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Agrobacterium-mediated transformation and regeneration of transgenic 'Chancellor' wine grape plants expressing the *tfdA* gene

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Summary

The establishment of grape vineyards in the Midwestern USA is greatly hampered by the use of the herbicide 2,4-D in grain crop fields. The overall goal of this study was to transform 'Chancellor' with the plant expressible bacterial *tfd*A gene to make it tolerant to 2,4-D. Embryogenic callus was infected and co-cultivated with an *Agrobacterium* construct (LBA4404: pAL4404::*tfd*A); transformed callus was selected with kanamycin; kanamycin resistant callus was bulked and transformed cell lines identified by PCR. Three PCR-positive embryogenic callus lines were used to regenerate transgenic plants. Analysis of the plant lines for the presence of the *tfd*A gene by PCR and southern hybridization confirmed its stable integration in their genomes. Transgenic 'Chancellor' grape plants regenerated from these calli proved to be resistant to up to 10 kg·ha^{-1} of a commercial ester-formulation of 2,4-D, indicating positive expression of the *tfd*A gene and affording them protection from 2,4-D injury.

K e y w o r d s : 'Chancellor' grapes, somatic embryogenesis, tfdA gene, genetic transformation, 2,4-D = 2,4-dichlorophenoxyacetic acid, herbicide resistance.

Introduction

To improve popular grape cultivars worldwide genetic engineering is currently being applied to incorporate novel traits. So far these efforts have yielded transgenic *Vitis* cultivars with improved disease resistance, fruit quality, environmental fitness and herbicide tolerance; these are at various stages of field testing prior to release for commercial production (Vivier and Pretorius 2002).

One of the major challenges facing grape growers in the Midwest and Southern United States is widespread vine damage caused by the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). 2,4-D is an effective, safe and widely used herbicide for the control of broadleaf weeds in grain crops. However, some formulations of the herbicide have a tendency to volatilize and drift over long distances from the target fields, causing damage in sensitive crops including grapes. Since there is no known 2,4-D resistance in grape germplasm, genetic engineering to incorporate 2,4-D breakdown genes in important adapted commercial grape cultivars could be used as an avenue to improve grape survival in these regions.

A soil bacterium, *Ralstonia eutrophus*, contains a plasmid (JMP134) that codes for a 2,4-D breakdown pathway which enables this organism to detoxify and utilize the herbicide as a carbon source (Don and Pemberton 1981). The first gene (*tfd*A) in the 2,4-D breakdown pathway of *R. eutrophus* was cloned (Don *et al*. 1985) and found to encode a 2,4-D α-ketoglutarate dioxygenase which catalyzes the removal of the acetate side chain of 2,4-D, yielding 2,4-dichlorophenol and glyoxylate (Fukumori and Hausinger 1993); these compounds are much less toxic to plant cells than 2,4-D. Expression of the *tfd*A gene in cotton and tobacco has conferred tolerance to up to ten and twenty times the recommended 2,4-D application rates in corn, respectively (Lyon *et al*. 1993; Last and Llewellyn 1999).

In a previous paper we described an efficient somatic embryogenesis (SE) system for regenerating 'Chancellor' grape plants. In this paper we report on genetic transformation studies to incorporate the *tfd*A gene of *Ralstonia eutrophus* into 'Chancellor' wine grape to produce transgenic plants that will tolerate accidental exposure to 2,4-D drift utilizing the SE system developed earlier.

Material and Methods

T a r g e t e x p l a n t s : Embryogenic callus from the *Vitis* complex hybrid 'Chancellor' cultivated in long-term maintenance medium (LTMM, Mulwa *et al*. in press [NN (Nitsch and Nitsch 1969) medium + 2 μM 2, 4-D + 0.2 μM TDZ + 4 μM IASP]) for 5-6 weeks was used as the target tissue for transformation. This embryogenic callus was originally initiated from ovary explants collected 10-14 d before anthesis on green house-forced cuttings.

D e t e r m i n a t i o n o f s e l e c t i o n p r e s s u r e : To determine an appropriate kanamycin concentration to select transformed embryogenic cells, embryogenic calli, approximately 5 mm in diameter, were cultivated in LTMM containing 250 mg·l^{-1} carbenicillin combined with either of 0, 100, 150, 200, 250, 300, 350 or 400 mg·l^{-1} kanamycin. Preliminary trials indicated that 'Chancellor' embryogenic callus grew uninhibited at 50 and 100 mg·l^{-1} kanamycin,

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respectively, so higher levels of kanamycin were tested. Growth of calli was assessed by measuring callus diameters at the surface of contact with the media 5 weeks after explanting. Three replicate plates per kanamycin treatment, each containing 10 calli, were set up in a completely randomized design (CRD). Mean callus diameters were computed and used to construct a kanamycin sensitivity regression curve. The highest kanamycin level that inhibited callus growth was selected and used in all subsequent transformation experiments to select transformed cells.

Preparation of Agrobacterium transformation vector: A glycerol stock of *A. tumefaciens* strain LBA4404:pAL4404::*tfd*A was streaked on MGIL-agar medium (Maniatis *et al*. 1982) containing 100 μg·ml^{-1} kanamycin and incubated for 2 d at 28 °C. A single colony was purified on similar medium under the same incubation conditions. Eight purified colonies were inoculated into tubes containing 2 ml of MGIL medium with 100 μg·ml^{-1} kanamycin and cultured overnight on a rotary incubator-shaker (120 rpm) at 28 °C. The cultures were centrifuged at 5,000 rpm (4 °C) for 10 min and resuspended in 5 ml of liquid NN medium containing 100 μg·ml^{-1} kanamycin and grown to an O.D. of 70-100 Klett Units (KU). Samples (500 μl) of each culture were inoculated into 25 ml of liquid LTMM in 50 ml plastic centrifuge tubes and grown to an $O.D._{600}$ of 0.6. Acetosyringone (final concentration of 200 μM) and 0.05 % v/v of Pluronic F-68 (Sigma-Aldrich, USA) were added to activate the *Agrobacterium* 2 h before infecting grape cells.

Infection and co-cultivation of grape embryogenic callus with Agrobacterium: Twenty embryogenic calli (4 mm diameter) were submerged in each of the 8 bacterial suspension cultures prepared above and incubated at room temperature with gentle swirling for 20 min. Calli were blotted dry on sterile paper towels and transferred into Petri plates (100 mm diameter) containing co-cultivation medium consisting of LTMM with 200 μM acetosyringone and 2.5 % w/v polyvinylpolypyrrolidone (PVPP) overlaid with filter paper bridges. Co-cultivation was carried out for 48 h at 27 °C in the dark.

Incubation of co-cultivated embryogenic callus on recovery medium and bulking of recovered callus: Prohibitively high rates of cell necrosis were observed within a few days of transferring co-cultivated embryogenic callus to selection media. To improve the survival of co-cultivated embryogenic cultures we compared the effects of the conventional direct transfer to selection media to use of a pre-selection stage in which *Agrobacterium*-co-cultivated calli were placed on media containing antioxidants as recommended by Perl *et al.* (1996). Co-cultivated calli were washed twice in sterile distilled water and transferred either directly to selection medium (no pre-selection treatment) containing 300 mg·l^{-1} kanamycin and 250 mg·l^{-1} each of carbenicillin and timentin (to kill *Agrobacterium*) or to embryogenic callus recovery media (CRM) consisting of gelled LTMM supplemented with 250 mg·l^{-1} each of carbenicillin and timentin and 2.5 % w/v PVPP (medium designated CRM1) or gelled LTMM containing 2.5 % w/v PVPP supplemented with 250 mg·l^{-1} each of carbenicillin and timentin and overlaid with filter paper bridges moist-saturated with a 5 ml solution of 0.01 % v/v dithiothreitol (DTT) and 250 mg·l^{-1} timentin (medium designated CRM2). Filter paper bridges were used to stabilize the callus colonies as earlier set-ups had problems with the callus dispersing in the liquid phase. Eight plates containing 10 callus colonies each were set up per treatment. Plates were sealed with parafilm® and incubated at 25 °C in the dark. Callus survival and growth were assessed after 3 weeks.

Calli recovered from the 8 CRM2 plates were transferred to fresh plates containing selection/ bulking medium consisting of LTMM supplemented with 250 mg·l^{-1} timentin and 300 mg·l^{-1} kanamycin. The cells surviving this initial selection process were collected from each plate and used to select and bulk up 8 putative transformed kanamycin resistant callus lines. The 8 cell lines were subcultured 7 times at 5-6 week intervals (35-42 weeks all tolled). Under this kanamycin pressure virtually all wild type cells died. Since our regeneration system involved somatic embryogenesis, with no obvious sign of organogenesis, we believe that each cell line was of single cell origin and not chimeral in nature.

PCR analysis of recovered embryogenic callus lines: Total genomic DNA was isolated from 100 mg of each of the putative transformed and wild-type control callus lines using DNeasy plant mini kits (Qiagen Inc., USA) according to the manufacturer's instructions. To identify transformed callus lines, 0.5 μg of DNA were subjected to the polymerase chain reaction (PCR) using *tfd*A gene-specific primers (Forward: 5'-CTAGACGACGGCATCGTCCAG-3'; Reverse: 5'-GTGAGCGTCGTCGCAAATCC-3'). PCR-positive callus lines were multiplied further by 4 serial subcultures in LTMM containing 350 mg·l^{-1} kanamycin and used to regenerate transgenic plants.

Plant regeneration from transformed callus: Three PCR positive embryogenic and control callus cell lines were placed in embryo development and maturation medium (EDMM, NN medium + 10 μM IASP + 8 μM NOA + 1 μM TDZ + 1 μM ABA + 2.5 g·l^{-1} activated charcoal [AC, DARCO-S5]). Mature embryos were germinated and converted into plantlets on ½ MS (Murashige and Skoog 1962) medium containing 0.5 μM benzyladenine (BA) + 0.025 μM naphthaleacetic acid (NAA) supplemented with 350 mg·l^{-1} kanamycin. Converted plantlets obtained from each of the cell lines were multiplied by clonal axillary proliferation in ½ MS medium supplemented with 0.5 mg·l^{-1} BA to develop populations for spray tests. Micropropagated plantlets were rooted in 30 ml of growth regulator-free ½ MS medium contained in baby food jars (50 x 100 mm) and acclimatized in a laboratory under cool white lights. All micropropagated plants tested positive in subsequent PCR tests with the *tfd*A-specific primers.

Southern hybridization: Total genomic DNA was isolated from one g of young expanding leaves harvested from 3 randomly selected plants representing

3 independently transformed regenerated plant lines (designated Ch1, Ch2 and Ch3) and a control plant using the method of Lodhi *et al*. (1994) with minor modifications. Ten micrograms of DNA were digested overnight with *Eco*RI (Life Technologies Inc, USA), separated on a 0.8 % agarose gel and transferred by capillarity onto a positively charged nylon membrane (Roche Diagnostics, Germany). The membrane was hybridized with a probe made from a PCR gel purified *tfd*A band labeled with digoxigen (DIG, Roche Diagnostics, Germany). Subsequent washes and chemiluminiscent detection of hybridized bands was performed according to the Roche Applied Science (2004) Instruction Manual.

Phenotypic 2,4-D spray tests: Two transgenic plant lines (Ch1 and Ch3) and wild-type controls were used in spray tests. The two plant lines were selected because sufficient plants had been regenerated from them to enable the setting up of a balanced spray trial. Four plants from each line were treated with each of 4 2,4-D rates (0, 0.5, 5, and 10 kg·ha^{-1}) of a commercial ester-formulation of 2,4-D (LV400, Growmark Inc., USA) in a spray chamber. Treated plants were allowed to air-dry and transferred to an isolation greenhouse where they were observed for damage over a period of three weeks.

Results and Discussion

Determination of selection pressure: Since transformation is a rare event, an antibiotic or herbicide resistance gene is usually incorporated in the transformation vector to aid the selection of transformed cells by culturing them in media containing appropriate antibiotics. In the present study the kanamycin resistance gene (*npt*II) incorporated in the T-DNA of vector pBIN19 was used as the selection marker gene. Thus all transformed cells should contain the *npt*II gene and be positively selected on media containing kanamycin.

To establish the optimum concentration of kanamycin for selection of transformed cells, 'Chancellor' embryogenic callus was cultured on LTMM containing various concentrations of the antibiotic and assessed for growth inhibition. Results indicated that growth of 'Chancellor' embryogenic callus was suppressed at kanamycin levels above 300 mg·l^{-1} (Fig. 1). Therefore in subsequent selection and bulking of transformed callus studies kanamycin was incorporated in the selection medium at 300 mg·l^{-1} for the first three subcultures and then increased to 350 mg·l^{-1} in the next 4 subcultures. Overall, these observations confirmed reports by Kikkert *et al.* (1996) that 'Chancellor' embryogenic callus can tolerate higher levels of kanamycin than other cultivars whose callus growth is suppressed at very low concentrations.

Effects of culture treatments on recovery of Agrobacterium-infected embryogenic cell lines: In conventional transformation experiments, co-cultivated explants are usually rinsed in sterile water and transferred directly to regeneration/ selection media. However, there is wide consensus

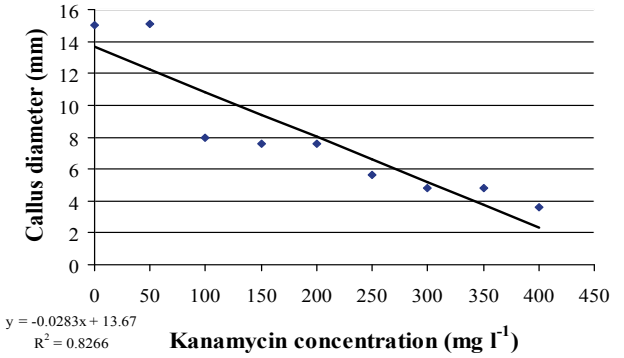

Fig. 1: Regression graph of the effect of kanamycin concentrations on the growth of 'Chancellor' grape embryogenic callus measured as colony diameter after 5-6 weeks of cultivation.

that *Agrobacterium* co-cultivated plant cells (Kuta and Tripathi 2005), and grape cells in particular (Deng *et al*. 1995; Perl *et al*. 1996), are negatively affected by *Agrobacterium* infection leading to considerably lower recovery rates of transformed plants. Perl *et al.* (1996) mitigated this difficulty by developing a double layer culture system incorporating various antioxidants. After co-cultivation, they placed the cell colonies in a liquid layer of DTT solution overlaying a semi-solid medium containing PVPP for 7 d then transferred them to selection media. In this manner they obtained up to 63 % recovery of transformed callus that regenerated into viable plantlets from several *V. vinifera* cultivars. Perl *et al*. (1996) also reported that the inclusion of antioxidants in their media interfered with kanamycin resistance selection because their grape cells appeared to gain tolerance to kanamycin when the antioxidants were present. However, both Motoike *et al.* (2002) and Das *et al.* (2002) have used their system to recover viable transformed callus of *Vitis* x *labruscana* and *V. vinifera,* respectively, but neither observed the kanamycin insensitivity problem reported by Perl *et al*. (1996).

In the present study, we sought to determine if antioxidants could also improve the recovery of 'Chancellor' embryogenic callus. To this end the conventional method of direct transfer of washed co-cultivated callus to selection media was compared with two modified culture regimes based on Perl *et al.* (1996). In one treatment co-cultivated embryogenic callus was placed on selection medium with PVPP only (designated CRM1); a second treatment had callus placed on selection medium with PVPP but overlaid with filter paper bridges saturated with 0.01 % DTT (designated CRM2). After 3 weeks a significant variation in the regrowth response of co-cultivated embryogenic callus was observed. On average 46.3 % of the calli incubated in CRM2 had initiated regrowth compared to 13.8 % and 5 %, respectively, from CRM1 and the conventional method (Fig. 3 a). Callus colonies placed in straight selection medium or pre-cultured on CRM1 became necrotic within a few days. Our results further confirm the usefulness of the technique by Perl *et al.* (1996) and show that the two antioxidants (PVPP and DTT) act synergistically to induce regrowth of grape embryogenic callus after co-cultivation. Thus this system provided adequate proliferation of the

transformed callus with no obvious increase in tolerance of 'Chancellor' embryogenic callus to kanamycin, *i.e.* 'Chancellor' showed no tendency to habituate to kanamycin tolerance during the course of our experiments.

The hypersensitive response of grape embryogenic cultures to *Agrobacterium* exposure could be a natural plant host/ pathogen interaction between *Agrobacterium* and grape cells. Plant cells respond to microbial infection by a defense mechanism that involves the generation of super-active oxygen species, the accumulation of which leads to cell death and tissue necrosis (Bolwell and Wojtszek 1997). Therefore, the addition of antioxidants in the medium could be quenching the highly reactive oxygen species, hence improving cell survival and subsequent transformed callus recovery.

Selection of transformed embryogenic callus and screening for *tfd*A gene: Callus recovered from CRM2 was successfully bulked in a stepwise selection process in media containing 300 and 350 mg·l^{-1} kanamycin, respectively, to develop 8 independently transformed callus lines. In the first step each callus line was multiplied in selection medium containing 300 mg·l^{-1} kanamycin for 3 serial subcultures at 4-week intervals followed by a second step selection in medium containing 350 mg·l^{-1} for 4 subcultures at 5-week intervals. DNA extracted from samples of each of the embryogenic callus lines and analyzed by PCR with primers specific to the *tfd*A gene revealed the presence of the gene in 3 out of the 8 putative kanamycin-resistant cell lines (Fig. 2). The three PCR positive embryogenic cell lines were designated Ch1, Ch2 and Ch3, respectively, bulked further in selection medium and used to regenerate transgenic plants.

Regeneration of transgenic plants: Embryogenic calli from the 3 transformed lines were transferred to EDMM containing 350 mg·l^{-1} kanamycin for somatic embryo development. A profusion of mature somatic embryos was observed within 4 weeks of transfer to this medium (Fig. 3 b). The kanamycin resistance status of the transformed embryos was determined

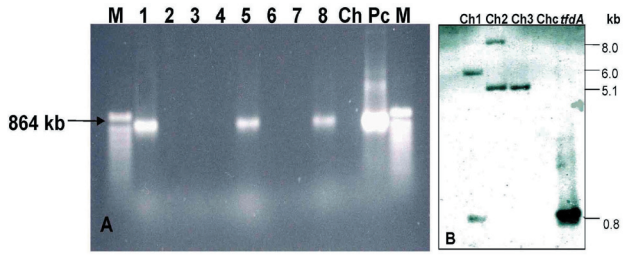

Fig. 2: **(A)** PCR Screening for the presence of the *tfd*A gene in 'Chancellor' grape embryogenic callus. DNA was extracted from 8 kanamycin-resistant embryogenic callus lines (lanes 1 to 8) and a non-transformed control line (lane Ch) and primed with *tfd*A-specific primers. Lane PC is plasmid pBIN19:pAL4404::*tfd*A positive control. Lanes marked M are 1 Kb ladder. **(B)** Southern hybridization of genomic DNA isolated from leaves of 3 independently transformed (Ch1, Ch2 and Ch3) and one non-transformed control (Chc) 'Chancellor' grape plants. DNA (10 µg) was digested with *Eco*RI and separated on 0.8 % agarose gels. Hybridization was performed with a DIG-labeled 864 bp *tfd*A-specific PCR gel-extracted fragment.

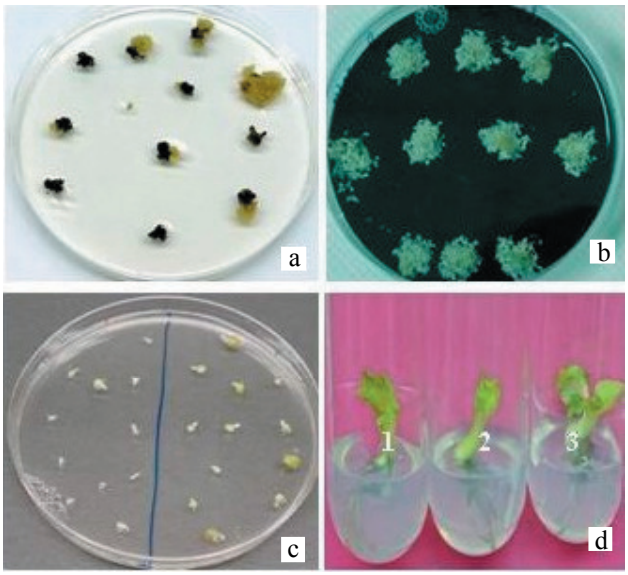

Fig. 3: Recovery of *Agrobacterium* transformed embryogenic callus and regeneration of transgenic 'Chancellor' grape via somatic embryogenesis. **a**) embryogenic callus on recovery medium overlaid with DTT-saturated filter paper showing re-growth; **b**) embryo development and maturation in medium containing activated charcoal; **c**) embryo germination on medium containing kanamycin: left- control embryos showing bleaching; right - transformed greening embryos; **d**) embryo teratologies: 1 - monocotyledonary development; 2 - fused cotyledons; 3 - failed meristem development.

by germination trials with both transformed and non-transformed somatic embryos in media with or without kanamycin. Up to 100 % of the transformed embryos germinated and displayed cotyledon expansion, greening, and radicle development (Fig. 3 c, right) while their non-transformed control counterparts only exhibited initial cotyledon expansion but they failed to develop chlorophyll, a normal response of non-transformed plant tissues to kanamycin (Fig. 3 c, left). Transformed somatic embryos developed into plantlets but non-transformed embryos showed no further development on germination and conversion medium containing kanamycin. However, non-transformed somatic embryos cultured in germination medium without kanamycin (control) germinated and developed into plantlets.

During embryo conversion several teratologies were observed among both transgenic and non-transgenic embryos. The most frequent of these abnormalities were monocotyledonary embryos, embryos developing fused cotyledons and the failure of converting embryos to develop functional shoot meristems (Fig. 3 d). These abnormalities have been cited as the most limiting factors in grape regeneration by somatic embryogenesis (Goebel-Tourand *et al*. 1993). Successfully converted embryos from both transgenic and control lines were multiplied by micropropagation to develop sufficient plants for further *ex vitro* analyses.

Analysis of transgene integration in regenerated plants: Transgene integration and copy number was assessed using southern blots of *Eco*RI digested DNA extracted from leaves of single randomly selected plants from transgenic and control plant

lines. Hybridization performed with a Dig-labeled *tfd*A PCR gel extracted band showed positive hybridization bands in transgenic plant lines only (Fig. 2 b). The *tfd*A probe produced two fragments in lines Ch1 (5.8 kb and 0.87 kb) and Ch2 (10 kb and 5.3 kb) and one fragment in line Ch3 (5.3 kb), respectively (Fig. 2 b). These results suggest the integration of two independent copies of the *tfd*A gene in lines Ch1 and Ch2 and one copy in line Ch3. All the hybridization bands obtained with the *tfd*A probe were larger than or equal to the total length of the *tfd*A gene, an indication that in all events intact copies of gene had been stably integrated into the plant genomes. Similar results had been obtained in independent preliminary southern hybridization analyses of the cell lines from which the plants were regenerated (data not shown), indicating that the cell lines had originated from single transformed cells.

Assessment of the 2,4-D resistance phenotype in transgenic and control lines: The results of the spraying trial for micropropagated T_o transformants of lines Ch1, Ch3 and control plants variously displayed tolerance and susceptibility to the herbicide. Whereas non-transformed wild-type plants were killed at all the 2,4-D rates tested within 7 d of treatment, transformed plants survived the highest 2,4-D rate (10 kg ha^{-1}). Control plants sprayed with 2,4-D began exhibiting symptoms two h after treatment. The early symptoms included wilting of leaves, which was followed 24 h later by the collapse of the growing tips of plants. All control plants were dead within one week of 2,4-D treatment (Fig. 4). Three Ch1 and two Ch3 transgenic plants, respectively, sprayed at the 5 and 10 kg·ha^{-1} 2,4-D rates, developed minor short-lived injury characterized by leaf epinasty for up to 7 d. However, these plants recovered from this initial shock and by the end of two weeks were growing normally.

Hellman (1987) reports that 'Chancellor' is "moderately sensitive" to 2,4-D under field conditions. In our greenhouse studies we found non-transformed control 'Chancellor' grapes were extremely sensitive to 2,4-D exposure as demonstrated by the quick death of plants even at the lowest rate of application. However, the transformed plants survived 2,4-D application at rates 20 times that recommended for application to corn (Anderson 1996). We presume these unique grapes will be able to grow and produce fruit normally without being damaged by 2,4-D spray drifts in the environment. To our knowledge, this is the first report of genetic engineering of 2,4-D resistance into grapes.

Acknowledgements

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References

Anderson, W. P.; 1996: Weed Science Principles and Applications. 3rd edition. West Publishing Company, New York.

Bolwell, G. P.; Wojtszek, P.; 1997: Mechanisms for the generation of reactive oxygen species in plant defense- a broad perspective. Physiol. Mol. Plant Pathol. **51**, 347-366.

Das, D.; Reddy, M.; Upadhaya, S.; Sopory, S.; 2002: An efficient leaf disc culture method for the regeneration via somatic embryogenesis and transformation of grape (*Vitis vinifera* L.). Plant Cell Rep. **20**, 999-1005.

Deng, W.; PU, X. A.; Goodman, R. N.; Gordon, M. P.; Nester, E. W.; 1995: T-DNA genes responsible for inducing a necrotic response on grape vines. Mol. Plant Microbe Interact. **8**, 538-548.

Don, R. H.; Pemberton, J. M.; 1981: Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. J. Bacteriol. **145**, 681-686.

Don, R. H.; Weightman, A. J.; Knackmuss, H. J.; Timmis, K. N.; 1985: Transposon mutagenesis and cloning analysis of the pathways for

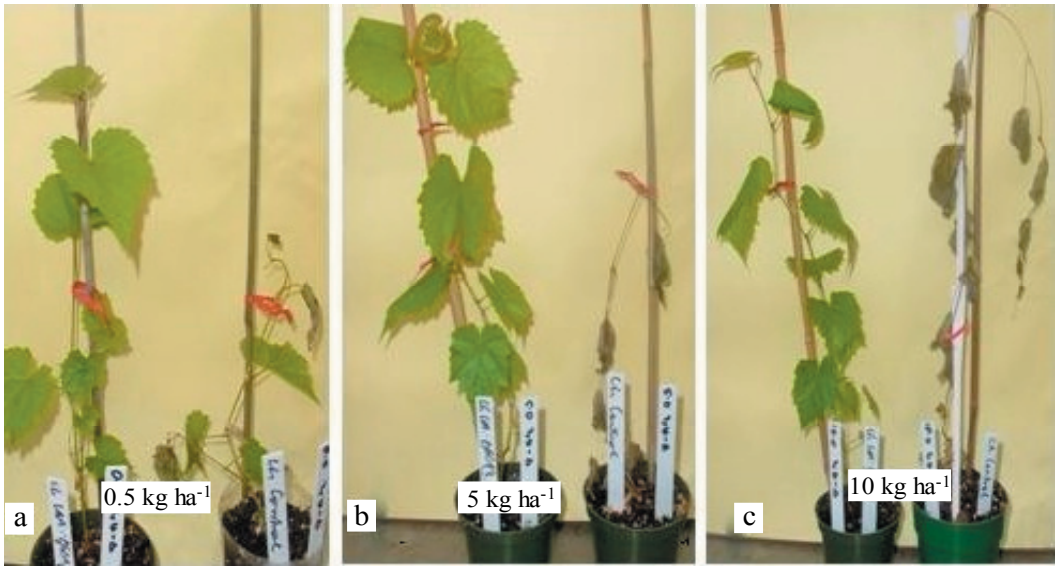

Fig. 4: Responses of transgenic and control 'Chancellor' grape plants to various 2,4-D spray rates 7 d after treatment. In each photographic set the plant on the left is a transgenic plant and the one on the right is a non-transformed control.

degradation of 2,4-dichlorophenoxyacetic acid and 3-chlorobenzoate in *Alcaligenes eutrophus* JMP134. J. Bacteriol. **161**, 85-90.

Fukumori, F.; Hausinger, R. P.; 1993: Purification and characterization of 2,4-dichlorophenoxyacetate α-ketoglutarate dioxygenase. J. Biol. Chem. **268**, 24311-24317.

Goebel-Tourand, I.; Mauro, M. C.; Sossountazov, L.; Miginiac, E.; Deloire, A.; 1993: Arrest of somatic embryo development in grapevine: histological characterization and the effect of ABA, BAP and zeatin in stimulating plantlet development. Plant Cell Tiss. Org. Cult. **33**, 91-103.

Hellman, E. W.; 1987: Cultivar sensitivity of grape to 2,4-D. Fruit Var. J. **41**, 111-114.

Kikkert, J. R.; Hébért-Soulé, D.; Wallace, P. G.; Striem, M. J.; Reisch, B. J.; 1996. Transgenic plantlets of 'Chancellor' grapevine (*Vitis sp*.) from biolistic transformation of embryogenic cell suspensions. Plant Cell Rep. **15**, 311-316.

Kuta, D. D.; Tripathi, L.; 2005: *Agrobacterium*-induced hypersensitive necrotic reaction in plant cells: a resistance response against *Agrobacterium*-mediated DNA transfer. Afr. J. Biotechnol. **4**, 752-757.

Last, D. I; Llewellyn, D. J.; 1999: A detoxification gene in transgenic *Nicotiana tabacum* confers 2,4-D tolerance. Weed Sci. **47**, 401-404.

Lodhi, M. A.; Ye, G. N.; Weeden, N. F.; Reisch, B. J.; 1994: A simple and efficient method for DNA extraction from grapevine cultivars and *Vitis* species. Plant Mol. Biol. Rep. **12**, 6-13.

Lyon, B. R.; Cousins, Y. L.; Llewellyn, D. J.; Dennis, E. S.; 1993: Cotton plants transformed with a bacterial degradation gene are protected from accidental spray drift damage by the herbicide 2,4-dichlorophanoxyacetic acid. Transgen. Res. **2**,162-169.

Maniatis, T.; Fritsch, E. F.; Sambrook, J.; 1982: Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Motoike, S. Y.; Skirvin, R. M.; Norton, M. A.; Otterbacher, A. G.; 2002: Development of methods to genetically transform American grape (*Vitis x labruscana* L. H. Bailey). J. Hortic. Sci. Biotechnol. **77**, 691-696.

Murashige, T.; Skoog, F; 1962: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15**, 473-497.

Nitsch, J. P.; Nitsch, C.; 1969: Haploid plants from pollen grains. Science **163**, 85-87

Perl, A.; Lotan, O.; Abu-Abeid, M.; Holland, D.; 1996: Establishment of an *Agrobacterium*-mediated transformation system for grape (*Vitis vinifera* L.): the role of antioxidants during grape-*Agrobacterium* interactions. Nat. Biotech. **14**, 1521-1525.

Roche Diagnostics; 2004: Dig DNA Labeling and Detection Kit - Instruction Manual. Version October 2004. Roche Applied Science, Germany.

Vivier, M. A.; Pretorius, J. S.; 2002: Genetically tailored grapevines for the wine industry. Trends Biotech. **20**, 472-478.

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