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Establishment of embryogenic cultures in several cultivars of *Vitis vinifera* and *V.* x *labruscana*

by

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S u m m a r y : Establishment of embryogenic cultures was examined for different tissue explants in 23 cultivars of grapevine (*Vitis* spp.). The explants were initially cultured on callus induction media (C media) for 2 months, and those producing calli were then transferred to an embryogenesis induction medium (E medium) containing 1 µM 2,4-dichlorophenoxyacetic acid (2,4-D), on which adventitious embryos or embryogenic calli were induced 4 to 6 months after transfer. C media containing 10 µM 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) in combination with 10 µM N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU) or N-(1,2,3-thiadiazol-5-yl)-N'-phenylurea (TDZ) were suitable for inducing subsequent adventitious embryogenesis from leaf explants of *V. vinifera* Koshusanjaku. Adventitious embryogenesis was more efficiently induced from immature ovary explants than leaf and anther ones. Among 23 cultivars examined, embryogenic cultures, such as embryogenic calli or adventitious embryos proliferating *via* secondary embryogenesis, were established in 10 cultivars including *V. vinifera* Sekirei, Rosario Bianco, Sémillon and Merlot, and *V.* x *labruscana* Delaware. These embryogenic cultures could be maintained without loosing a high regeneration capacity for over 20 months by subculturing onto fresh E medium. They could be useful as a target material for *Agrobacterium*- or particle gun-mediated genetic transformation.

K e y w o r d s : adventitious embryos, embryogenic calli, grapevine, ovary culture, plant regeneration.

A b b r e v i a t i o n s : CPPU: N-(2-chloro-4-pyridyl)-N'-phenylurea, 2,4-D: 2,4-dichlorophenoxyacetic acid, DIC: 3,6-dichloro-o-anisic acid (dicamba), MS: MURASHIGE and SKOOG (1962), NOA: 2-naphthoxyacetic acid, PIC: 4-amino-3,5,6-trichloropicolinic acid (picloram), 2,4,5-T: 2,4,5-trichlorophenoxyacetic acid, TDZ: N-(1,2,3-thiadiazol-5-yl)-N'-phenylurea (thidiazuron).

Introduction

Grapes (*Vitis* spp.) are a major fruit crop throughout the temperate regions of the world. Although improvement of this crop is possible by conventional breeding, it is rather difficult and time-consuming because of the 2-3-year generation cycle, high levels of heterozygosity, and inbreeding depression which prohibits selfing (GRAY and MEREDITH 1992). An alternative approach by using genetic transformation techniques is, therefore, particularly desirable for grapevines (SCORZA 1991; COLBY and MEREDITH 1993).

Although grapevines have been considered a recalcitrant crop for establishing genetic transformation systems (COLBY and MEREDITH 1993), several recent papers demonstrated the successful production of transgenic plants in both scions and rootstocks by using embryogenic cultures, such as embryogenic calli, embryogenic cell suspensions, and proliferating adventitious embryos *via* secondary embryogenesis (RAEMAKERS *et al.* 1995), as tissues to be transformed (MULLINS *et al.* 1990; MARTINELLI and MANDOLONO 1994; NAKANO *et al.* 1994; MAURO *et al.* 1995; SCORZA *et al.* 1995, 1996; KIKKERT *et al.* 1996; PERL *et al.* 1996). Thus, in grapevines, embryogenic cultures appear to be the most suitable target material for transformation; they have been shown to have high proliferation and regeneration abilities (GRAY and MORTENSEN 1987; STAMP and MEREDITH 1988 a, b; MATSUTA and HIRABAYASHI 1989; GRAY 1992; MARTINELLI *et al.* 1993; PERL *et al.* 1995); and adventitious embryos have been shown to arise from single cells (KRUL and WORLEY 1977; FAURE *et al.* 1996), which may avoid the regeneration of chimeric transformants frequently occurring *via* adventitious shoot regeneration (BARIBAULT *et al.* 1990; BERRES *et al.* 1992).

Although in grapevines many papers have so far been published on the induction of adventitious embryogenesis (GRAY and MEREDITH 1992), establishment of embryogenic cultures has rather been restricted to a few genotypes (GRAY and MORTENSEN 1987; STAMP and MEREDITH 1988 a, b; MATSUTA and HIRABAYASHI 1989; GRAY 1992; MARTINELLI *et al.* 1993; PERL *et al.* 1995). For wide application of genetic transformation techniques in grapevines, however, it is necessary to establish embryogenic cultures in various cultivars. In the present study, we aimed to induce embryogenic cultures in several grapevine genotypes including table grape cultivars widely cultivated in Japan. The effect of plant growth regulators and explant source on induction of embryogenic cultures are specifically examined.

Materials and methods

Twenty-three grapevine cultivars (11 cultivars of *Vitis vinifera*, 11 cultivars of *V.* x *labruscana*, and one *V. rupestris* cultivar), listed in Tab. 1, were used in the present study. Seven to 15 days before anthesis fully expanded leaves and flower buds were harvested from field-grown plants. Leaves were surface-disinfected with a sodium hypochlorite solution (1 % active chlorine) for 10

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Table 1

Comparison of adventitious embryogenesis from leaf, anther, and ovary explants of several grapevine cultivars (*Vitis* spp.)

Species and cultivars	Adventitious embryogenesis (%)[1]		
	Leaf	Anther	Ovary
V. vinifera			
Cabernet Sauvignon	0	-	-
Cabernet franc	0	0	0
Koshusanjaku	28.3 ± 5.6	-	-
Merlot	2.3 ± 1.5	-	-
Neo Muscat	0.3 ± 0.3	2.7 ± 0.7	12.9 ± 4.3
Riesling	0.3 ± 0.3	-	-
Riesling Lion	15.6 ± 3.3	-	-
Rosario Bianco	0	1.5 ± 0.8	3.7 ± 0.6
Ruby Okuyama	0	0.1 ± 0.1	2.3 ± 1.3
Sekirei	0	2.1 ± 0.5	6.7 ± 2.5
Sémillon	1.2 ± 0.4	-	-
V. x *labruscana*			
Benifuji	0	0	0
Benizuiho	0	0	0
Cabernet Suntory	0	0	0
Campbell Early	0	0	0
Delaware	0	0.3 ± 0.3	1.3 ± 0.4
Early Steuben	0	0	0
Kyoho	0	0	0
Muscat Bailey A	0	0	0
Steuben	0	0	0
Takasumi	0	0	0
Tano Red	0	0	0
V. rupestris			
St. George	0	0	0

Explants were initially cultured on the C medium containing 10 µM each of 2,4,5-T and CPPU for 2 months, and explants which formed calli were then transferred to the E medium. Data were recorded 6 months after transfer.

[1]) (Number of explants producing adventitious embryos or embryogenic calli on the E medium / Number of explants initially inoculated on the C medium) x 100. Values represent the mean ± SE of 3 independent experiments.

-: not examined.

min and rinsed 3 times with sterilized distilled water. Disinfected leaves were cut into pieces (ca. 1 cm^2) and used as explants. Flower buds were surface-disinfected in a 70 % ethanol solution for 30 s and then in a sodium hypochlorite solution (2 % active chlorine) for 20 min followed by 3 rinses with sterilized distilled water. Immature anthers and ovaries were dissected from the buds and used as explants.

Adventitious embryogenesis from cultured explants was induced according to the method of MATSUTA and HIRABAYASHI (1989) with several modifications. Explants were initially cultured on callus induction media (C media) containing half-strength MS basal medium, 3 % sucrose, 0.25 % gellan gum, and 10 µM auxin (2,4-D, DIC, NOA, PIC, or 2,4,5-T) with or without 10 µM cytokinin (CPPU or TDZ) (Tab. 2). All culture media used were adjusted to pH 5.8 prior to autoclaving. Petri dishes (90 x 20 mm) containing 30 ml of the C medium were used for the culture of 20 leaf explants, 50 to 60 anther explants, or 20 ovary explants per dish. Cultures were maintained at 25 °C in the dark for 2 months.

Explants which formed calli were then transferred to an embryogenesis induction medium (E medium) containing half-strength MS basal medium, 1 µM 2,4-D, 3 % sucrose, and 0.25 % gellan gum. Petri dishes (90 x 20 mm) containing 30 ml of E medium were used for the culture of 20 to 25 explants per dish, and cultures were maintained at 25 °C in the dark. Percentage of adventitious embryogenesis 6 months after transfer was calculated as follows: (number of explants which produced adventitious embryos or embryogenic calli on E medium / number of explants initially inoculated on C medium) x 100. Adventitious embryos and embryogenic calli were monthly subcultured to the fresh E medium under the same conditions.

Embryogenic calli and adventitious embryos were transferred for embryo induction and germination, respectively, to test tubes (2.5 x 12 cm) containing 10 ml of half-strength MS basal medium lacking plant growth regulators but with 3 % sucrose and 0.25 % gellan gum. Cultures were incubated at 25 °C under continuous illumination (35 µmol m^{-2} s^{-1}) with fluorescent lamps. Plantlets (i.e., germinated adventitious embryos with roots and shoots) were carefully washed with tap water to remove gellan gum and transferred to pots (6 x 6 cm) containing vermiculite. They were acclimatized in a plastic cabinet covered with a polyethylene bag at 25 °C under continuous illumination (50 µmol m^{-2} s^{-1}) with fluorescent lamps. After 2 to 3 weeks, plants were transferred to the greenhouse.

Results

Induction of adventitious embryogenesis: We first examined the effect of plant growth regulators in the initial callus induction medium (C medium) on the induction of subsequent adventitious embryogenesis using leaf explants of the 4 cultivars listed in Tab. 2. All combinations of plant growth regulators tested in the present study allowed to induce calli within two months on C medium from leaf explants of all 4 cultivars (data not shown). Explants which produced calli were transferred to the E medium, on which adventitious embryogenesis, i.e. development of adventitious embryos and/or embryogenic calli on the primary calli, occurred 4-6 months after transfer. Adventitious embryos were white and exhibited various stages of development, whereas embryogenic calli were white to pale yellow and had a granular appearance (Fig. 1). Both adventitious embryos and embryogenic calli often developed concurrently from the same explants. Adventitious embryogenesis was obtained only with *V. vinifera* cultivars Koshusanjaku and Neo Muscat (Tab. 2). In both cultivars, cytokinins in the C medium were necessary for inducing subsequent adventitious embryogenesis, and neither adventitious embryos nor embryogenic calli were produced from leaf explants initially cultured on C media con-

Table 2

Effect of plant growth regulators in the C medium on the induction of subsequent adventitious embryogenesis from leaf explants of 4 grapevine cultivars (*Vitis* spp.)

Plant growth regulators (μM)							Adventitious embryogenesis (%)[1]			
							V. vinifera		*V.* x *labruscana*	
2,4-D	DIC	NOA	PIC	2,4,5-T	CPPU	TDZ	Koshusanjaku	Neo Muscat	Delaware	Kyoho
10	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	10	0	2.8 ± 0.7	0.3 ± 0.3	0	0
10	0	0	0	0	0	10	3.2 ± 1.6	0	0	0
0	10	0	0	0	0	0	0	0	0	0
0	10	0	0	0	10	0	4.1 ± 2.1	0	0	0
0	10	0	0	0	0	10	5.6 ± 1.6	0.3 ± 0.3	0	0
0	0	10	0	0	0	0	0	0	0	0
0	0	10	0	0	10	0	4.8 ± 2.1	0	0	0
0	0	10	0	0	0	10	9.6 ± 2.7	0	0	0
0	0	0	10	0	0	0	0	0	0	0
0	0	0	10	0	10	0	0.8 ± 0.3	0.3 ± 0.3	0	0
0	0	0	10	0	0	10	2.3 ± 1.3	0	0	0
0	0	0	0	10	0	0	0	0	0	0
0	0	0	0	10	10	0	31.6 ± 4.5	0.5 ± 0.3	0	0
0	0	0	0	10	0	10	23.7 ± 8.9	0.3 ± 0.3	0	0

Leaf explants were initially cultured on C media containing various plant growth regulators for 2 months, and explants which formed calli were then transferred to the E medium. Data were recorded 6 months after transfer.

[1]) (Number of explants producing adventitious embryos or embryogenic calli on the E medium / Number of explants initially inoculated on the C medium) x 100. Values represent the mean ± SE of 3 independent experiments.

taining only auxins. No difference in the percentage of adventitious embryogenesis was observed between the culture on the C medium containing CPPU and that containing TDZ. However, adventitious embryos tended to be induced from leaf explants initially cultured on CPPU-containing media, while embryogenic calli were preferentially induced from those initially cultured on TDZ-containing media (data not shown). In Neo Muscat, no difference in the percentage of adventitious embryogenesis (0 to 0.5 %) was observed among the auxins tested in the C medium. On the other hand, in Koshusanjaku, 2,4,5-T in the C medium most efficiently induced subsequent adventitious embryogenesis, and 31.6 and 23.7 % of leaf explants initially cultured on C media containing 10 μM 2,4,5-T in combination with 10 μM CPPU or TDZ, respectively, produced adventitious embryos or embryogenic calli.

We then made a comparison of adventitious embryogenesis from different explant sources using the 23 cultivars listed in Tab. 1. Leaf, anther, and ovary explants were initially cultured on the C medium containing 10 μM each of 2,4,5-T and CPPU. Within two months of culture on this medium, most of the leaves and ovaries produced calli, whereas many anthers turned brown and only a few anthers produced calli (data not shown). After transfer of the explants which produced calli to the E medium, adventitious embryos or embryogenic calli were induced in 9 *V. vinifera* cultivars (Koshusanjaku, Merlot, Neo Muscat, Riesling, Riesling Lion, Rosario Bianco, Ruby Okuyama, Sekirei, and Sémillon) and one *V.* x *labruscana* cultivar (Delaware). In the cultures of leaf explants, the highest percentage of adventitious embryogenesis was obtained in *V. vinifera* Koshusanjaku (28.3 %) among the 23 cultivars examined. Efficiency of the induction of adventitious embryogenesis was also affected by the explant source. Anthers and ovaries were more suitable for inducing adventitious embryogenesis than leaves, and adventitious embryogenesis was induced only when anther and ovary explants were used in *V. vinifera* Rosario Bianco, Ruby Okuyama, and Sekirei, and *V.* x *labruscana* Delaware. In general, percentage of adventitious embryogenesis from ovary explants was slightly higher than that from anther explants.

Maintenance and plant regeneration of embryogenic cultures: Most of the embryogenic calli could proliferate after transfer to the fresh E medium, and some of them occasionally developed globular embryos on this medium. On the other hand, adventitious embryos transferred to the fresh E medium exhibited two types of growth pattern: one was the proliferation of adventitious embryos *via* direct secondary embryogenesis and the other was the development of embryogenic calli after browning of the embryos. Adventitious embryos which were derived from explants initially cultured on CPPU-containing C media tended to exhibit the former growth pattern.

Embryogenic calli subcultured on the E medium produced numerous adventitious embryos 2-3 weeks after transfer to a plant growth regulator-free medium (Fig. 2). Adventitious embryos both derived from embryogenic calli and proliferated with secondary embryogenesis on the E medium germinated into plantlets on this medium (Fig. 3). In addition, development of secondary adventi-

tious embryos from both adventitious embryos and adventitious embryo-derived plantlets was frequently observed on the plant growth regulator-free medium. Adventitious embryo-derived plantlets could readily be transferred to the greenhouse (Fig. 4), and they exhibited a normal phenotype with respect to leaf shape and growth habit at early stages of development.

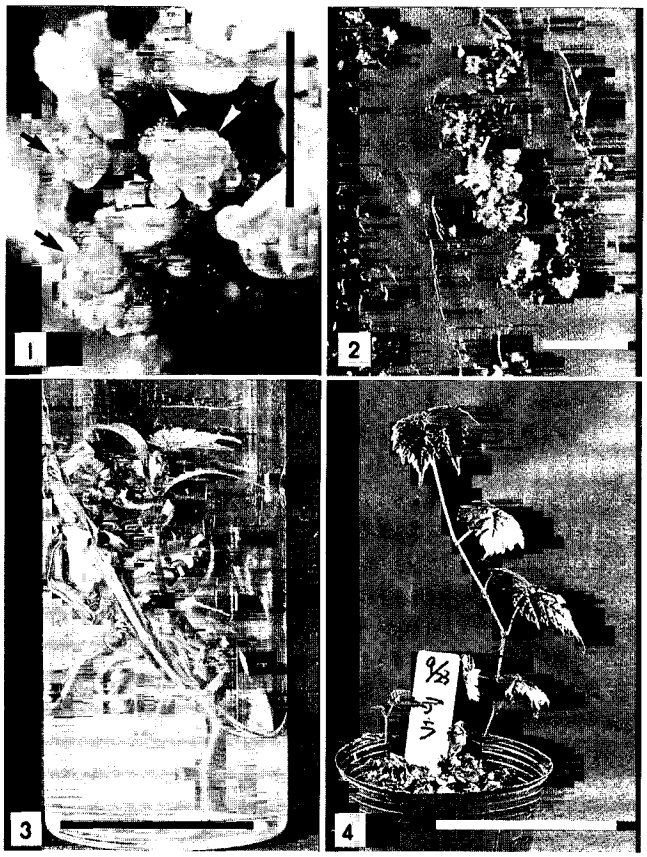

Fig. 1: Adventitious embryos (arrows) and embryogenic calli (arrow heads) arising from a leaf explant of *Vitis vinifera* Neo Muscat. Bar = 3 mm.

Fig. 2: Numerous adventitious embryos developed from embryogenic calli of *V.* x *labruscana* Delaware. Bar = 1 cm.

Fig. 3: Germination of adventitious embryos of *V.* x *labruscana* Delaware. Bar = 2 cm.

Fig. 4: An adventitious embryo-derived plant of *V.* x *labruscana* Delaware growing in the greenhouse. Bar = 5 cm.

Embryogenic cultures established in 10 cultivars, *V. vinifera* Koshusanjaku, Merlot, Neo Muscat, Riesling, Riesling Lion, Rosario Bianco, Ruby Okuyama, Sekirei, and Sémillon and *V.* x *labruscana* Delaware, have so far been maintained with a high regeneration capacity for over 20 months through both callus proliferation and direct secondary embryogenesis on the E medium.

Discussion

To date, embryogenic cultures of grapevines have been established only in a limited number of genotypes. In the present study, we examined several factors affecting adventitious embryogenesis from cultured explants of grapevines, and newly established embryogenic cultures in several commercially important grapevine cultivars, such as the wine cultivars Merlot and Sémillon, and the table grape cultivars widely cultivated in Japan, Neo Muscat, Rosario Bianco, Ruby Okuyama, Sekirei, and Delaware.

Both auxins (MATSUTA 1992; PERL *et al.* 1995; TORREGROSA *et al.* 1995) and cytokinins (MATSUTA and HIRABAYASHI 1989) have been reported to affect adventitious embryogenesis from cultured explants of grapevines. Among auxins, 2,4-D has been preferentially used for inducing adventitious embryogenesis in grapevines (GRAY and MEREDITH 1992). However, other auxins such as NOA (PERL *et al.* 1995) and 2,4,5-T (MATSUTA 1992) have occasionally been used and, in some cases, provided better results than 2,4-D. In the present study, 2,4,5-T was most efficient for inducing adventitious embryogenesis from leaf explants of Koshusanjaku, which is in agreement with a previous report (MATSUTA 1992). However, no difference in the percentage of adventitious embryogenesis from leaf explants of Neo Muscat was observed among the auxins tested in the present study, and furthermore, 2,4-D has previously been reported to be superior to 2,4,5-T for inducing adventitious embryogenesis from leaf explants of intergeneric hybrid grapevines (TORREGROSA *et al.* 1995). Therefore, combinations and concentrations of plant growth regulators optimal for inducing adventitious embryogenesis may vary among the genotypes and also among explant sources as suggested by STAMP and MEREDITH (1988 b).

Adventitious embryogenesis of grapevines have successfully been induced from various explants including anthers, ovules, ovaries, zygotic embryos, and leaves (KRUL and WORLEY 1977; GRAY and MORTENSEN 1987; STAMP and MEREDITH 1988 a, b; MATSUTA and HIRABAYASHI 1989; GRAY 1992; GRAY and MEREDITH 1992; MATSUTA 1992; MARTINELLI *et al.* 1993; PERL *et al.* 1995; TORREGROSA *et al.* 1995; FAURE *et al.* 1996). However, only few information is available on the effect of explant sources on adventitious embryogenesis due to the limited number of comparable studies using different explants (GRAY and MORTENSEN 1987; STAMP and MEREDITH 1988 b). In the present study, immature anthers and ovaries were proved to be superior to leaves as explant sources for inducing adventitious embryogenesis in a range of grapevine cultivars. Especially, ovaries showed a slightly higher percentage of adventitious embryogenesis than anthers and seem to be more useful than anthers because of the easiness to prepare explants. Suitability of immature ovaries as an explant source has also been reported for *V. longii* Microsperma (GRAY and MORTENSEN 1987). Although the origin of adventitious embryos and embryogenic calli induced from immature anthers and ovaries was not clarified in the present study, a somatic rather than gametic origin has been demonstrated for adventitious embryos and embryogenic calli derived from anthers (RAJASEKARAN and MULLINS 1983; FAURE *et al.* 1996) and unfertilized ovules (MULLINS and SRINIVASAN 1976). Therefore, it is natural to consider that the embryogenic cultures obtained in the present study also originated from somatic tissues.

It is clear from the present and previous studies (STAMP and MEREDITH 1988 b) that there is a marked difference in the ability for adventitious embryogenesis among differ-

ent grapevine genotypes. Results from the present study show that *V. vinifera* genotypes generally have a relatively high embryogenic ability as compared to the genotypes of *V.* x *labruscana*, in which adventitious embryogenesis cannot yet be obtained with most cultivars such as Kyoho, a predominant cultivar in Japan. Therefore, continued effort is needed to identify the factors which will increase the efficiency and broaden the genotypic range for inducing adventitious embryogenesis.

Embryogenic cultures established in the present study have high proliferation and regeneration abilities for over 20 months. They seem to be suitable materials not only for micropropagation of desired genotypes but also for *Agrobacterium*- or particle gun-mediated genetic transformation.

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