RESEARCH PAPER

GM Crops & Food: Biotechnology in Agriculture and the Food Chain 5:1, 27–35; January/February/March 2014; © 2014 Landes Bioscience

Improved regeneration and transformation protocols for three strawberry cultivars

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Keywords: agrobacterium, direct organogenesis, Fragaria sp., plant transformation, strawberry

Strawberry (*Fragaria* × *ananassa*) is an economically important soft fruit crop with polyploid genome, which makes the breeding of new cultivars difficult. Simple and efficient methods for transformation and regeneration are required for cultivars improvement in strawberries. In the present study, adventitious shoot regeneration has been investigated in three cultivated strawberry plants, i.e., Festival, Sweet Charlie, and Florida via direct organogenesis using the in vitro juvenile leaves as explants. Explants were collected after sub-culturing on a propagation medium composed of MS supplemented with 0.5 mg/l BA; 0.1 mg/l GA3 and 0.1 mg/l IBA. To select the suitable organogenesis, the explants of the three cultivars were cultured on MS medium supplemented with different concentrations of TDZ (1, 2, 3, and 4 mg/l), then incubated at a temperature of 22 °C ± 2. Medium containing 2 mg/l TDZ revealed the best regeneration efficiency with the three cultivars (72% for Festival and 73% for Sweet Charlie and Florida). After 4 weeks the produced shoots were cultured on MS medium with different concentrations of BA and Kin to enhance shoot elongation. Results showed that the medium containing 1.5 mg/l BA and 0.5 mg/l Kin revealed highest elongation efficiency (88% and 94%) for Festival and Sweet Charlie, respectively. On the other hand, medium media containing 1.5 mg/l BA and 0.1 mg/l Kin showed highest elongation efficiency (90%) in Florida. Elongated shoots were successfully rooted on MS medium containing 1.5 mg/l NAA. Furthermore, transformation of the two cultivars, Festival and Sweet Charlie, has been established via *Agrobacterium* strain LBA44404 containing the plasmid pISV2678 with *gus*-intron and *bar* genes. Three days post-co-cultivation, GUS activity was screened using the histochemical assay. The results showed 16% and 18% of the tested plant materials had changed into a blue color for Festival and Sweet Charlie, respectively. Out of 120 explants only 13 shoots were developed on bialaphos medium for each cultivar, representing 10.8% bialaphos-resistant strawberry shoot. The presence of both the genes *bar* and *uid* A was detected by PCR and Northern, giving a transformation efficiency of 5%.

Introduction

The cultivated strawberry (*Fragaria* × *ananassa* Duch.), a member of the *Rosaceae*, is an economically important berry crop with high demand for fresh fruits and from the processing industry.[1] The global strawberry production is nearly 4.1 million metric tons from almost 255 thousand hectares (FAO 2008). Losses in Strawberry production are caused by several factors that involve a complex interaction between abiotic factors (temperature, soil type, and moisture) and pathogenic infections.

The handiness of regeneration systems, coupled with the ability of infection by *Agrobacterium* species,[2] makes the strawberry well suited for *Agrobacterium*-mediated genetic transformation. This allows introduction of a novel gene(s) with new desired traits in strawberry plants via *Agrobacterium*-mediated strategy.

The application of in vitro techniques and genetic engineering could be implemented to overcome the limited potential of strawberry improvement through traditional breeding methods.[3] Efficient in vitro shoot proliferation and regeneration systems used in conjunction with classical breeding methods could accelerate cultivar development programs.[4,5] Several developments of the in vitro techniques are important tools for modern plant improvement programs that introduce new traits into selected plants, clonal multiplication, germplasm improvement, and gene conservation of this flavorful and nutritious berry crop, which may develop suitable cultivars in the minimum time.[6,7] Plant regeneration is a crucial aspect of plant biotechnology and tissue culture techniques that facilitate the production of genetically engineered plants, somaclonal variants, and the rapid multiplication of difficult-to-propagate species.[5,7]

Establishment of an efficient regeneration system for each strawberry cultivar is an essential pre-requisite for the successful development of transgenic plants. Adventitious buds and shoots regeneration has been previously reported from different explants such as: leaves,[8-16] petioles,[11,12,15,17,18] peduncles/peduncular base of the flower buds,[17,18] stems,[19] stipules,[11,20] stolon,[18] roots,[11,20] runners,[9] and anther cultures.[21] Furthermore, shoot regeneration

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Submitted: 06/10/2013; Revised: 11/14/2013; Accepted: 11/15/2013; Published Online: 11/08/2013
http://dx.doi.org/10.4161/gmcr.27229

was produced directly from field,[10] greenhouse-grown strawberry plants,[12,15,22] or from in vitro-grown shoots.[1,11]

Transformation in strawberries has been previously documented using different constructs in various genotypes.[23-27] Strawberry genetic transformation has made outstanding progress. The transformation of *Fragaria* was focused on establishing the system and using genes with potential for pest and herbicide resistance, cold tolerance, and ripening.[22,28-36] The aim of this study was to develop an efficient and reproducible *Agrobacterium*-mediated protocol for the strawberry cultivars using the juvenile leaf explants.

Results and Discussion

Optimization of strawberry regeneration

Plant regeneration from adventitious strawberry explants are accomplished through: (1) formation of viable adventitious shoots from the explant, (2) elongation of the shoots, and (3) rooting of the shoots to form whole plants.

Adventitious shoots formation

Pieces from fully juvenile leaves and petioles of 3–4 wk-old from in vitro cultures were used as explants. It was reported that the explants taken from field-grown plants are difficult to sterilize in order to establish in vitro cultures due to high degree of contamination. Thus, it is usually recommended to take explants from plants grown under controlled conditions such as a growth room or greenhouse or from buds that flush from dormant shoots stored indoors.[7,37]

To evaluate the best media for the shoot formation, the juvenile leaf explants of the three cultivars (Festival, Sweet Charlie, and Florida) were cultured on MS medium with TDZ at concentrations of 1, 2, and 3 mg/l, individually. Cultures were incubated at dark for a week, then transferred to light for at least three weeks. It was observed that shoots started to develop 10 d post-culturing. Four weeks later the produced shoots were ready for transferring into the elongation medium. Medium containing 2 mg/l TDZ produced the highest regeneration efficiency among the three cultivars. Sweet Charlie recorded the highest percentage of regeneration response among the three cultivars (73.3%) followed by 71.6% for Festival and Florida (**Table 1**; **Fig. 1**). In addition, juvenile leaves of Sweet Charlie reached the highest number of shoots per explant (4 shoots /explant) compared with other cultivars under analysis. These results are in consensus with previous reports,[38,39] indicating that chemical composition of the media is one of the factors determining the success of in vitro. In addition, selection of the proper hormones is the key to regeneration success in strawberries.[40-43]

Organogenesis relies on the inherent plasticity of plant tissues and is regulated by altering the components of the medium. It is well known that to induce shoot formation we need to increase the cytokinin to auxin ratio of the culture medium. Thidiazuron (TDZ), with its cytokinin- and auxin-like effects, was used as a substitute to phenylurea (Nphenyl- N'-1,2,3-thidiazol-5-ylurea and is now among the most active cytokinin-like substances for plant tissue cultures; it has been used to induce shoot organogenesis of strawberries.[12] Using TDZ alone[12] or in combination with 2,4-dichlorophenoxy-acetic acid (2,4-D)[11] or (indol-butyric acid) IBA[14] was found to be effective in strawberry shoot regeneration when leaves are the explants.

Incubation in dark is well-known[44] to solve the problem of the browning of the surface of plant tissues of in vitro cultured strawberry explants. Browning is produced from the oxidation of phenolic compounds that produce the quinones that react with plant tissues leading to poisonous tissue. Therefore, dark incubation is recommended for reducing the tissue browning by inhibiting enzymatic activity that is responsible for tissue oxidation.[45,46] In strawberries, dark incubation of leaf explants for at least a week is required to enhance organogenesis in strawberry explants.[9,20,22,47-51]

Elongation of the shoots

After shoot development, produced shoots in cluster were transferred to different elongated media and incubated for 4 wks. The most efficient elongation medium was selected upon reaching shoots height to approximately 1 cm. Results showed that medium SE6 containing 1.5 mg/l 6-benzyladenine (BA) and 0.5 mg/l kinetin (Kin) was the best for elongated shoots in both Festival and Sweet Charlie, while SE5 containing 1.5 mg/l BA and 0.1 mg/l Kin showed the best elongation in Florida (**Table 2**; **Fig. 2**). It was known that cytokinins including BA and kinetin play an integral role in controlling the adventitious shoot formation, promote cell division, and leaf cell enlargement that stimulates leaf expansion. Our results indicate that the BA with a concentration of 1.5 mg/l, combined with low concentration of kin (0.1 and 0.5 mg/l), have a positive impact in elongation and proliferation of strawberry plants. It was previously reported that the BA was comparatively more effective than Kin in proliferation of shoots. Similarly, Debnath[52] and Haddaidi et al.[53] reported that high concentration of cytokinin with no or low auxin concentration promotes the branching of lateral buds from leaf axis. On the other hand, in contradiction with our results, Biswas et al.[54] reported that low concentration (0.1 mg/l) of BA is more effective for mass propagation in the studied strawberries. Debnath[15] reported that the success in shoots proliferation and roots development was observed when explants were cultured in medium supplemented with zeatin in cultivar Bounty.

Rooting stage of the produced shoots

In order to study root formation, the elongated shoots were transferred to different rooting media. Roots started to appear after about 10 d and completed formation during one month. It was shown that the in vitro-formed roots were thick, possessed no hairy roots, and grew horizontally. It was observed that the number of the rooted shoots increased when the concentration of IBA and NAA was increased. Additionally, the highest concentration of either IBA or NAA, which is 1.5 mg/l, revealed the highest percentage of forming roots. Furthermore, IBA revealed higher response than NAA. Among the three cultivars, Festival showed the highest response (**Table 3**; **Fig. 3**).

In previous articles it was recommended to use a single step for shoot multiplication and rooting ,which takes place in the same culture medium.[15,50] Different media composed of MS with or without auxins that have been used for shoot proliferation in strawberry produced roots at the same time.[55]

Table1. Effect of different TDZ concentration on adventitious shoot regeneration development of the three strawberry cultivars (Festival, Sweet Charlie, and Florida)

TDZ Concentration (mg/L)	Total number of explants	Festival	Sweet Charlie	Florida
		% of shoots		
1	R1 = 100	13	20	10
	R2 = 100	12	19	13
	R3 = 100	11	19	15
	Mean: 100	12	19.3	12.7
2	R1 = 100	72	74	70
	R2 = 100	71	73	72
	R3 = 100	72	73	73
	Mean: 100	71.6	73.3	71.6
3	R1 = 100	39	48	25
	R2 = 100	38	45	23
	R3 = 100	37	45	22
	Mean: 100	38	46	23.3

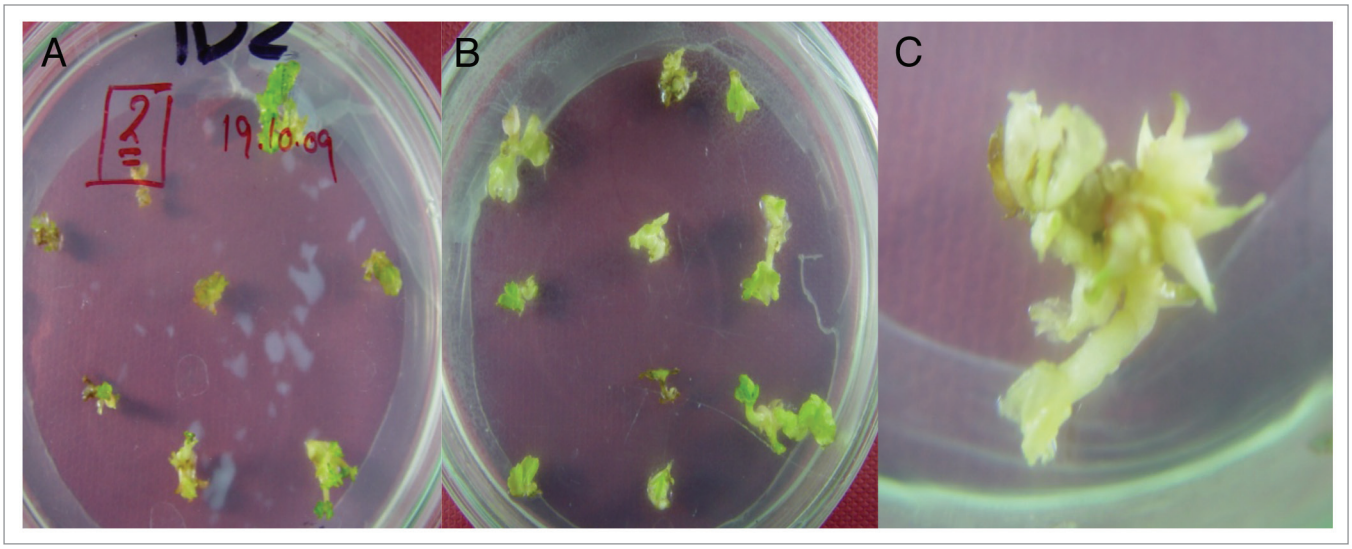

Figure 1. Direct shoots regeneration from the juvenile leaf explants after, (**A**) 10 d, (**B**) 3 wk, and (**C**) 4 wk.

Auxin-free half-strength MS medium[55] with activated charcoal at a concentration of 0.6 g/l and IAA at a concentration of 1 mg/l have been also used.[56] Recent studies using IBA proved superior to (1-Naphthaleneacetic acid (NAA) in-root formation frequency. Positive effects of IBA in root formation were also reported in the cases of Centellasiatica[57] and Acacia sinuate.[58] On the other hand, Ying et al.[59] reported that roots were successfully formed at low concentrations of NAA (0.02mg/l). Zeatin is also recommended as a suitable hormone for root development.[16]

Plant transformation

After optimizing in vitro regeneration of strawberry cultivars, two of them (Sweet Charlie and Festival) were subsequently used for establishing *Agrobacterium*-mediated transformation. Genetic transformation via *Agrobacterium* with different constructs in various strawberry genotypes has been previously reported.[23-27,60]

In the present study, *Agrobacterium*-mediated transformation of strawberry plants has been established using the juvenile leaf as explant, and the plasmid pISV2678 harbors the *gus*-intron and *bar* genes. Transformation in strawberries has been performed in two independent experiments with a total of 120 explants for each cultivar (Sweet Charlie and Festival). Explants were soaked O/N *Agrobacterium* suspension for 5 and 10 min, co-cultivated for three days on selected regeneration medium, and then transferred onto the selective medium (regeneration medium with 1.5 mg/l bialaphos and 300 mg/l carbincillin). It was observed that explants that were immersed in the *Agrobacterium* culture for 10 min were more efficient for regeneration than 5 min (data not shown). After about 15 d, 22 out of 120 explants of Festival and 27 out of 120 explants of cultivar Sweet Charlie were bialaphos resistant. Six weeks later, the bialaphos resistant

Table 2. Effect of the different media composition on strawberry shoots elongation

Media	Festival		Sweet Sherry		Florida	
	Elongated shoots	%	Elongated shoots	%	Elongated shoots	%
SE1	24	48	20	40	19	38
SE2	29	58	23	46	25	50
SE3	32	64	31	62	34	68
SE4	35	70	37	74	33	66
SE5	39	78	42	84	45	90
SE6	44	88	47	94	42	84
SE7	36	72	31	62	33	66
SE8	25	50	27	54	23	46

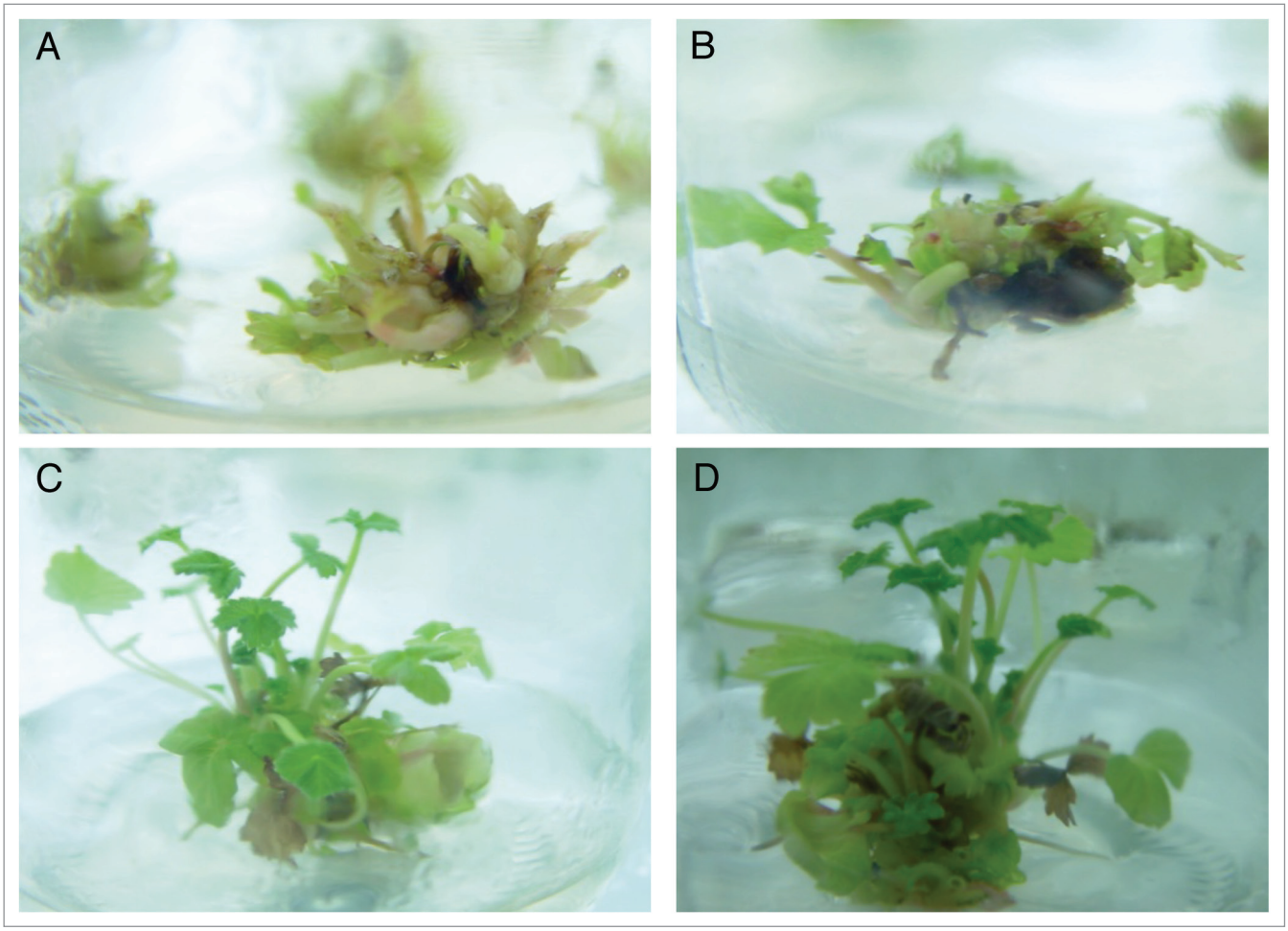

Figure 2. Elongation stage at (**A**) zero incubation time, (**B**) 1 wk, (**C**) 3 wk, and (**D**) 4 wk.

explants produced only 13 shoots developed from each cultivar. Shoots were subsequently transferred to the elongation medium with 300 mg/l carbincillin for 4 wk and then transferred to the rooting medium for at least 4 wk (**Table 4**).

Rapid shoot regeneration is requested to minimize the risks that are associated with strawberry transformation, including escapes, formation of chimeric shoots, and somaclonal variation.[7,61] In this work the regeneration period took only 12–14 wk, which is less than the previously reported protocols of approximately 2 wks.[60]

GUS activity screening was performed three days after co-cultivation using the histochemical assay in treated plant

Table 3. The percentage of rooted shoots on different auxins concentrations after 4 wk

Hormone	Concentration (mg/l)	Festival		Sweet Charlie		Florida	
		No of rooted shoots	%	No of rooted shoots	%	No of rooted shoots	%
IBA	0.5	18	60	17	56	15	50
	1.0	23	77	22	73	22	37
	1.5	27	90	26	86	24	80
NAA	0.5	13	43	15	50	17	56
	1.0	21	70	17	56	15	50
	1.5	24	80	19	63	21	70

Figure 3. Rooted strawberry shoot before and after incubation for 4 wk on rooting medium.

leaves. Results showed that 16% and 18% of the tested plant materials were stained with blue color for Festival and Sweet Charlie, respectively (**Fig. 4**).

The strawberry plants with positive expression of GUS were further confirmed for transgene (*bar* and *gus* genes) integration by PCR. The expected 540 bp and 2070 bp bands corresponding to *bar* and *uid* A genes, respectively, were detected in 6 plants out of 13 putatively transgenic shoots (representing 46% transgenic plants) with Festival and Sweet Charlie (**Fig. 5A1 and 5A2**). In order to make clear whether there is any relation between the level of expression of *gus* genes and the level of transcription, Northern hybridization was done with GUS-positive plants and transcription levels, as can be seen in **Figure 5B**.

The transformation efficiency was calculated as the number of bialaphos resistant strawberry plants developed on separate shoots with respect to the total number of explants infected, which was 10.8% for both cultivars. However, the transformation efficiency based on the PCR results with respect to a total number of infected explants recorded 5%. Our obtained protocol for strawberry transformation via *Agrobacterium* is in the same consensus as previous transformation protocols with different cultivars where the transformation ratio ranged between 4–11%. A transformation frequency of 0.95% has been reported for the cultivar "Rapella"[62] and 6.5% for "Red Coat"[30,31] and an efficiency of 4.22% and 10% with cultivar Chandeler[22,60] and 11% for "Firework."[63]

Materials and Methods

Plant materials

Strawberry cultivars Festival, Sweet Charlie, and Florida were used in this study; these cultivars were kindly provided as in vitro cultures from Agricultural Modern Company (PICO). All cultures were incubated at 22 °C ± 2 °C with a 16 h photoperiod under white fluorescent lamps or in dark in a growth room.

Bacteria and plasmid

The *Agrobacterium tumefaciens* strain LBA4404 that harbors the plasmid pISV2678 with the *gus*-intron under the control of 35S promoter and *nos* terminator, as well as the *bar*, fused the AMV Leader gene under the control of *nos* promoter; pAg7 terminator[64] was used for optimizing the transformation system in strawberry via *Agrobacterium*-mediated transformation.[65] The plasmid was kindly provided by Dr P Ratet, Institut des Sciences Vegetales (ISV), Centre National de la Recherche Scientifique (CNRS), Gif-Sur-Yvette, France.

Table 4. Transformation efficiency of juvenile leave explants of two strawberry cultivars (Festival and Sweet Charlie)

Festival			Sweet Charlie		
Total No. of explants	No. of survived explants	Produced shoots	Total No. of explants	No. of survived explants	Produced shoots
60	10	5	60	12	7
60	12	8	60	15	6
Total (120)	22	13	Total (120)	27	13
% of survived explants	18.3		% of survived explants	22.5	
% of produced shoots	10.8		% of produced shoots	10.8	

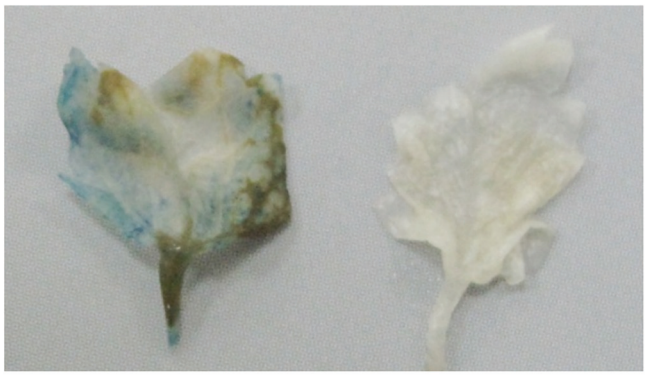

Figure 4. GUS assay of treated leaf explants (left) and control strawberry leaves (right).

Table 5. Composition of different elongation media

Media	Hormone concentration	
	BA (mg/l)	Kin (mg/l)
SE1	0.5	0.1
SE2	0.5	0.5
SE3	1.0	0.1
SE4	1.0	0.5
SE5	1.5	0.1
SE6	1.5	0.5
SE7	2.0	0.1
SE8	2.0	0.5

Plant regeneration

Regeneration systems of strawberries has been developed using in vitro juvenile leaves as explants. Explants were collected for the three cultivars, i.e., Festival, Sweet Charlie, and Florida, from the in vitro cultures on propagation medium, which was composed of MS basal medium (Murashige and Skoog,[66] supplemented with 0.5 mg/l BA; 0.1 mg/l gibberelic acid [GA3] and 0.1 mg/l IBA as described by Boxus[67] and Litwinczuk[42]).

In order to optimize the proper regeneration medium, the explants were cultured on solid MS medium supplemented with different concentrations (1, 2, 3, and 4 mg/l) of Thidiazuron (TDZ). The cultures were incubated in the growth chamber under dark for one week and then transferred to light. This experiment was repeated three times, and each time there were 100 explants. Data were analyzed based upon the percentage of means.

Subsequently, the 4-wk-old shoots produced in clusters from the previous stage were transferred to the medium for elongating and proliferating. To select the best elongation medium, different concentrations and combinations of the hormones BA and Kin were tested (**Table 5**). Data were analyzed based upon the percentage of means.

Four weeks later in the elongation stage, shoot heights of approximately 1 cm were excised and transferred to different rooting media based on MS basal medium with naphthalene acetic acid (NAA) and IBA in different concentrations (0.5, 0.1, and 1.5 mg/l, individually) and incubated for 3 wk. Each treatment had a total number of 30 shoots. Data were analyzed based upon the percentage of means.

Transformation system of strawberry

Primary cultures of *Agrobacterium* were prepared by inoculating a single colony from a freshly streaked plate in 5 ml of autoclaved liquid YEP medium supplemented with 25 mg/l streptomycin, 10 mg/l rifampicin, and 50 mg/l kanamycin. The culture was incubated overnight in the dark at 28 °C. Subculture was performed in a 100 ml YEP medium supplemented with the same antibiotics grown under similar conditions. Once the OD_{600} reached 0.8, *Agrobacterium* cells were pelleted by centrifugation and were re-suspended in MS re-suspension medium containing 150 μM acetosyringone to adjust the O.D.600 of the bacterial suspension to ~0.3. In vitro juvenile leaf explants of the two cultivars (Festival and Sweet Charlie) were soaked in the *Agrobacterium* culture for 5 and 10 min. This was performed through two replicates, and there were 60 explants in each replicate for a total of 120 explants. The treated explants were cultured onto selected regeneration medium for 3 d. Subsequently, they were transferred to the selection medium (regeneration medium supplemented with 1.5 mg/L bialaphos and 300 mg/L carbinicillin) and incubated for 2–3 wk. Developed shoots were excised from their original explants and then transferred to the root formation medium. Plantlets were then acclimatized in the greenhouse.

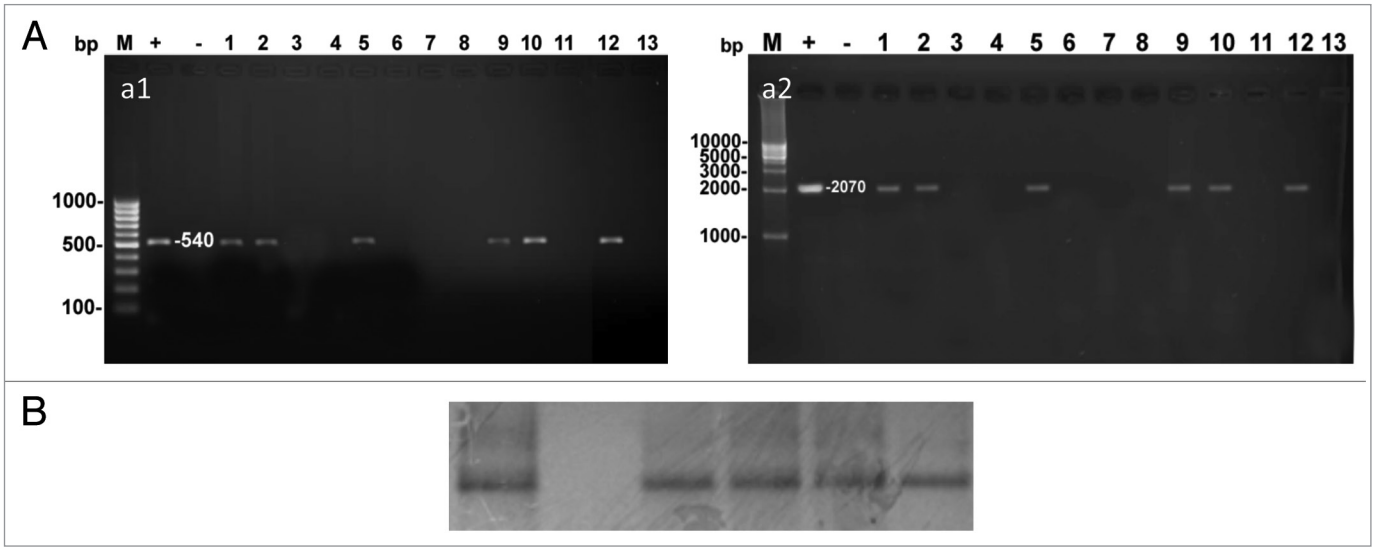

Figure 5. (**A**) PCR analysis of putative transgenic strawberry plants (Lanes 1–13) using, (**A1**) *bar*-specific primers amplifying 540 bp and (**A2**) *gus*-specific primers amplifying 2070 bp. (**B**) Northern blot analysis to detect the presence of *gus* transcription in transformed strawberry plants.

Molecular confirmation of putative transgenic plants

Histochemical GUS assay

GUS expression was visually determined using the histochemical assay according to Jefferson et al.[68] The GUS reaction was performed three days after transformation by incubating the samples with GUS buffer solution containing 1 mM X-gluc (5-bromo-4-choloro-3-indolyl-β-glucuronic acid) overnight at 37 °C. The blue color was detected visually by naked eye and using a light microscope after bleaching the chlorophyll and fixation in 70% ethanol.

PCR

To confirm the presence of transgenes in bialaphos-resistant plants, these plants, along with wild type (WT) lines, were analyzed through PCR analysis. Total genomic DNA from various independent young leaves of putative transgenic plants was extracted as described by Dellaporta et al.[69] and was used for PCR using the *gus*-specific primers (forward 5′ AACAATGCGC GGTGGTCAGT CCC 3′ and reverse 5′ ATTCATTGTT TGCCTCCCTG CTGC 3′), designed to amplify the full *gus*-intron of 2070 bp, and the *bar* specific primers (forward 5′ CCACCATGAG CCCAGAACGACG 3′ and reverse 5′ TCAGATCTCG GTGACGG 3′), designed to amplify 540 bp. The PCR products were analyzed on 1% agarose gel containing ethidium bromide and visualized under a gel documentation unit.

The PCR reactions were performed in a total volume of 25 µl, containing 1 µl DNA, 20 pmol of each primer, 200 µM of each dNTP, 0.5 unit *Taq* DNA polymerase, and 3 µl 10× PCR buffer. The PCR temperature profile was as follows: initial denaturation of DNA at 94 °C for 5 min, 35 cycles comprised of 1 min denaturation at 94 °C, 1 min annealing at 55 °C, extension for 2 min at 72 °C, and a final extension step at 72 °C for 7 min. Amplification products were analyzed by electrophoresis on 1% agarose gels and detected by staining with ethidium bromide.

Northern hybridization of transgenic strawberry plants

Total RNA was isolated from various transgenic plants using a modification of a CTAB-based method and was separated with electrophoresis in 1.2% denaturing gel, then transferred to Hybond nylon membrane for Northern hybridization.[70]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Thank you to the Science and Technology Development Fund (STDF) for funding this investigation under a project entitled "Developing strawberry plants resistant to whitefly transmitted geminivirus using siRNA strategy."

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