformation, 500 µl of an *Agrobacterium* (strain LBA4404) aliquot stored at −80°C was added to 50 ml of Luria Broth (LB) in a 150 ml flask. The appropriate antibiotics, 50 µl of spectinomycin (100 mg/ml stock), and streptomycin (100 mg/ml stock) were added and the flask placed on a shaker at 28°C overnight.

On the morning of the transformation, the *Agrobacterium* were removed from the 28°C shaker and replenished with LB (i.e. 50 ml of LB was added to the *Agrobacterium* and swirled before 50 ml of this dilute solution was tipped back into the empty conical flask). The antibiotics were also replenished by adding half the aliquot initially to the overnight culture (i.e., 25 µl each of spectinomycin [100 mg/ml stock] and streptomycin [100 mg/ml stock]). Then 10 µl of acetosyringone stock (500 mM in DMSO) was added to give a final concentration of 0.1 mM. These flasks were then put back on the 28°C shaker for at least 3 h prior to co-cultivation. Once all the leaf sections had been isolated (see below), the *Agrobacterium* were removed from the 28°C shaker. An aliquot of this *Agrobacterium* was transferred to a 50-ml falcon tube and centrifuged at ~4,000 rpm (590 r.c.f.) for 5 min at room temperature. The supernatant was then poured off and discarded. The pellet was resuspended in liquid P5 (Eady et al. 1998, briefly BDS macronutrients, micronutrients, and vitamins (Dunstan and Short 1977), plus 50 mg/l casein enzymatic hydrolysate, 5 mg/l Picloram, 3% sucrose and pH6.0) to give an OD of between 0.7 and 0.9 when reading the absorbance at 550 nm. Then 0.8 µl/ml of acetosyringone stock solution (500 mM in DMSO) was added to this solution to give a final concentration of 0.4 mM.

Equipment

Stereo microscopes, a light source, scalpels and tweezers were placed in a laminar flow hood. Then 200 µl of liquid P5 medium (Eady et al. 1998) was dispensed into sterile 1.5-ml microfuge tubes and placed in the laminar flow hood. P5 (P5 liquid plus 4 gm/l Phytagel, Sigma) solid medium in Petri dishes were taken out of the refrigerator and opened up in the back of a laminar flow hood to dry for at least 1 h prior to use.

Isolation of immature leaf sections

The garlic cloves were cut horizontally about 1 cm above the basal plate and the top section was discarded. The remaining storage leaf and some of the largest outer most immature leaves were then excised and discarded. The remaining leaf tissue was sliced horizontally from the clove to create thin transverse leaf sections (<1 mm thick) starting ~0.5 cm from the base plate and finishing at the base plate/leaf interface. Leaf sections from either one or two cloves (depending on clove size) were placed in each microfuge tube with 200 µl of liquid P5 medium.

Co-cultivation

Agrobacterium suspension (200 µl) was added to each microfuge tube containing leaf sections. These tubes were then vortexed for 30 s, after which the microfuge tube was 'flicked' so that leaf sections moved down into the solution. The lids of each microfuge tube were then pierced with a scalpel blade and placed in a vacuum chamber at 635 mmHg for 30 min.

After the vacuum treatment, the *Agrobacterium* solution was pipetted out of the microfuge tube and the leaf sections gently transferred onto sterile filter paper (Whatman 1 90 mm Diameter). Each leaf section was then carefully separated and both cut edges were blotted dry. Leaf sections were transferred to a Petri dish containing solid P5 media. The cultures were then kept at 23°C in the dark for 5 days.

Selection of transgenic material

After 5 days on P5 media, leaf sections were subcultured onto P5 + 4FPA (50 µM) medium, containing selection agents Timentin (200 mg/l) and hygromycin (5 mg/l). After 3 weeks on this media, leaf sections were subcultured onto P5 + 4FPA media containing Timentin (200 mg/l) and hygromycin (10 mg/l). These leaf sections were then subcultured on this media every 3 weeks until they had spent 12 weeks on selection. During this period leaf sections remained in the dark at 23°C.

GFP screening

Leaf sections were screened by observation under a fluorescence microscope using 475 nm excitation and 510 nm emission (Haseloff et al. 1997) before being subcultured onto fresh media. Any material not expressing GFP was discarded. Some leaf explants were cut into smaller sections during this period to enable more complete contact with the selection media.

Shoot regeneration

After 12 weeks, actively growing material was transferred to K-7S (Shahin and Kaneko 1986), briefly BDS macro nutrients and vitamins (Dunstan and Short 1977), MS (Murashige and Skoog 1962) micronutrients and iron, Adenine sulfate 84 mg/l, Kinetin 1 mg/l, 3% sucrose, 4gm/l Phytagel and pH 5.8. To kill latent *Agrobacterium* and select transgenic material 150 mg/l Timentin and 5 mg/l hygromycin was added. At this point, nodular (meristematic or embryogenic) growths produced on the leaf explants were broken up. Any tissue derived from a single explant was grouped together and defined by its plate number and a letter to avoid any duplication of transformation events. Shoot cultures were subcultured onto fresh media every 3–4 weeks.

Root regeneration

GFP-expressing shoot cultures were transferred onto ½ MS (Murashige and Skoog 1962) media containing 15 g/l sucrose and 5 mg/l hygromycin. Shoot cultures were subcultured every 3–4 weeks or until good root growth was established. Cultures that did not root were discarded after 2 months.

Ex-flasking

Healthy in vitro plantlets were transferred to small (~5–10 cm) pots containing a generic seed raising potting mix which were placed in trays containing 2–3 cm of water. The trays had perforated plastic covers. The trays were transferred to a glasshouse and the plants left for 1 week to harden off. Following that, plants and pots were removed from the trays and placed on a bench in the glasshouse, and the plants grown to maturity.

Analyses of transgene expression

Tissue GFP expression was detected as previously described (Eady et al. 2000). Growth and regeneration, especially root production on media containing hygromycin, was used as an indication of *hpt* gene expression.

PCR detection of transgenes

PCR reactions to confirm the presence of the *mgfp-ER* transgene in garlic DNA were performed on DNA isolated using a urea extraction method (Shure et al. 1983). Primers, forward (GFPA 5′ACGTCTCGAGGATCCAAGGAGATATAACA3′), and reverse (GFPB 5′ ACGTCTCGAGCTCTTAAAGCTCATCATG3′) were used to amplify an 834 bp fragment. Each 25 µl PCR reaction contained ~100 ng template DNA, 2.5 µl 10× buffer (Roche), 2.5 µl 200 nM of each dNTP, 100 µM of forward and reverse primers, and 0.2 µl Taq (1 U/µl) polymerase (Roche). PCR reactions were for 5 min at 94°C, followed by 35 cycles at 94, 60 and 72°C for 1 min each, then 72°C for 7 min.

Southern analysis

The Southern procedure described by Eady et al. (2000) was used to reconfirm the integrated nature of the transgenes and to determine the copy number. Genomic DNA was digested with *HindIII* which cuts once within the T-DNA between the corresponding *mgfp-ER* probe sequence and the right border. A 900-bp P^{32}-labeled sequence from

the *mgfp-ER* gene was used as probe DNA, this lies between the single *HindIII* digestion site within the T-DNA and the left border, such that each integration event when digested with *HindIII* should have a unique size when probed.

Ploidy evaluation

Leaf samples from transgenic and control plants were analyzed by flow cytometry for nuclear DNA content by comparison with nuclei from a reference sample of *Bellis perennis* (Otto 1990). This was performed by finely chopping healthy leaf material and analyzing the released nuclei by DAPI fluorescence in a Partec PAII flow cytometer according to the manufacturer's instructions.

Results and discussion

Tissue source

Immature leaf material close to the inner whorls and near the baseplate interface gave rise to the best transformation and regeneration tissue (Fig. 1). This was determined by separating out the inner, middle, and outer leaf pieces with respect to each other and their position, bottom, mid, or top, relative to the base plate. Transformation and culture of these separated pieces then allowed easy determination of which material was best suited for T-DNA transfer by observation of GFP expression on the various leaf pieces. The inner leaf sheaf pieces adjacent to the base plate gave the best GFP transfer and were most amenable to regeneration. Material further out from the central leaf sheaf or distant from the baseplate was still amenable to transformation, but generally failed to develop normally and regenerate. This older tissue was considered to have undergone greater differentiation, and as such was probably less plastic and so less capable of undergoing redifferentiation into embryogenic or meristematic totipotent cell types.

Regeneration

Initial regeneration experiments were undertaken to determine the best method for regenerating shoot cultures from the immature leaf sections following co-culture on P5 medium. The results in Table 1 demonstrated that P5 + 2ip or P5 + 4FPA performed best. P5 + 4FPA was chosen for the initial culture selection phase as this produced the greatest frequency of nodular cultures, which is the type of material most likely to form shoot cultures. K-7S was chosen as the standard regeneration media. The effect of 4FPA and 2ip were investigated as these have been used previously to successfully regenerate from onion tissue (Tanikawa et al. 1996).

GFP expression

Initial GFP expression after 5 days of co-culture demonstrated that many of the cells within the immature leaf pieces, particularly the epidermal layer and cells around the vasculature, were susceptible to T-DNA transfer and transient gene expression (Fig. 2 top left). Many transient

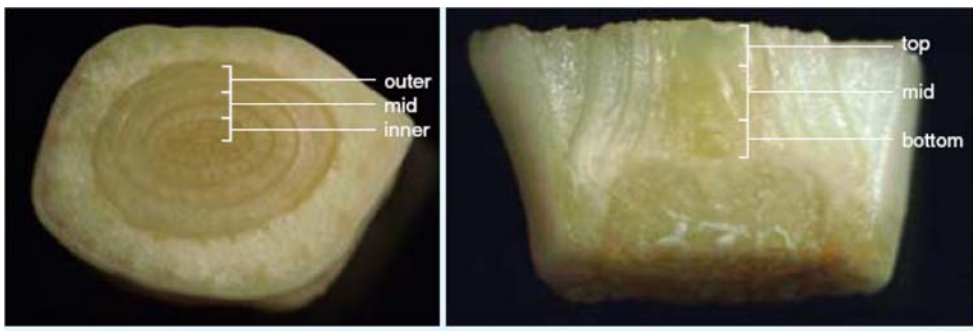

Fig. 1 Transverse and longitudinal section of a garlic clove to show the immature leaf material. The table shows GFP expression in leaf sections from this material after cocultivation with *Agrobacterium*

Treatment	Position on the Horizontal axis	Position on the Vertical axis	Nº of explants	Nº exp GFP at 5 days	GFP % of explants at 5 days
1	Outer	Top (3.4-5 mm from base)	15	0	0
2	Mid	Top (3.4-5 mm from base)	19	1	5
3	Inner	Top (3.4-5 mm from base)	29	9	31
4	Outer	Mid (1.6-3.4 mm from base)	14	0	0
5	Mid	Mid (1.6-3.4 mm from base)	15	7	47
6	Inner	Mid (1.6-3.4 mm from base)	42	20	48
7	Outer	Bottom (0-1.6 mm from base)	35	0	0
8	Mid	Bottom (0-1.6 mm from base)	46	14	30
9	Inner	Bottom (0-1.6 mm from base)	89	50	56

Table 1 The effect of various culture media on immature leaf nodular culture formation and subsequent shoot regenearation

Culture media	No. of immature leaf explants	Percentage of explants forming nodular cultures after 8 weeks on culture media	Percentage of nodular cultures forming shoots following 2 weeks subculture onto K-7S
P5	80	1	0
P5 + 2ip (1 μM)	92	22	41
P5 + 4FPA (50 μM)	94	57	24
P5 + 2ip (1 μM), 4FPA (50 μM)	90	38	11

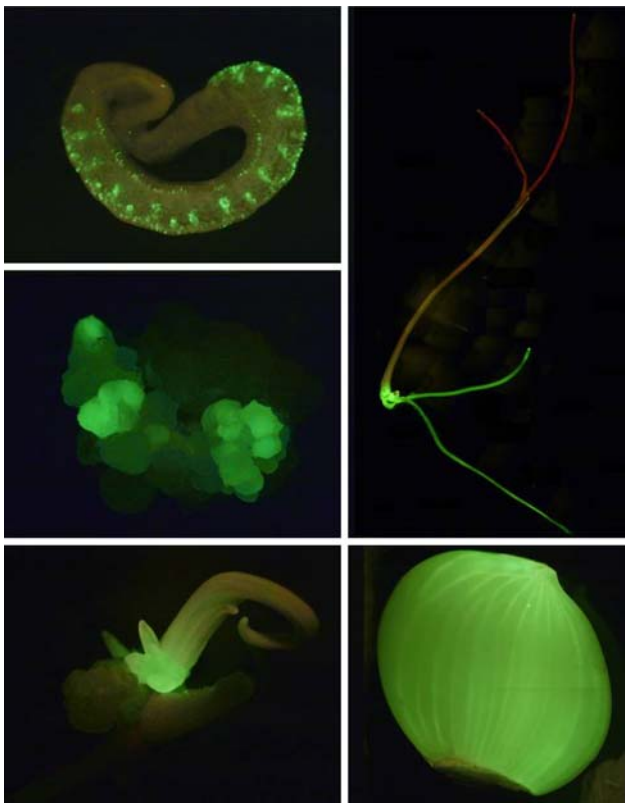

Fig. 2 GFP expression in immature leaf sections (*top left*), selected nodular cultures (*middle left*) regenerating shoots (*bottom left*) whole plants (*top right*) and mature cloves (*bottom right*)

expressing cells rapidly progressed to become stable GFP expressing nodular multicellular cultures (Fig. 2 middle left) and upon transfer to regeneration media many of these regenerated into GFP expressing shoot cultures (Fig. 2 bottom left). Mature ex-flasked plants exhibited GFP throughout the tissue, including in mature cloves (Fig. 2 top and bottom right).

Transformation efficiency

Three transformation experiments were undertaken with the model *mgfp-ER* construct. The results from this are presented in Table 2. However, it should be recognized that between experiments 1 and 2 it was noticed that slice thickness and blotting time on filter paper were important factors in transient GFP activity. Thicker slices had relatively fewer GFP expressing cells and material that was not blotted dry and retained liquid around it when placed onto the media was more susceptible to die during co-cultivation, and so in experiment 3, this was carefully monitored so that slices were <1.0 mm thick and each slice was blotted to remove excess *Agrobacterium* suspension before transfer to solid media.

Many subsequent transformation experiments (data unpublished) have been undertaken with constructs, ranging from simple (e.g. selectable marker) to complex (three or more genes) containing different traits, some of which may be detrimental to survival and regeneration. In these experiments it has been noted that construct, operator skill, and tissue condition all affect transgenic plant recovery. However, for simple constructs with traits that are not detrimental and easily selectable (e.g. herbicide tolerance), a similar frequency of transgenic plants has been recovered (26 lines from 42 cloves) to that reported in experiment 3 above. This confirms the reproducibility of this system in the hands of a skilled operator and that high garlic transformation frequencies are routinely possible using the above method.

Ex-flasking and growth in the glasshouse

All healthy plant material that was ex-flasked survived in the glasshouse and matured into healthy garlic plants that produced bulbs containing phenotypically normal (observation only) cloves.

PCR analysis

Ex-flasked garlic plants were analysed for the presence of the *mgfp-ER* transgene by using *gfp* primers (Fig. 3). All 55 lines tested were PCR-positive for the gene, indicating their transgenic status.

Southern analysis

Southern analysis of a representative sample of the transgenic lines clearly demonstrated that the transgenic events

Table 2 Transformation efficiency data from three garlic immature leaf transformation experiments

Experiment no.	No. cloves	No. leaf pieces	Transgenic lines recovered to the glasshouse	Transformed lines/leaf piece/clove (%)
1	29	571	5	0.9 (17.2)
2	16	662	3	0.5 (18.8)
3	24	1,143	47	4.1 (195.8)
Total	69	2,376	55	2.3 (79.7)

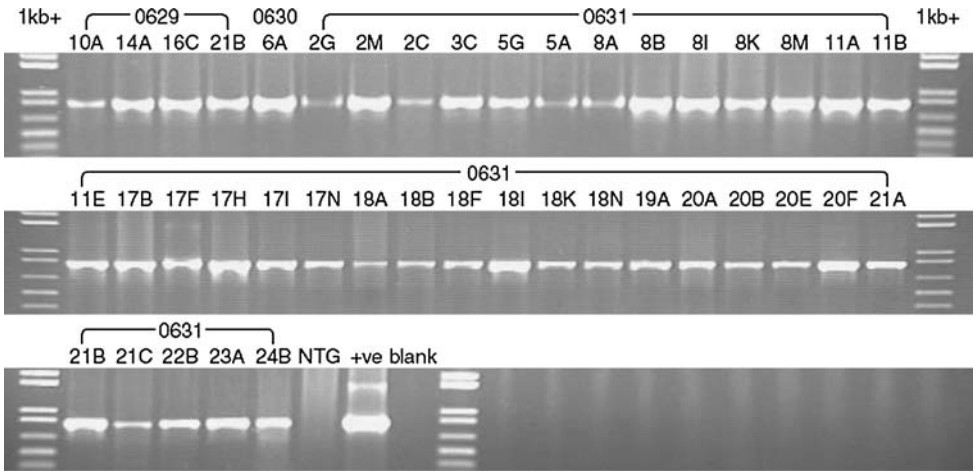

Fig. 3 Ethidium bromide stained electrophoretic gel of PCR-amplified DNA samples from transgenic garlic lines from three experiments (0629, 0630, and 0631). Amplified product represents a 834 bp fragment of the *m-gfpER* gene. 1Kb+ = DNA marker, NTG = non transgenic garlic DNA, +ve = positive plasmid control DNA, blank = PCR reagents only no DNA added

recovered to healthy ex-flasked plants in the glasshouse were derived from independent transformation events (Fig. 4). Interestingly, 9 of 39 events were single copy (lane 3,4,7,8,12,14,23,31,41), while 30 were multiple copy (42 is multiple copy at a single locus). This frequency of multiple (two or more) copies is higher than that observed in onion immature embryo transformations, which seem to routinely produce only single or double copy integration events (Eady et al. 2008, plus unpublished data). The high frequency of multiple integrations is more similar to that observed in potato and tobacco (Meiyalaghan et al. 2006; Subr et al. 2006). This may reflect the tissue source being transformed, as both tobacco and potato routinely use leaf discs and the garlic uses an immature leaf material, whilst the onion uses immature embryo material.

Nuclear DNA content

Analyses of leaf tissue from 10 transgenic lines and 3 control non-transgenic garlic plants (Fig. 5) showed that the transgenic material, when compared with a reference sample (*Bellis perennis*), was similar to the control. Sample:reference ratios for the control garlic ranged between 11.46 and 11.79, while that of the transgenic plants ranged from 11.07 to 11.53. This indicates that the nuclear content and thus ploidy level of the transgenic plants was not changed by transformation or passage through tissue culture. Phenotype observations supported this conclusion as the transgenic lines also appeared indistinguishable from control garlic plants.

Conclusions

The garlic immature leaf transformation system developed here is repeatable and workable for the conditions outlined above and as yet unpublished data would indicate that it also works with similar efficiencies with different selective agents and constructs. It is very efficient compared with other published allium transformation systems. The transformation system for onion, garlic and leek developed by Eady et al. (2000, 2005) works routinely but is inefficient, putting a huge resource limit on the amount of transformation research that can be undertaken. The maximum transformation frequency reported for onion was 2.7% of starting embryos in one experiment (Eady et al. 2000). However, the average figure, calculated after 8 years of experimentation on ~160,000 immature embryos is much lower at about 0.05%. The highly improved garlic transformation system reported here has produced garlic at a maximum frequency of 4.1% of leaf pieces or an average of 2.3%, approximately 50-fold higher than the average for onion immature embryos. In addition, the process is much easier, as isolating and processing approximately 800

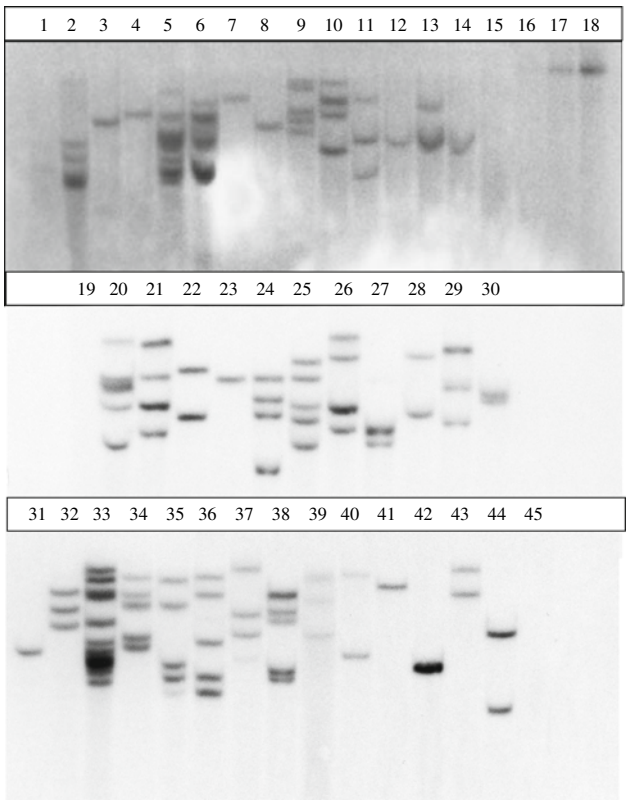

Fig. 4 Southern analyses of transgenic garlic plants probed with the *gfp* gene probe. *Lanes* 1–14 and 20–44 HindIII digested DNA from leaf material from different transgenic garlic lines. *Lanes* 15, 19, 45, non-transgenic control garlic leaf DNA. *Lanes* 16–18 plasmid controls approximately equivalent to 1, 2 and 5 copies of the transgene within a garlic genome

immature leaf pieces of garlic represents only 3–4 h of endeavor per experiment, while isolating and processing approximately 800 immature embryos requires about 8–10 h per experiment. This will allow garlic to become a model species for allium transformation research.

Transformation frequency data is an unreliable measure of the effort required to transform a plant. The measure does not include the time taken to obtain the transformation competent material, or post-transformation manipulations and selections, or the resources required. Further, the definition of starting material also affects the transformation frequency (e.g., 20 garlic cloves is equivalent to ~800 immature garlic leaf pieces, yet recovery of a single transformant when represented as a frequency from this material would appear as 5 or 0.125%, respectively). Kondo et al. (2000) are ambiguous in this way as the research claims 20 lines from 1,000 callus pieces, yet upon closer inspection not all recovered lines ex-flasked successfully and some were vitrified and presumably failed to survive ex-flasking. Only two lines were actually analyzed by Southern analysis. In addition, the effort required to maintain fresh totipotent callus is large and would likely result in a much greater risk of generating a somatic mutation than the direct regeneration protocol described here. The previously published garlic transformation system of Zheng et al. (2004), although reproducible, was still inefficient, and from that publication only five lines survived ex-flasking to the greenhouse. In our current immature onion embryo transformation protocol, a skilled operator can produce ~10–20 transgenic events in a flowering season, which is approximately 3 months in duration. It then takes a further 6 months to regenerate these to plants in the glasshouse. For the immature leaf garlic protocol described here, over 40 transgenic events were produced from cloves isolated from eight bulbs of garlic by one operator in just three experiments, or approximately 2 weeks work, followed by 6 months of subculture and selection. Thus the transformation effort required is now comparable to that required for model plants such as tobacco. The major barrier to more rapid transformation in garlic is now the time taken to regenerate and ex-flask. As garlic is a relatively slow-growing biennial it is unlikely that this characteristic can be altered. However, overall this system now makes feasible a rational iterative

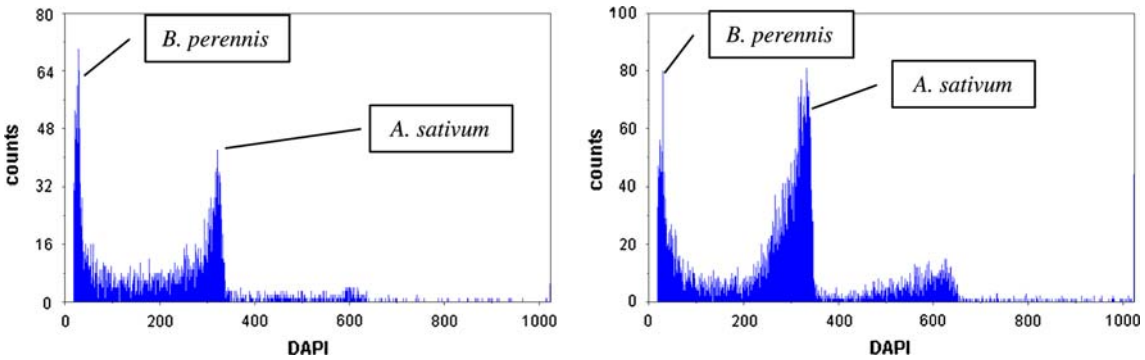

Fig. 5 Flow cytometry profiles of representative control garlic (*left* graph) peak 321, and transgenic garlic (*right* graph) peak 332 compared with an internal reference (*Belli perenniss*), peaks 28 and 30, respectively, to give sample to reference ratios of 11.46 and 11.07, respectively, indicating that the ploidy of both samples is the same

approach to the manipulation of traits in allium through the use of transgenics.

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