Vitis **39** (3), 99–102 (2000)

Influence of culture technique and genotype on the efficiency of *Agrobacterium*-mediated transformation of somatic embryos (*Vitis vinifera*) and their conversion to transgenic plants

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Summary

Somatic embryos originating from anther cultures of *Vitis vinifera* cvs Dornfelder, Müller-Thurgau and Riesling cultured on solid or in liquid medium were used for transformation via *Agrobacterium tumefaciens* strain LBA 4404. After two days of cocultivation the embryos proliferated in liquid NN69 medium containing kanamycin for selection and cefotaxim for removal of bacteria. Subsequent cultivation of larger embryos was carried out on solid NN69 medium supplemented with kanamycin. Highest conversion rates of selected germinated embryos to rooted plantlets were obtained from embryos of suspension cultures of Dornfelder and Riesling. Transgenic plants of both varieties were adapted to glasshouse and field conditions.

Key words: *Agrobacterium tumefaciens*, somatic embryogenesis, suspension culture, transformation.

Introduction

Most transformation protocols for grapevine are based on gene transfer via *Agrobacterium tumefaciens* which infects grapevine in the field (LE GALL *et al.* 1994, MARTINELLI and MANDOLINO 1994, MAURO *et al.* 1995, PERL *et al.* 1996). However, necrotic reactions of treated tissues and the lack of sufficient regeneration protocols often limit regeneration. An important prerequisite for successful gene transfer is the availability of suitable starting material such as homogeneous embryogenic material. Although many protocols on gene transfer for grapevines outline the use of somatic embryos as target material for transformation purposes (NAKANO *et al.* 1994; KRASTANOVA *et al.* 1995; KIKKERT *et al.* 1996; SCORZA *et al.* 1996), the present study concentrated on the induction and establishment of suitable embryogenic starting material of economically important varieties, and on high conversion rates of germinated embryos to transgenic plants.

Material and Methods

E m b r y o c u l t u r e : For induction of secondary embryos of *Vitis vinifera* L. cvs Dornfelder, Müller-Thurgau and Riesling in liquid culture somatic embryos at different developmental stages originating from anther culture were used as donor material (HARST-LANGENBUCHER and ALLEWELDT 1993). First experiments focused on the optimization of anther cultures for high-yielding and homogeneous production of secondary embryos either on solid NN69 medium without hormones or in suspension cultures. The protocol for cultivation of somatic embryos in liquid culture was elaborated by BORNHOFF and HARST (2000). For continuous embryo induction solid and liquid media were replaced monthly. Both cultures were kept in incubation chambers at 27 °C and permanent darkness. For regeneration into rooted plants the germinated embryos >2 mm were transferred to culture tubes with solid LINSMAIER and SKOOG medium (1965) and cultured at 27 °C and 16 h light (50-60 µmol quanta·m^{-2}·s^{-1}).

T r a n s f o r m a t i o n : The most common *A. tumefaciens* strain LBA 4404 (OOMS *et al.* 1981) was used in all gene transfer experiments. For transformation of somatic embryos (cvs Dornfelder and Müller-Thurgau) the *A. tumefaciens* carried the plasmid p35Sgusint with β-glucuronidase (GUS) and neomycinphosphotransferase II (NPT II) for screening and selection (VANCANNEYT *et al.* 1990). To transfer foreign genes to Riesling and Müller-Thurgau the *Agrobacterium* strain contained either plasmid pGJ40 harbouring the anti-fungal genes glucanase and chitinase or plasmid pGJ42 encoding chitinase and RIP (ribosome inactivating proteine), both under control of the CaMV 35S RNA-promotor (JACH *et al.* 1995). Bacterial cultures were grown overnight in LB medium (AUSUBEL *et al.* 1987) with 100 µM acetosyringone at 28 °C on a rotary shaker (200 rpm).

Transformation of explants on solid medium was obtained by incubating somatic embryos of Dornfelder for 20 min in a bacterial suspension (OD_{550}= 1.2) of *A. tumefaciens*. After cocultivation (2 d) with 100 µM acetosyringone the somatic embryos proliferated on a modified solid hormone-free NN69 medium containing 100 µl·ml^{-1} kanamycin for selection and 300 µg·ml^{-1} cefotaxim for removal of bacteria. The explants were cultivated in an incubation chamber at 27 °C in permanent darkness. Subcultivation of the explants with the same concentration of antibiotics was carried out at two-week intervals. After three months cefotaxim was removed and the medium was replaced monthly. The embryos were cultivated in permanent darkness until germination. For further conversion to rooted plants the germinated embryos were transferred to rooting medium (LS medium) in culture tubes and cultivated at 27 °C and 16 h light (50-60 µmol quanta·m^{-2}·s^{-1}).

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For transformation of suspension cultures somatic embryos of Dornfelder, Riesling and Müller-Thurgau were cocultivated with *A. tumefaciens* for 2 d in liquid NN69 medium after incubation for 20 min in the bacterial suspension (OD_{550}= 1.2). For proliferation the embryos were transferred to liquid NN69 medium containing 100 $\mu g \cdot ml^{-1}$ kanamycin and 300 $\mu g \cdot ml^{-1}$ cefotaxim. Five weeks after cocultivation newly developed embryos (>2 mm) were removed from the suspension and subcultured on solid NN69 medium supplemented with kanamycin and cefotaxim. About 13 weeks post initiation of cocultivation cefotaxim was removed and 4 weeks later the kanamycin concentration was reduced from 100 to 50 $\mu g \cdot ml^{-1}$. Further cultivation steps followed the protocol on solid medium. Germinated embryos were transferred to test tubes with kanamycin-containing LS medium for rooting. Control experiments were carried out on media without agrobacteria and antibiotic.

Transformation efficiency was examined by growth and rooting assays on kanamycin-containing medium throughout *in vitro* cultivation. The transfer of foreign genes into Dornfelder and Müller-Thurgau was confirmed by histochemical GUS assay (JEFFERSON 1987). Successfully introduced genes in transgenic plants of Dornfelder and Riesling were detected by PCR.

In 4 years of anther excision (1995-1998) the average of three excisions per year and genotype was used to determine the degree of anther regeneration. For each of the three tested genotypes the experiments on solid and liquid medium carried out in 1998 were repeated three times.

The level of secondary embryogenesis in suspension cultures was determined from the increase of induced globulary embryos per g fresh weight of embryogenic starting tissue.

Results and Discussion

Regeneration of embryogenic tissue: The rate of regeneration and transformation highly depends on the ability of suitable starting material. Regeneration experiments were carried out with three varieties which showed a high regeneration capacity of anther explants over 4 years of anther excisions (1995-1998) and sufficient induction of somatic embyros on anther explants (Fig.1). Twelve weeks post initiation of anther culture the rate of embryogenic anthers was determined for an average of 6 experiments per year, each with more than 3,000 anthers per variety. To lower the high annual rate of variation of the embryo induction the embryogenic competence of initial explants was maintained by subsequent secondary embryo induction. Due to the variation of embryo yield and quality by subculturing the mother explants suspension cultures might be useful for continuous embryo production. The availability of homogeneous embryogenic starting material is an important prerequisite for many biotechnological experiments, *e.g.* it has been recommended for the purpose of gene transfer by KRASTANOVA *et al.* (1995), MAURO *et al.* (1995) and HOSHINO *et al.* (1998). Only a few protocols are based on embryogenic suspension cultures for biolistic and *Agrobacterium*-mediated transformation (MARTINELLI and MANDOLINO 1994; MAURO *et al.* 1995; KIKKERT *et al.* 1996). In 1998 anthers were used for primary embryo induction. Primary embryos were harvested from the anther calli and transferred to suspension cultures. A genotype-specific reaction in embryo induction on the mother explant and a subsequent induction of secondary embryos in suspension was observed (Tab. 1).

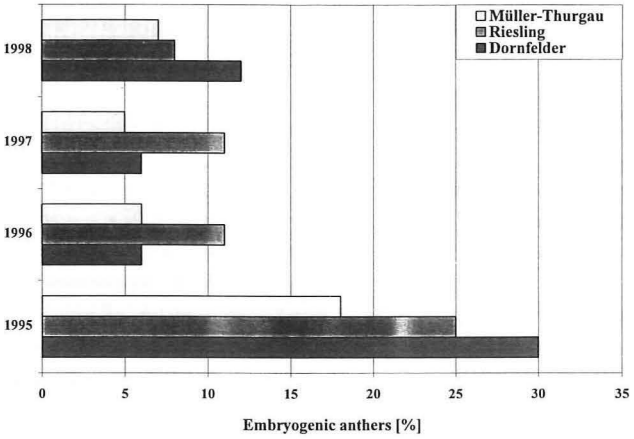

Fig. 1: Induction of somatic embryogenesis on anther explants.

Table 1

Induction of primary embryos on anther explants and secondary embryogenesis in suspension cultures

Variety	Number of excised anthers	Induction rate of primary somatic embryos on anthers[1] (%)	secondary somatic embryos in suspension cultures[2] (%)
Dornfelder	1,200	11	36
Riesling	900	24	8
Müller-Thurgau	1,070	6	40
Average	1,057	14	28

[1] Values determined 13-14 weeks after inoculation of anthers on solid induction medium as percentage of embryogenic anther explants.

[2] Values determined 12 weeks after culture initiation, measured as an increase in weight relative to the initial culture.

Compared with the cultivation on solid medium the embryo suspension culture is advantageous due to a higher rate of homogeneous embryogenic material. Moreover the uptake of nutrients as well as the release and distribution of metabolites from the whole embryos are improved under the condition of suspension culture (BORNHOFF and HARST 2000).

Conversion of transgenic germinated embryos: First transformation experiments were initiated with cv. Dornfelder which showed high embryo production rates in subsequent culti-

vation of the initial anther explants in suspension cultures. Embryos originating from suspension cultures revealed a higher conversion rate than embryos which were subsequently cultured on solid medium (Fig. 2). Transformation procedures considerably decreased the induction of secondary embryos and the conversion of germinated embryos to intact plants in both culture methods.

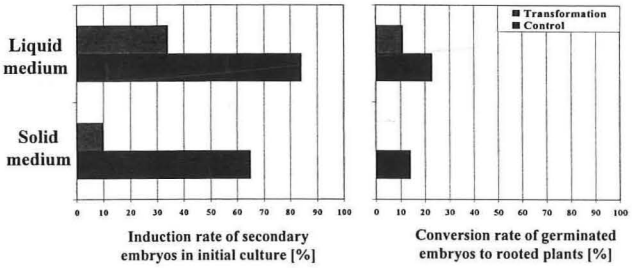

Fig. 2: Regeneration and transformation of cv. Dornfelder explants on liquid or solid medium. Secondary embryo induction was recorded 20 weeks after inititation of culture as an increase of weight relative to the initial culture. Conversion rate was determined 8 weeks after transfer of germinated embryos on a rooting medium under light conditions.

Further investigations focused on the adaptation of the transformation procedure of Dornfelder to Riesling and Müller-Thurgau. In control experiments highest plant regeneration rates were obtained from germinated embryos of Müller-Thurgau which almost completely regenerated to shoots 12 weeks after transfer to the rooting medium and to light conditions. However, transformation reduced the conversion rates of the germinated embryos to insufficient low values for Dornfelder and Riesling while Müller-Thurgau failed completely to regenerate (Tab. 2). The overall regeneration time increased in transformation experiments depending on the genotype tested (Tab. 3).

Table 2

Conversion rate of germinated embryos to rooted plants ca.12 weeks after transfer to rooting medium and 16 h light

Variety	Conversion rate (%) Control	Transformation
Dornfelder	28	11
Riesling	59	13
Müller-Thurgau	94	0
Average	55	8

The present results demonstrate that suspension cultures are preferred to solid medium for the production of homogeneous embryogenic tissue and for further transformation studies due to the better induction of secondary embryos. Conversion of germinated embryos to rooted plants was improved if explants originated from suspension cultures. However, transformation procedures lowered the total regeneration rate considerably. Therefore suspension cultures should be preferred as initial cultures because regeneration was totally suppressed when explants from solid cultures were used. To our knowledge there are no reports on the influence of different culture techniques on the improvement of gene transfer to *V. vinifera* cultivars.

Table 3

Time of regeneration of transgenic grapevine plants

Regeneration steps	Time of regeneration, months Dornfelder Control	Dornfelder Transf.	Riesling Control	Riesling Transf.
Transformation of suspension cultures	0	0	0	0
Transfer to solid medium, darkness	1.0	1.5	1.0	1.0
Transfer to test tubes, light	4.5	7.5	2.5	2.5
Adaptation to greenhouse conditions	8.0	12.0	6.0	17.0

Analysis of transformed explants: To select transgenic tissue the explants were cultured continuously on kanamycin-containing medium (50 $mg \cdot ml^{-1}$) according to the protocol of Kikkert *et al.* (1996) and Perl *et al.* (1996). On kanamycin-containing medium most of the embryogenic explants showed necrotic reactions (Fig. 3 a). Only white embryos were harvested from the embryogenic calli and were successfully differentiated to germinated embryos under selective conditions (Fig. 3 b). One year post cocultivation transgenic shoots could be selected and converted into rooted plantlets on kanamycin containing LS medium without hormones. Regenerated transgenic plants were transferred to the glasshouse for adaptation to field conditions. The first field release of transgenic grapevines in Germany was carried out in July 1999 in order to test the plants under natural conditions. Despite the growth under selective conditions the gene transfer was recorded by PCR amplifications (Fig. 4). Histochemical GUS assays at different developmental stages of transgenic explants of Dornfelder and Müller-Thurgau showed a strong uniform blue colour (Fig. 5).

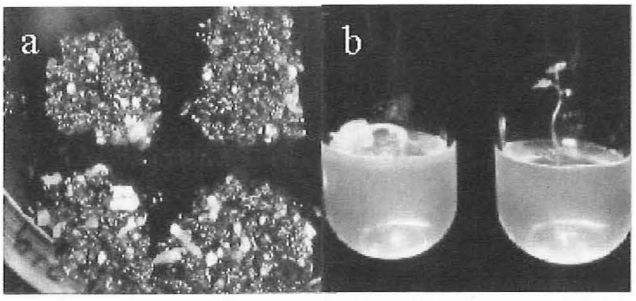

Fig. 3: Selection of transgenic explants by continuous growth on kanamycin-containing medium (50 $mg \cdot l^{-1}$). (**a**) somatic embryos of cv. Riesling on NN69 medium cultured in permanent darkness, 8 weeks post transfer on solid medium; (**b**) germinated embryos of cv. Riesling on LS medium, 6 weeks after transfer to test tubes and in 16 h light. Left: non-transformed germinated embryo; right: transformed germinated embryo. (Coloured version of these pictures can be found on the front cover.)

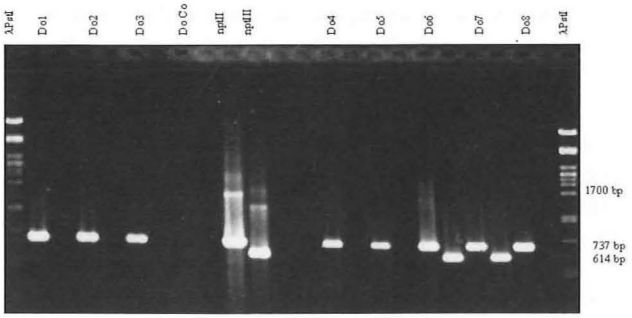

Fig. 4: PCR amplification products of foreign genes in Dornfelder.

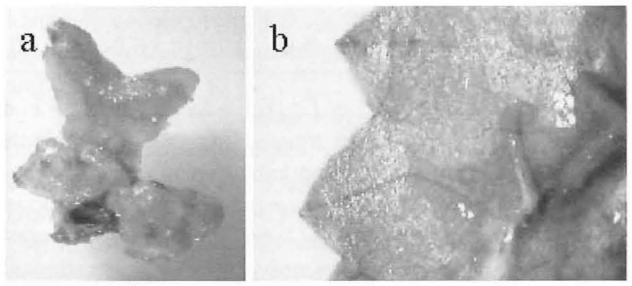

Fig. 5: GUS-assay of transgenic *in vitro* explants. (**a**) somatic embryo clusters of cv. Müller-Thurgau; (**b**) leaf segment of an *In vitro* plant of cv. Dornfelder. (Coloured version of these pictures can be found on the front cover.)

Conclusion

From two out of three economically important grapevine varieties transgenic grapevines have been obtained. The first transgenic plants of the red variety Dornfelder contain genes which are of scientific interest. However, the results obtained with cv. Riesling are especially promising for further attempts to improve this very old and commercially important variety. The regenerated transgenic Riesling plants need to be analysed in more detail to determine the effect of inserted genes with regard to their contribution to possible fungal resistance. The protocol will be of high value for further tests of interesting gene constructs.

Acknowledgements

We gratefully acknowledge the excellent technical assistance of EDITH FICK and RUTH TRAUTH and the provision of the plasmids pGJ40 and pGJ42 by GUIDO JACH, Max-Planck-Institut für Züchtungsforschung, Köln, Germany.

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Received March 28, 2000