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Studies on gene transfer of shoot apical meristems by *Agrobacterium*-mediated genetic transformation in a progeny of Chinese wild *Vitis pseudoreticulata*

Xin Guan[*),1),2),3)], Heqing Zhao[*),1),2),3)], Yan Xu[1),2),3)] and Yuejin Wang[1),2),3)]

[1)] College of Horticulture, Northwest A&F University, Yangling, Shaanxi, China
[2)] Key Laboratory of Horticultural Plant Biology and Germplasm Innovation in Northwest China, Ministry of Agriculture, Yangling, Shaanxi, China
[3)] State Key Laboratory of Crop Stress Biology in Arid Areas, Northwest A&F University, Yangling, Shaanxi, China

Summary

Shoot tips and/or single-bud-internodes, containing shoot apical meristems (SAM) were presented, of *Vitis* '6-12-2', derived from a Chinese wild *V. pseudoreticulata* 'Baihe-35-1' × *V. vinifera* 'Carignane' cross, were used for *Agrobacterium*-mediated genetic transformation. In order to achieve Glyoxal oxidase (VpGLOX) over-expressing plants, the propagation and gene transfer system of shoot tips and/or single-bud-internodes undergoing either micropropagated (starting with micro-shoot-tips) or callus induced (starting with stems with single bud) *in vitro* plants were optimized. The results show that the most effective way to gain shoot tips and/or single-bud-internodes undergoing micropropagation procedure was to keep micro-shoot-tips in liquid C_2D4B medium at 80 rpm constant orbital shaking with light, then placed on solidified C_2D4B medium with 2.9 µM Gibberellic acid 3 (GA_3) for elongation. *In vitro* stems with single buds gave best results for callus formation and adventitious buds induction on half-strength MS medium with 9.0 µM Thidiazuron (TDZ) and 2.9 µM mg·L^{-1} α-Naphthaleneacetic acid (NAA). The highest gene transfer frequency was obtained when explants were infected for 10 min with the concentration of *Agrobacterium tumefaciens* with an optical density at 600 nm of 0.4, and then co-cultivated for 3 days. Incubation of shoot tips and/or single-bud-internodes in darkness for 3 days is helpful for enhancing gene transfer efficiency. Polymerase chain reaction (PCR) and PCR-Southern blot analyses were utilized to confirm putative transgenic plants. Up to 45 clones have proven to be transformed, and one of them has been planted out. This method opens a door for the gene transfer of recalcitrant Chinese wild *V. pseudoreticulata*.

Key words: Grapevine; Gene transfer; Shoot apical meristems; *Glyoxal oxidase*.

Introduction

Transgenic grapevines were obtained by *Agrobacterium*-mediated genetic transformation originating from different organs *via* organogenesis or via somatic embryogenesis since 1990 (Colby *et al.* 1990, Mullins *et al*. 1990, Martinelli *et al.* 1993, Gambino *et al.* 2007, Fan *et al.* 2008). However, it is still a hard approach for some *Vitis* species, respectively varieties and accessions that are recalcitrant to regeneration. For instance, Chinese wild *Vitis pseudoreticulata* representing a valuable genetic resource for grapevine disease resistance breeding is widely studied for some of their resistance genes in our laboratory (Xu *et al*. 2011, Li *et al*. 2010, He *et al*. 2012); meanwhile many attempts have been made to establish a system for somatic embryo gene transfer and regeneration. However no transgenic plants or even regenerated leaf-like structures could be obtained so far after agrobacteria inoculation. At present, the best advance is an optimized regeneration system from anthers of Chinese wild *Vitis pseudoreticulata* accession Guangxi-2 (Zhi *et al*. 2010).

Shoot apical meristems (SAM) could be attractive for gene transfer due to their potential ability for regeneration. The activity of the pluripotent stem cell in the SAM is dynamically controlled by complex, overlapping signaling networks that include the feedback regulation of meristem maintenance genes and the signaling of plant hormones (Ha *et al.* 2010). Findings obtained from *in vitro* or *in situ* suggest that cells of the shoot apical meristem/tunica (smaller fragments of leaf primordial, but not larger fragments of meristem itself) have the ability to: 1) produce leaf-like structures regenerated directly from primoridial cells, without the need for dedifferentiation and callus formation (Barlass and Skene 1978, Barlass and Skene 1980), and 2) give rise to adventitious buds on transfer to solid medium (Dutt *et al.* 2007) or develop to differentiated cell types (Sussex 1952). The gene transfer and regeneration efficiency could be enhanced with SAM as target material. The former reports of using SAM for gene transfer and providing technical details were successfully conducted in *Vitis vinifera* 'Silcora' by Mezzetti *et al.* (2002), in 'Thompson Seedless' by Mezzetti *et al.* (2002) and Dutt *et al.* (2007). Besides, transient expression was obtained with other *V. vinifera* and *V. rotundifolia* varieties by Dutt *et al.* (2007).

It needs to be mentioned that, unlike 'Thompson Seedless' which has been routinely transformed *via* somatic embryogenesis, from Chinese wild *Vitis* no transgenic plants have been obtained via somatic embryogenesis so far. Concerning the gene transfer efficiency, the reason apart from

Correspondence to: Dr. Yuejin Wang, College of Horticulture, Northwest A&F University, Yangling, Shaanxi 712100, China. Fax: +86-29-8708-2803. E-mail: wangyj@nwsuaf.edu.cn

[*)] Xin Guan and Heqing Zhao contributed equally to the experimental part of this work.

the numerous well studied aspects (e.g. modified binary vectors, suitable *Agrobacterium* strains, transgene selection techniques, gene targeting and homologous recombination) probably depends on the host, for instance, proteins involved in the initial bacterium-host contact, nuclear import of the T-complex and intranuclear transport, uncoating, and integration (for review see TZFIRA and CITOVSKY 2006). Studies focused on genetic manipulation of the host genome and protein gene transfer processes in recalcitrant species successfully improved gene transfer efficiency (MYSORE *et al*. 2000, TZFIRA *et al*. 2001). Concerning the regeneration potential between different wild species, again, besides the widely studied aspects (genotype, explant type, environment and culture medium) (LÓPEZ-PÉREZ *et al*. 2005), somaclonal variation can play a role as it was determined (SCHELLENBAUM *et al*. 2008). There is growing evidence that the existence of sophisticated regulation on the molecular level during somatic embryogenesis differs between species. Therefore gene transfer occuring in meristematic tissue provided by explants as shoot tips and/or single-bud-internodes from this Chinese wild *Vitis* species was tested as an alternative strategy from the somatic embryogenesis.

In our previous work, the sequence of the *Vitis pseudoreticulata glyoxal oxidase gene* (*VpGLOX,* GenBank accession no. DQ201181) was isolated from the Chinese wild *Vitis pseudoreticulata* W. T. Wang accession 'Baihe-35-1' during a screen for genes being up-regulated in response to infection with *Erysiphe necator*, the causal agent of grapevine Powdery Mildew (PM) (WANG, 2004). *Vp*GLOX was transiently overexpressed in leaves of '6-12-2' and some accessions of *Vitis pseudoreticulata*. After inoculation with *E. necator*, only sparse growth of hyphae on leaves with transient *Vp*GLOX-overexpression was observed compared to the profuse fungal growth on control leaves. Real-time PCR and Western blot were utilized to test the transcriptional and translational expression levels. Moreover we found this gene to be involved in H_2O_2-production mediating resistance to *E. necator* (GUAN *et al.* 2011, ZHAO *et al*. 2012).

In the present study, aiming to gain abundant SAM, the methods of propagation starting from micro-shoot-tips through micropropagation (CHEE *et al*. 1984) and from stems with single bud by callus induction were optimized, which were then used as target explants for gene transfer to study several parameters influencing the transgenic efficiency. The goal of this work was to develop a SAM-based protocol on Chinese wild *Vitis pseudoreticulata* that could be also widely adoptable for different grapevine cultivars resp. species.

Material and Methods

Plant material: This study use an accession belonging to a segregating population derived from a 'Baihe-35-1' × *V. vinifera* 'Carignane' cross, referred to as '6-12-2' which was employed in our laboratory to study the genetic basis of the resistance to grapevine diseases. It was maintained in the grape germplasm resources orchard of Northwest A&F University, located in Yangling, Shaanxi, the People's Republic of China.

Plasmid construct: The construct *pWR306-VpGLOX* was produced in our laboratory (XU *et al*. 2005, SUN and WANG 2006). *VpGLOX* was driven by an enhanced cauliflower mosaic virus 35S promoter in combination with a 5'-untranslated leader sequence from tobacco mosaic virus Omega (designated as ED35S/Ω). The gene *htpII* coding for hygromycin phosphotransferase II (HPT II) was used as selectable marker and *mgfp5*' (ZERNICKA-GOETZ *et al*. 1996) was used as reporter gene for green fluorescent protein expression studies. This construct was then transferred in to *A. tumefaciens* strain GV3101.

Propagation and rooting of micro-shoot-tips and stems with bud

Micro-shoot-tips (approximately 3 mm in length) were dissected from *in vitro* plants and placed, cut surface down, on solid C_2D medium (CHEE *et al.* 1984) containing 1 $g \cdot L^{-1}$ activated charcoal (AC), 4 µM benzyladenine (BA), C_2D4B (DUTT *et al.*, 2007). One week later, 1 cm on top of the explants were excised, and cultured in liquid C_2D4B for three weeks with constant orbital shaking (HWY-100C, Shanghai, China) at 80 or 120 rpm respectively, in order to form bud clusters.

Shoot-tips and nodes dissected from these bud clusters were transferred to half-strength MS solid medium (MURASHIGE and SKOOG 1962) supplemented with 9.0 µM Thidiazuron (TDZ) and 2.9 µM α-Naphthaleneacetic acid (NAA) (GUAN, 2010). Two parameters, variation of light intensity (36 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 4 $\mu mol \cdot m^{-2} \cdot s^{-1}$), and different Gibberellic acid (GA_3) concentrations in the medium (0, 1.5, 2.9 and 5.8 µM) were tested. Non-etiolated shoot-tips and nodes were harvested after two weeks, and subcultured in solid C_2D4B under 36 $\mu mol \cdot m^{-2} \cdot s^{-1}$ white light. Gene transfer was performed 1 week later.

In parallel to shoot tips/internodes via micropropagation, stems with single buds were maintained on MS medium containing AC (1 $g \cdot L^{-1}$), TDZ (4.5, 9.0, 13.5 or 18.0 µM), and NAA (0.29, 0.58, 0.87 or 1.74 µM) for callus formation (indirect organogenesis, KURMI *et al*., 2011) and/or adventitious buds induction (due to its potential in the L2 layer cells, via auxiliary bud proliferation, MEZZETTI *et al*. 2002, CADAVID-LABRADA *et al*. 2009). The weight of healthy shoot-tips/internodes was measured after four weeks of culture. Three explants were placed per Erlenmeyer flask, 20 Erlenmeyer flasks for each treatment, three independent experiments were conducted.

The media in this study, if not otherwise indicated, were supplemented with 6.5 $g \cdot L^{-1}$ plant agar (Walson, Japan), 30 $g \cdot L^{-1}$ sucrose, to the final pH of medium 5.8. During selection the plantlets were kept at 25 °C under a light regime of 16 h light/8 h darkness using cool white fluorescent light (36 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

Agrobacterium-mediated genetic transformation: Shoot tips/internodes originated from micro-shoot-tips by micropropagation and from stems with single bud by callus induction were further dissected into 8 mm in size from tip-tops, then pre-cultured for 3 d with

stem side down on C_2D solid medium supplemented with 0.5 $g \cdot L^{-1}$ casein hydrolysate (CH), 1 $g \cdot L^{-1}$ polyvinylpyrrolidone (PVP) at a low light intensity (4 $\mu mol \cdot m^{-2} \cdot s^{-1}$). *Agrobacterium* suspensions, induction and dilution buffer were prepared as previously described (Guan *et al.* 2011). Shoot-tips/internodes wounded by blade-cutting were immersed in 20 ml *Agrobacterium* suspensions with optical densities at 600 nm of 0, 0.4, 0.6, 0.8 and 1.2 for 10 to 30 min, and then dried on sterile filter paper. After co-cultivation for 3 d in darkness on solid C_2D4B containing 20 $\mu mol \cdot L^{-1}$ Acetosyringone (AS), GFP-fluorescence was monitored with an epifluorescence stereomicroscope (BX51+DP70). Each treatment was repeated three times, with 20 tissue samples for each replicate.

The inoculated tissue samples were then transferred to solid C_2D4B medium containing AC (1 $g \cdot L^{-1}$), GA_3 (0, 1.5, 2.9 or 5.8 µM), and the antibiotics Cefotaxime (Cef, 350 $mg \cdot L^{-1}$), and Carbenicillin (Carb, 200 $mg \cdot L^{-1}$) to eliminate the agrobacteria. When the tips reached 3 to 5 cm in length, they were transferred to half-strength MS containing 0.7 µM IBA and 0.12 µM NAA for rooting (Zhang *et al*. 2004). Subsequently, before the start of Hygromycin B (Hyg B) selection, a killing curve was performed in order to determine the effect of Hyg B on growth. Hereby, the rooted plantlets were cultured on solid C_2D4B medium containing 0, 6, 9, 12 or 15 $mg \cdot L^{-1}$ Hyg B. The plantlets were monitored 30 and 45 d after start of selection, and it was found that 20-30 % survived under Hyg B selection. Each treatment was replicated three times with each replicate consisting of 20 plantlets. Based on this monitoring, the inoculated plantlets were subjected to stepwise enhanced selection (3, 6, 9, 12 $mg \cdot L^{-1}$) by increasing Hyg B-concentrations every two weeks.

Analysis of transgenes: Genomic DNA was isolated from leaves of putative transgenic plantlets based on a rapid DNA extraction protocol for *Arabidopsis thaliana* (Kasajima *et al.* 2004). PCR with specific primers 5' CACTGTGCCCAGGATGTA 3' (forward primer binds to *VpGLOX-sequence*) and 5' GTATAATTGCGGGACTCTAA 3' (reverse primer binds to *nos*-terminator-sequence in order to exclude the amplification from the endogenous *VpGLOX* gene) amplified a 633 bp fragment. For polymerase chain reaction (PCR), a hot start of 5 min at 94 °C was followed by 30 cycles of 30 s at 94 °C, 30 s at 54 °C and 60 s at 72 °C, and 10 min for final extension at 72 °C. Genomic DNA extracted from plants tested positive was transferred to a positively charged nitrocellulose membrane (Roche, Germany) for PCR-Southern blot detection according to the manufacturer's protocol.

Ex vitro acclimation of transgenic plant: After 3 months of culture on selection medium (12 $mg \cdot L^{-1}$ Hyg B), the transgenic plantlet was acclimated as described by Zhang *et al.* (2004). At first the transgenic plantlets *in vitro* (still in flasks) were placed in greenhouse for 1 week at 25 °C, under a light regime of 16 h light/ 8 h darkness using cool white fluorescent light (102.8 $\mu mol \cdot m^{-2} \cdot s^{-1}$). After washing out the agar attached to the roots, shoots and roots were cut back keeping 4-5 nodes and 3-4 cm in length respectively. Plantlets were cultivated in potting soil (Xintiandi, China). In order to prevent direct sunshine the plantlets were covered with a plastic lid and gauze. After 2 d plantlets were sprayed with Ronilan DF (BASF, Germany). The plastic lids were removed after one week.

Results

Propagation of micropropagated shoots from micro-shoot-tip: Micro-shoot-tips of 3 mm in size were cultured on solid C_2D4B (Fig. 1A). After growth to approx. 1 cm in length, shoot-tips were harvested and transferred to liquid C_2D4B (Fig. 1B). The bud clusters, when cultured at 80 rpm, were green with more nodes formed and compact stems (Fig. 1C) compared to those kept at 120 rpm (Fig. 1D). After dissection for subsequent culture (Fig. 1E), the majority of the plantlets developed as shown on half-strength MS solid medium supplemented with 9.0 µM TDZ and 2.9 µM NAA (Fig. 1F); tips and internodes were collected to solid C_2D4B for gene transfer.

The effect of orbital shaking rate for micropropagated shoots: The 1 cm-sized shoot-tips maintained at 120 rpm constant orbital shaking showed a dying-off rate of 87 %, with few branches and narrow leaves; in contrast the plantlets cultured at 80 rpm proved to be more vigorous. Additionally, the proliferation was found to be faster than on solid medium (Data not shown).

Optimization of basic solid propagation media, GA3, and light conditions for single bud stems propagation from micropropagated shoots: Resulting bud clusters from orbital shaking were cut into single bud stems, and placed on half-strength MS medium supplemented with 9.0 µM TDZ and 2.9 µM NAA, or on C_2D4B medium. The results obtained after 4-week culture demonstrated that half-strength MS (TDZ and NAA) medium was better than C_2D4B concerning the rooting rate (100 %) and 50 % of the cultures showed a root length of more than 10 cm. In contrast, the rooting rate for C_2D4B medium was only 54 %. On C_2D4B more lateral buds formed than on half-strength MS (supplemented with TDZ and NAA) medium, from 43 % of the single bud stems germinated more than 4 lateral buds (Tab. 1). This progress was aimed at proliferating shoot-tips and nodes. In summarization the C_2D4B medium was preferred to 1/2 MS.

Light intensity had no effects on shoot elongation (data not shown). The most rapid growth rate of single bud stems as well of their green leaves, was obtained at a concentration of 2.9 µM GA_3. The single bud stems gave rise to 3-4 internodes after four weeks. Rooting and survival rate were highly influenced by light conditions, especially the light intensity. Precisely, under higher light intensity (36 $\mu mol \cdot m^{-2} \cdot s^{-1}$), there was a lower die-off rate (lowest at 9.5 %, with GA_3 2.9 µM) and a higher rooting rate (Tab. 2). Therefore, these conditions were used for all further studies.

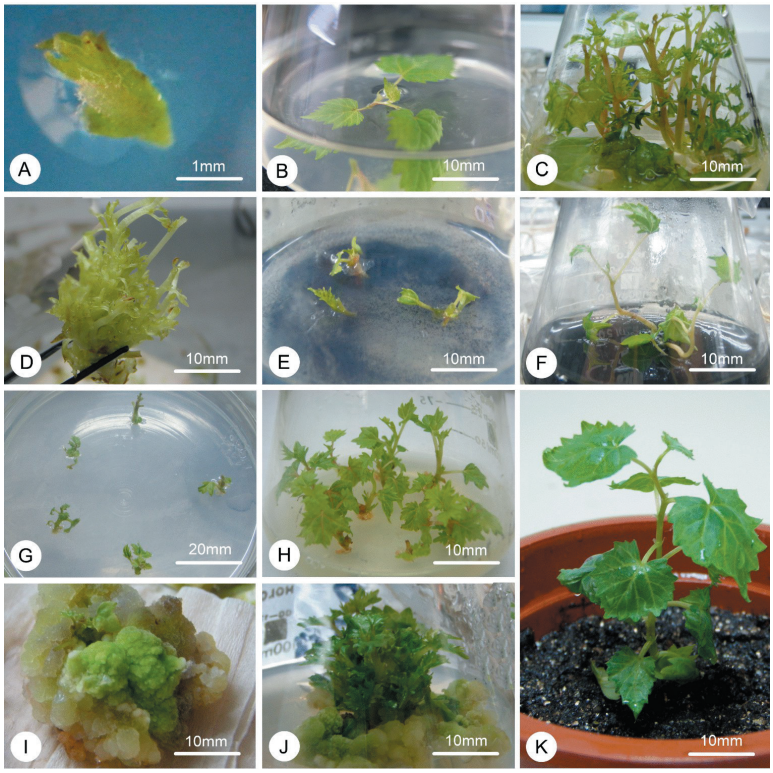

Fig. 1: Steps in the micropropagation culture, multiple shoot clusters production and rooting of '6-12-2'. **A**: Shoot-tips 3 mm in size on solid medium; **B**: Shoot 1 cm in size in liquid medium; Proliferation of shoot-tips and nodes after four weeks of transfer into liquid medium at 80 rpm (**C**) and 120 rpm (**D**) constant orbital shaking; **E**: Shoot-tips and nodes transferred to solid medium; **F**: Single shoots grown on half-strength MS solid medium supplemented with 9.0 µM TDZ and 2.9 µM NAA for shoot formation; **G**: Callus induction and shoot regeneration from stems with bud; **H**: Elongation of multiple shoot clusters; **I**: Shoot-tips and/or single-bud-internodes collected from two regeneration systems. **J**: Hygromycin-resistant shoots; and **K**: Planting out of transgenic plants. **A-F:** Propagation of micropropagated shoots from micro-shoot-tip; **J** and **H**: Induction of shoot clusters from stem with single bud.

Table 1

Effect of different media on rooting and shoot formation of '6-12-2' micropropagated shoots. Data were monitored one month after the onset of culture of micropropagated shoots. The same letter within the same columns was not significantly different at $p < 0.05$ (DUNCAN 1955)

Medium	Rate of different root length classes [%]				Rate of shoot production [%]		
	None	< 5 cm	5-10 cm	> 10 cm	No. of nodes 1-2	No. of nodes 3-4	No. of nodes > 4
1/2MS + TDZ 9.0 µM + NAA 2.9 µM 0	0 a	36.7 a	13.3 a	50 a	30 a	43.3 a	26.7 a
C_2D4B	54.4 b	23.3 b	8.9 a	14.4 b	28.9 a	28.9 a	43.3 a

Induction of shoot clusters from stem with single bud: Callus genesis occurred within 1 week at the wounded surfaces of stems with bud on different light conditions and all of the hormone combinations. Lower concentrations of TDZ and NAA resulted in the formation of light green friable soft calli. An increase of the concentration of TDZ yielded more compact calli (Fig. 1G) and four weeks thereafter, the shoot clusters were formed regardless of the hormone combinations. Whereas, at the highest concentration of TDZ 18.0 µM (NAA 0.58 µM), under both high (Fig. 1H) and low light conditions, shoot cluster growth was enhanced. The fresh weight of shoots under the above mentioned cultivation parameters reached 0.50 g and 0.66 g, respectively (Tab. 3). This medium was used for the following transgenic approach.

Effects influencing the gene transfer. Effect of Hyg B concentration on plantlet growth: Hyg B in a concentration of 6 mg·L^{-1}, led to the base of the plantlet stems to become slightly browned, and the amount of healthy shoot-tips was reduced. After 30 d 33 % of the plantlets died off, the number increased to 66.7 % after 45 d. The mortality increased with an increasing of Hyg B concentration. All the plantlets became brown and died off after 30 d on the medium supplemented with Hyg B 15 mg·L^{-1} (Fig. 2). Therefore, the stepwise selection method by increasing Hyg B concentrations from

Table 2

Effect of different light intensities and GA_3 concentrations on rooting and shoot formation of '6-12-2' micropropagated shoots. Data were monitored one month after the onset of culture of micropropagated shoots. The same letter within the same columns was not significantly different at $p < 0.05$ (Duncan 1955)

photon fluence rate [µmol·m^{-2}·s^{-1}]	GA_3 [µM]	Rate of rooting [%]	Mortality [%]
36	0	35.7 abc	13.2 cd
	1.5	33.3 abc	27.8 bcd
	2.9	46.2 ab	9.5 d
	5.8	66.6 a	33.3 bcd
4	0	66.7 ab	34.8 bc
	1.5	33.3 bc	76.9 a
	2.9	38.5 ab	44 b
	5.8	25 c	40.5 bcd

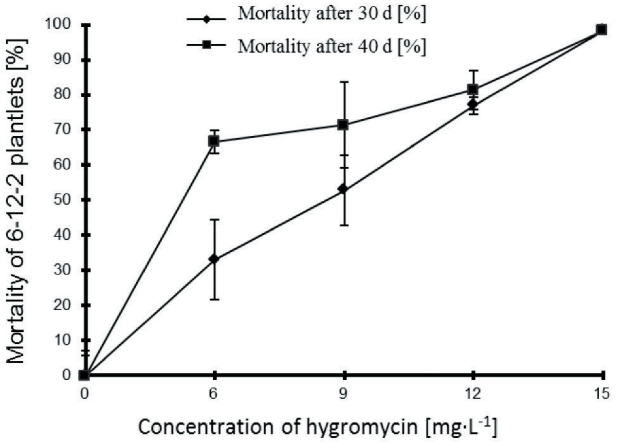

Fig. 2: Effect of Hyg B concentrations on '6-12-2' plantlets. Die-off rate of '6-12-2' plantlets after 30 d (diamonds) and 45 d (squares) in Hyg B-selection medium of increasing concentration. Result shown are means ± s.e.m. (n = 3)

3 to 12 mg·L^{-1} was utilized. This stepwise selection method was also run in case of *Vitis vinifera* L. gene transfer by Fan *et al.* (2008).

GV3101/pWR-VpGLOX inoculation optimization: Transient expression of GFP could be detected both in shoot-tips and node tissue after a period of infection with vacuum infiltration or orbital shaking. The efficiency was improved by increasing the concentration of agrobacteria (with an optical density at 600 nm of 0.2 to 0.4), infection time (10 to 30 min), and vacuum infiltration application. Some of the parameters causing different effects were combined to compare the different efficiencies of agrobacteria inoculation (Fig. 3). However, an excess of bacteria on the surface of the explants gave rise to inefficient elimination of the *agrobacteria*, leading to browning and dying-off of the explants. Hence, an agrobacterium OD (600 nm) of 0.4 and orbital shaking for 10 min, was chosen for inoculation. After 3 d of co-cultivation in darkness at 26 °C, explants were transferred to solid C_2D4B medium containing AC (1 g·L^{-1}), GA_3 (2.9 µM), Cef (350 mg·L^{-1}), and Carb (200 mg·L^{-1}) to eliminate agrobacterium for 2 d before being transferred to fresh medium.

Table 3

Effect of different light intensities and TDZ, NAA concentrations on multiple shoot clusters formation of '6-12-2' stems with bud. Data were monitored one month after the onset of culture of stems with bud. The same letter within the same columns was not significantly different at $p < 0.05$ (Duncan 1955)

TDZ [µM]	NAA [µM]	Fresh shoot weight [g]	
		36 µmol·m^{-2}·s^{-1}	4 µmol·m^{-2}·s^{-1}
4.5	0.29	0.35 abc	0.24 b
9.0	0.29	0.30 abc	0.38 ab
13.5	0.29	0.35 abc	0.47 ab
18.0	0.29	0.34 abc	0.20 b
4.5	0.58	0.14 c	0.37 ab
9.0	0.58	0.28 abc	0.24 b
13.5	0.58	0.48 ab	0.25 b
18.0	0.58	0.50 a	0.66 a
4.5	0.87	0.30 abc	0.36 ab
9.0	0.87	0.21 bc	0.34 ab
13.5	0.87	0.39 abc	0.47 ab
18.0	0.87	0.49 a	0.26 b
4.5	1.74	0.25 abc	0.37 ab
9.0	1.74	0.25 abc	0.27 b

Selection and analyses of transgenic plants: A stepwise Hygromycin resistance selection for twelve weeks yielded 89 transgenic plants (Fig. 1J) from a total of 5400 co-cultivated shoot-tips and nodes. Total DNA was extracted from the top 1-3 leaves. Forty-five independent transgenic clones were proven by PCR yielding a specific 633 bp fragment which was absent in the negative control (Fig. 4). PCR-Southern blot analysis was performed on 45 *VpGLOX*-PCR-positive lines. Transgenic integration was confirmed by bands of the expected length obtained (Fig. 5).

Ex vitro acclimation of transgenic plant: Most of the above mentioned lines maintained on Hyg B-containing medium for a 3-month selection period, rooted slowly, 11 out of 45 turned to become brown and died-off. Only one of the transgenes was vigorous and survived. It was cultivated in potting soil (Fig. 1K). This plant showed no morphological alterations concerning vegetative growth compared to wild type. Our future work will be focused on this stable gene transfer line which is considered as an important material to study the gene function of *Vitis pseudoreticulata glyoxal oxidase* in disease resistance.

Discussion

In the present study, SAM of *Vitis* '6-12-2' (*V. pseudoreticulata* 'Baihe-35-1' × *V. vinifera* 'Carignane') were used as target material for *Agrobacterium*-mediated gene transfer. SAM proliferation from stems with single bud was easy to process; besides, a large number of SAM could be obtained after four weeks of cultivation (To be mentioned here, it is true that the meristematic tissue contributed to the formation of adventitious bud, but we can't exclude the possibility that the adventitious bud regenerated from

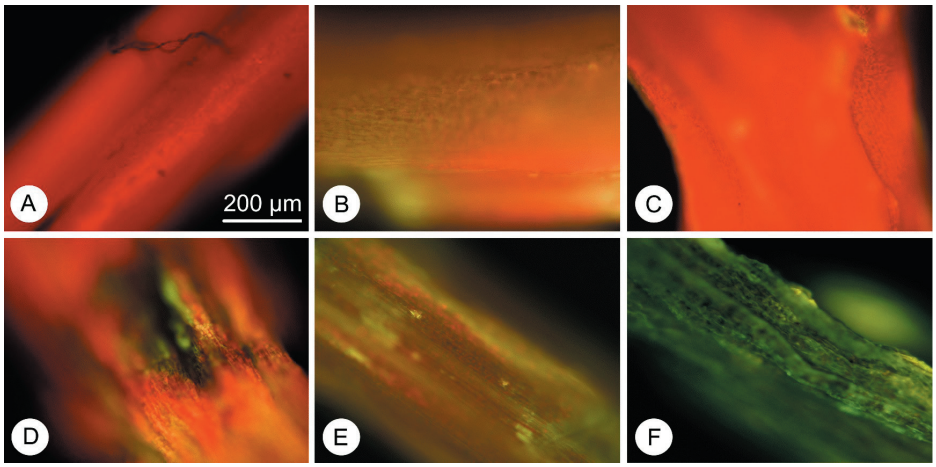

Fig. 3: Transient expression of GFP in '6-12-2' micropropagated shoots 3 days after the end of co-cultivation. Influence of different concentrations of *Agrobacterium tumefaciens*, with and without infiltration. Representative results are shown of three independent experiments with 30 micropropagated shoots in each replication. **A**: Induction buffer, infiltration 30 min; **B**: OD_{600} of 0.4, shaking 20 min; **C**: OD_{600} of 0.3, shaking 20 min; **D**: OD_{600} of 0.4, shaking 10 min; **E**: OD_{600} of 0.4, agro-infiltration 10 min; **F**: OD_{600} of 0.6, agro-infiltration 30 min.

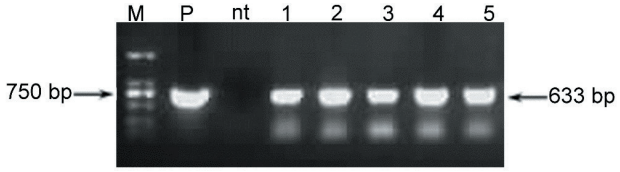

Fig. 4: PCR analysis of the '6-12-2' plants transformed with *Agrobacterium* strain GV3101 harboring pWR306-VpGLOX. M: DNA size marker (DL2000); P: pWR306-VpGLOX plasmid (positive control); nt: non-transgenic (negative control) plant; 1-5: transgenic plants.

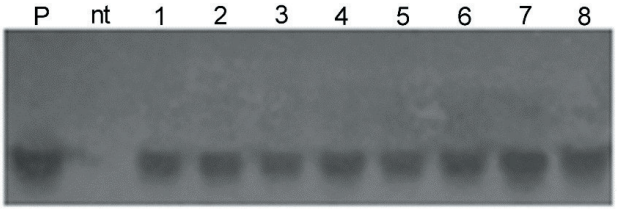

Fig. 5: PCR-Southern blot analysis of '6-12-2' transgenic plants. P: pWR306-VpGLOX plasmid (positive control); nt: non-transgenic (negative control) plant; 1-8: transgenic plants.

the callus was induced by wounding the stem. Our goal is to obtain the efficient way to collect buds as much as possible, either the final outcome is benefited from one of the factor discussed above or the combination of these two factors that can be a good direction in further studies.). In comparison, after an 8-week period, the number of SAM from micro-shoot-tips undergoing micropropagation was 4 to 5-fold enhanced compared to the trial started from stems with bud underwent callus induction **(**Fig. 6**)**. This shows that the micropropagated shoots approach was much more suitable for large scale proliferation. The *Agrobacterium*-mediated gene transfer efficiency was 0.83 %. Gene transfer of inner tunica layer (L2) cells (which can form undifferentiated, totipotent germline cells) was believed to generate non-chimeric transgenic plants (DUTT *et al.* 2007, SATINA *et al.* 1940). In our case, wounding and the addition of AS ensured the success of agrobacterium infection of L2 layer cells, although the effects of wounding varied between plant species, e.g. a large positive impact arose on leaves of *Nicotiana benthamiana* treated with a wire brush prior to infiltration (ANDREWS and CURTIS, 2005); and on shoot apices of *Vitis vinifera* L. cv. 'Thompson Seedless' by cutting through to expose the apical meristem and tunica cells (DUTT *et al*. 2007). On the contrary, however, no effect of an enhanced gene transfer efficiency either of leaves from *N. tabacum* or *N. glutinosa* after treatment with a

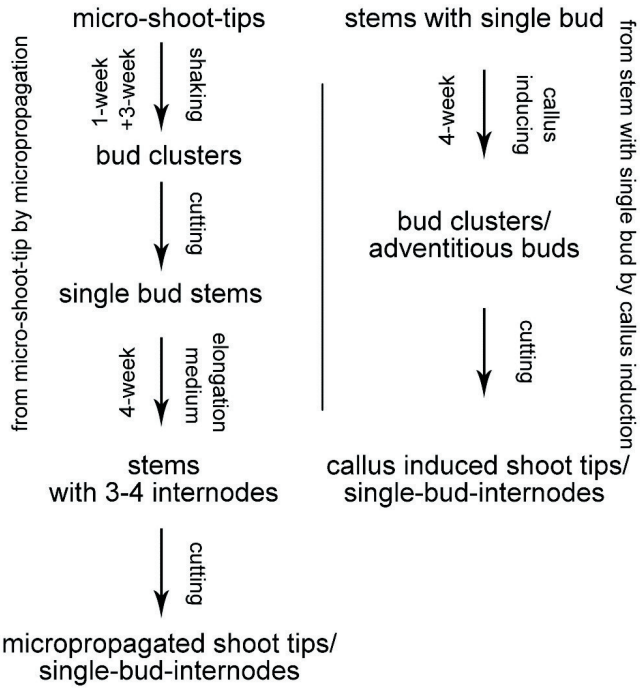

Fig. 6: A brief overview of the propagation progress of shoot tips/ internodes originated from micro-shoot-tips by micropropagation and from stem with single bud by callus induction. Left: micropropagation progress; right: callus induction progress.

wire brush (Andrews and Curtis 2005) was observed or by bead beating of isolated leaves of *Panicum virgatum* by (Vandergheynst *et al.* 2008). Zottini *et al.* (2008) and Fan *et al.* (2008) could enhance gene transfer efficiency by AS-addtion and the growth conditions of shoot-tips and/or single-bud-internodes of bud clusters originated from stems with single buds were strongly influenced by light intensities after the orbital shaking process. A high light intensity was considered to be essential. Dutt *et al.* (2007) found that only shoots were accessible for gene transfer which etiolated after a 4-week lack of light. However, our results could not be compared with their results. In our work a 4-week etiolation treatment resulted in a lowered rooting and decreasing survival rate. Without the application of 4-week etiolating treatment, but a 3-day pre-culture of non-etiolated material before co-culture was found to enhance gene transfer efficiency in this study. Kuta and Tripathi (2005) demonstrated that the pre-culture process could eliminate the effect of injury on agrobacterium induction; on the other hand, phenolic compounds produced by cuttings were prone to enhance the gene transfer rate by activating *vir* genes (Joubert *et al.* 2004). Previously we found a relatively high rate of false positive transformants for Chinese wild *Vitis* when the *nptII* antibiotic resistance gene for kanamycin selection was utilized, which may be due to different sensitivities to kanamycin among *Vitis* species (Colby *et al.* 1990, Levenko *et al.* 2000). Therefore, in the present study, Hyg B was applied for selection. Grapevine was found to be hypersensitive to Hyg B, even if Hyg B was added stepwise as selection agent. Fan *et al.* (2008) believed that Hyg B has notable inhibitory effects on leaf callus formation, adventitious bud differentiation and plant growth of 'Thomson Seedless' grapes. In our experiments, Hyg B was supplemented by stepwise enhancing the concentration after the plantlets rooted, in order to reduce the impairment of the transgenic cells by surrounding non-transgenic browning cells.

After three months of Hyg B selection (with subculture intervals of every four weeks), some of the PCR-Southern blotting positive plantlets were still lacking vigor, and could not reach the requirements for *ex vitro* cultivation. The studies for increasing the growth rate of transgenic cells in chimeric plantlets have been explored (Mauro *et al.* 1995). Dutt *et al*. (2007) also mentioned that it is most likely that kanamycin selection inhibited surrounding non-transgenic cells and tissues is one of the possibilities to death of chimeric shoots. The difficulty of selection of non-chimeric plantlets also existed in this study; however, optimization of propagation and genetic gene transfer gave rise to enhanced work efficiency.

This optimized method, to propagate numerous shoot tips and/or single-bud-internodes from micro-shoot-tips, has been used successfully for shortening the period in our present study on propagation of *Vitis vinifera* 'Chardonnay' shoots harbouring the second actin-binding domain of *Arabidopsis thaliana* fimbrin (to be published). Since the growth rate of plantlets with the propagation method in solid medium (here three weeks shaking in liquid medium) was relatively slow, probably due to confined AFs activity would lead to a reduced cell growth rate (Holweg, 2007). In the present study the gene transfer efficiency was 0.83 %, 45 transgenic events had been obtained, slightly lower but comparable to gene transfer efficiency of 1 % reported on *Vitis vinifera* L. 'Thompson Seedless' by Dutt *et al.* (2007). However, most of our positive transgenic events grew very slowly and died-off after several months of subculture. Further investigations are necessary to improve this specific step. However this method will be of importance for the gene transfer of Chinese wild *Vitis*, since the number of successfully transformed plants from SAM can be larger due to its potential of rapid proliferation and facilitates the examination of expression of other interesting genes also in other grapevine cultivars, recalcitrant to gene transfer and regeneration.

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