Regeneration and genetic transformation in the *Vitis* genus

Regeneratie en genetische transformatie in het genus *Vitis*

Rigenerazione e trasformazione genetica nel genere *Vitis*

Istituto Agrario di San Michele all'Adige
Laboratorio Biotecnologie

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Regeneration and genetic transformation in the *Vitis* genus

Proefschrift
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Stellingen

Propositions - *Massime*

1. Biotechnology is a innovative opportunity for genetic improvement in agriculture; it certainly will allow, in the near future, the solution of crucial world wide human questions. Among these being the adequacy of the food resources and the development of an agricultural practice more respectful of environmental issues.

   Le biotecnologie rappresentano un'opportunità innovativa per il miglioramento genetico in agricoltura e certamente daranno un contributo, nell'immediato futuro, alla soluzione di questioni cruciali per l'umanità quali, tra l'altro, l'adeguatezza delle risorse alimentari e lo sviluppo di un'agricoltura più rispettosa dell'ambiente.

2. Grape is one of the fruit crops with the highest "cultural" content, and studying this fascinating plant takes to explore fields far beyond the agricultural practice.

   La vite è una delle colture con il maggior contenuto "culturale" e studiare questa pianta affascinante porta ad esplorare campi molto lontani dalla pura pratica agricola.

3. While dessert grape is a dynamic product, the wine market, at the opposite end of the spectrum, is very conservative and the acceptance of new varieties has been determined by customs, law, and even traditional and emotional criteria.

   Mentre le uve da tavola sono un prodotto dinamico, la produzione enologica, al contrario, è molto più restia a modificare sicurezze già affermate e l'accettazione di nuove varietà è assoggettata alle regole del mercato, alla legislazione e perfino ad aspetti culturali ed emotivi.

4. Genetic transformation offers unique perspectives for the genetic improvement of *Vitis* genus. The most relevant features of this strategy are concerned with the possibility to overcome disadvantages associated with conventional breeding, and to modify specific characteristics in already superior genotypes.

   La trasformazione genetica offre prospettive uniche per il miglioramento genetico nel genere Vitis. Le aspettative più interessanti riguardano la possibilità di superare svantaggi associati al miglioramento genetico classico e di modificare caratteri specifici in genotipi già provati.

5. Is a genome a musical score and each gene is a simple note? Maybe... I have just began to play.

   Un genoma può essere una partitura musicale e ogni singolo gene una semplice nota? Può darsi... ho appena cominciato a suonare.

6. SCUBA Diving: discipline of breath, discipline of thought, discipline of technique. Together, a huge, unique, unrepeatable freedom.

   Subacquea: disciplina del respiro, disciplina del pensiero, disciplina della tecnica. Insieme, una grande, unica, irripetibile libertà.

7. Every significant earthquake exposes the weak points of structures and destroys them.

   Ogni terremoto serio mette a nudo le parti deboli delle strutture e le distrugge.

8. We are often so absorbed by our own "culture" that we are unable to capture the either offensive or racist aspect of a cliché, of an expression, of a joke.

   Talvolta siamo talmente impregnati dalla nostra "cultura" da non poter cogliere l'aspetto offensivo o razzista di un luogo comune, un'espressione o una battuta di spirito.

9. We often feel it our duty to live like workerants; but if cricket did not exist, life would be dull.

   Spesso il nostro dovere ci pare quello di vivere come formiche, ma se non esistessero le cicale la vita sarebbe insopportabile.

10. The radio is a great magical box, an ethereal fantasy that materializes.

    La radio è una grande scatola magica, eterea fantasia che si materializza.

11. The trickiness of the telephone is that a charming voice not necessary belongs to a beautiful person.

    Il trucco del telefono è che non sempre una voce affascinante appartiene ad una bella persona.

12. It is not important to be original at all costs, but it is relevant to enhance the cultural wealth which spans the whole world in time and space with personal and life-like hues.

    L'importante non è essere originali a tutti i costi, ma è dare una sfumatura personale e vissuta a quella dinamica rete nello spazio e nel tempo che è il patrimonio culturale che abbiamo a disposizione.

Stellingen behorende bij het proefschrift - Propositions from the Ph.D. thesis - Massime della tesi di dottorato: *"Regeneration and genetic transformation in the Vitis genus."*

Lucia Martinelli
22 december 1997, te Wageningen.

Uno scienziato si affida al suo agnostico baricentro galleggiante: il metodo scientifico. Questo è appena un salvagente nel mare grande della limitatezza, esposto ai moti imprevedibili dell'atmosfera, dell'irrazionalità. Uno scienziato è un umile naufrago intelligente.

Maurizio Indirli

A scientist entrusts himself to his agnostic floating center of gravity: the scientific method. This is only a life jacket in the vast sea of limitation, exposed to the unforeseeable movements of the atmosphere, of irrationality. A scientist, is a humble, intelligent, castaway.

Maurizio Indirli

This research has been carried out at the "Laboratorio di Biotecnologie" of the Istituto Agrario di San Michele all'Adige (Trento, Italy), and was also supported by the Italian Ministry of Agriculture (Piano Nazionale "Sviluppo di Tecnologie Avanzate Applicate alle Piante", Project N. 3) and by the National Research Council of Italy (Special Project "Ricerche Avanzate per Innovazioni nel Sistema Agricolo", Sub-project N. 2). In the latter framework, chapters 2 to 7 were published in scientific journals with RAISA Paper Numbers 509, 263, 767, 1185, 2778 and 2777, respectively.

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Abstract

This work is a contribution to the development of regeneration systems and genetic transformation in the *Vitis* genus and opens interesting perspectives to the application of molecular techniques for study interesting traits, as well as for genetic improvement of grape.

Somatic embryogenesis from leaf and petioles has been obtained in *V. rupestris* S. and the most crucial steps of this morphogenic event have been characterized through a two-dimensional electrophoretic analysis of the total proteins and a study of isozyme patterns of five enzymes.

In this genotype, the co-culture of isolated somatic embryos during secondary embryogenesis induction with *Agrobacterium tumefaciens* (LBA4404), which contains the plasmid pBI121 carrying the neomycin-phosphotranspherase and the β-glucuronidase genes, proved to be a valuable strategy for the production of stably transgenic plants.

The regeneration potential in the *Vitis* genus during direct organogenesis from young leaves was conducted on 18 different *Vitis* genotypes, including cultivars of *Vitis vinifera* L., wild species, rootstocks and a hybrid variety was investigated, opens the possibility to employ *in vitro* strategies for molecular studies and genetic improvement to valuable grape genotypes too.

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Preface

I became aware of my passion for genetics during my years of study at the University and it developed further during the preparation of my thesis. Just in those years, biotechnology started establishing itself. I turned at once to this fascinating and innovative field of research where theoretical studies and leading technologies - such as molecular biology, in vitro tissue culture and recombinant DNA techniques - intertwined. The branch where I met the best possibilities of growth and work in Italy and abroad was agrobiotechnology. It thus became my interest.

Biotechnology is a deeply innovative opportunity for genetic improvement in agriculture; it certainly will allow, in the near future, the solution of crucial world wide human questions. Among these being the adequacy of the food resources and the development of an agricultural practice more respectful of environmental issues. These great potentialities could be completely accomplished if correctly managed by the scientific community and the institutions, so that public acceptance would be increased and an equal distribution of the worldwide food resources would be ensured.

As for my dedication to this field of research, a crucial step resulted in the setting up the Biotechnology Laboratory at the Agricultural Institute of San Michele all'Adige (Trento, Italy) which was born in the October 1988 and officially inaugurated on June 8, 1990 during the meeting "Genetic strategies for the improvement of the resistance to stress in the fruit crops" organized with the Italian Society of Agricultural Genetics and the Italian Academy of the Vine and Wine. The laboratory, dedicated to the experimenter and geneticist of Trentino Rebo Rigotti, is an optimal facility for research in the field of plant biotechnology, and is the outcome of a far-sighted and strategic choice of the Agricultural Institute of San Michele all'Adige.

The research presented in this book devoted to the grapevine which is the

main subject of the activity has been developed here.

The study of grapevine gave me the great opportunity to become familiar with both complex scientific and experimental questions as well as in reaching knowledge at cultural, social and productive levels.

Furthermore, the activity of these past years has given me the opportunity of building solid and interesting cooperations with researchers and colleagues.

Preparing this publication represented an important moment for me. It has given me the possibility to think over the research conducted thus far, studying the various themes related to the world of viticulture. Furthermore, the cross cultured professional exchange with other researchers involved in the same studies has been overwhelming.

The opportunity of achieving the degree of doctor of agricultural and environmental sciences at the Agricultural University of Wageningen (The Netherlands) is also thanks to Prof. Maarten Koornneef. This degree is for me an accomplishment of an important result for my scientific development. I would also hope that this will bring a consolidation of contacts with the Dutch colleagues already started in 1985, during my "stage" at the group of Prof. Koornneef at the Department of Genetics of this University which has been a fundamental step toward my scientific and personal experience.

Lucia Martinelli

August, 1997

Prefazione

La passione per la genetica è nata nel corso dei miei studi universitari e si è sviluppata durante la preparazione della tesi di Laurea. Proprio in quegli anni, cominciavano ad affermarsi le biotecnologie, settore di ricerca affascinante e innovativo a cui subito mi sono rivolta, perché intreccia approfondimento teorico con tecnologie di avanguardia, quali la biologia molecolare, le colture in vitro dei tessuti e le tecniche del DNA ricombinante. La scelta di specialità si è indirizzata alle agrobiotecnologie poiché in quel campo ho incontrato le migliori possibilità di maturazione e di lavoro in Italia e all'estero.

Queste tecniche rappresentano un'opportunità profondamente innovativa per il miglioramento genetico in agricoltura e certamente daranno un contributo, nell'immediato futuro, alla soluzione di questioni cruciali per l'umanità quali, tra l'altro, l'adeguatezza delle risorse alimentari e lo sviluppo di un'agricoltura più rispettosa dell'ambiente. Queste grandi potenzialità possono trovare una completa realizzazione se gestite correttamente dalla comunità scientifica e dalle istituzioni in modo da favorirne l'accettabilità nella pubblica opinione e garantire l'equilibrata distribuzione delle risorse alimentari a livello mondiale.

Per quanto riguarda il mio impegno in questo settore, un momento decisivo è risultato essere la realizzazione del Laboratorio di Biotecnologie presso l'Istituto Agrario di San Michele all'Adige (Trento), nato nell'ottobre 1988 e inaugurato ufficialmente l'8 giugno 1990 nel corso del convegno "Strategie genetiche per il miglioramento delle resistenze agli stress nelle piante da frutto", organizzato in collaborazione con la Società Italiana di Genetica Agraria e l'Accademia Italiana della Vite e del Vino. Il laboratorio, intitolato alla memoria dello sperimentatore e genetista trentino Rebo Rigotti, è una struttura ottimale per ricerche nel campo delle biotecnologie vegetali ed è il risultato di una scelta lungimirante e strategica da parte dell'Istituto Agrario di San Michele all'Adige. Qui si è sviluppata la ricerca illustrata in questo libro, dedicata interamente alla vite, che riguarda gran

parte dell'attività svolta.

Lo studio della vite si è rivelato per me una grande opportunità; infatti, mi ha permesso di affrontare problematiche complesse dal punto di vista scientifico e sperimentale e di avvicinarmi anche a conoscenze più generali a livello culturale, sociale e produttivo.

L'attività svolta in questi anni mi ha dato inoltre l'occasione di costruire solidi e interessanti rapporti di collaborazione e amicizia con altri studiosi e colleghi.

Poi, la realizzazione di questa pubblicazione ha rappresentato per me un importante momento di riflessione sulla ricerca fin qui svolta, di studio e di approfondimento delle molteplici tematiche collegate al mondo della viticoltura e di confronto con le esperienze di altri ricercatori impegnati negli stessi studi.

Infine, la possibilità di conseguire con questo lavoro, grazie al Prof. Maarten Koornneef, il titolo di Dottore in Scienze Agrarie ed Ambientali presso l'Università di Agraria di Wageningen (Olanda), significa per me il raggiungimento di un risultato importante per la mia formazione scientifica e l'opportunità di un consolidamento dei contatti di collaborazione con i colleghi olandesi. Infatti, questa esperienza di lavoro comune è cominciata già nel 1985, presso il gruppo del Prof. Koornneef al Dipartimento di Genetica di questa Università, nel corso di un soggiorno di studio e lavoro che ha rappresentato per me una tappa fondamentale della mia maturazione scientifica e umana.

Lucia Martinelli
Agosto 1997

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I would like to thank the Agricultural Institute of San Michele all'Adige (Trento, Italy) for setting up the Biotechnology Laboratory and supporting the development of its activity and the present research.

I'm grateful to Prof. Maarten Koornneef who followed enthusiastically each step of this book's production. He has also played an important role in my scientific development since my "stage" at his group.

A special thanks to my closest co-workers of the laboratory, p. e. Valentino Poletti and p. e. Paola Bragagna, for their precious contribution to the present work. My acknowledgment also goes to Sig. Savina Minotto for her assistance; to the trainees, scholarship holders and visiting scientists who joined the laboratory (of them - in chronologic order - Dr. Mame Ourey Sy and Mrs. Salimata Sembene Niang of the Dakar University and the biologists Laura Biasetto, Daniela Mott, Elisa Poznanski and Daniela Costa); to the colleagues of the Agricultural Institute of San Michele all'Adige who offered their assistance in the laboratories, in the greenhouses, in the field collections, in the library and in the computer centre; to Mr. Taddeo Bontempelli for his photographical help; to the co-authors of the papers presented in this publication with whom I shared my scientific growth: in particular to Prof. Attilio Scienza (from the laboratory's beginning) and to Dott. Giuseppe Mandolino e Dott. Elisabetta Gianazza, who are also my dearest friends; to my friends Lucia Cardone, author of Figures 8 and 15, and Ann Bassi for the linguistic advice; to Dott. Norberto Pogna for the information on transgenic grape releases.

I also would like to acknowledge the two institutions which contributed to founding the research presented here in the framework of specific projects, and the coordinators and colleagues whom I encountered during the development of the related research. With regard to the Italian Ministry of Agriculture with the National Plane "Development of Advanced Technologies Applied to Plants",

Prof. Francesco Salamini who always offered his suggestions during the preparations of articles, and Prof. Pasquale Rosati. Among the above mentioned, I also thank the National Research Council of Italy with the Special Project "Advanced Researches for Innovation in Agriculture", Prof. Enrico Porceddu, Prof. Paolo Alghisi and Prof. Eddo Rugini who always encouraged my work.

I would also like to give mention to my unforgettable colleagues, despite their being spread through the world they are united in the same field of research: I had valuable exchanges of suggestions and materials; Prof. Domenico Palenzona and Prof. Rita Alicchio who transmitted me the passion for the research; the managers of Agrimont R&S (Montedison group) and of The Plant Cell Research Institute of Dublin (California) who permitted my "American adventure" and the colleagues who shared it with me. Among them, I remember with fondness Lilia Formica for the meaningful discussions and Lucynda Smith also for the linguistic help. I also thank my colleagues of the "Dutch" times, among the many, the friends Jelle Wijbrandi and Maria Bijlsma who lets me feel "at home" in Wageningen; the "Istituto Trentino di Cultura" and the Commission of the European Union, DGXII, who permitted my "Dutch adventure". I certainly can not forget to thank my relatives, for their constant support. Among them I appreciate the "American cousin" Gloria Daffner for her linguistic teachings and Augusto Goio for his editorial advise. Finally, I thank Maurizio, my life-companion who always backed my professional choices and lent his poetic touch for the preparation of this book.

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Una riconoscenza particolare va al Prof. Maarten Koornneef per aver seguito con entusiasmo ogni passo della realizzazione di questo libro e per il suo ruolo rilevante nella mia formazione scientifica, a cominciare dai tempi della mia permanenza presso il suo gruppo.

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Salamini, che ha sempre offerto i suoi suggerimenti nella preparazione delle pubblicazioni e il Prof. Pasquale Rosati, ed il Consiglio Nazionale delle Ricerche, nel corso del Progetto Speciale "Ricerche Avanzate per Innovazioni nel Sistema Agricolo", con i Prof. Enrico Porceddu, Paolo Alghisi e Eddo Rugini, che hanno sempre incoraggiato il mio lavoro.

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Infine, un grazie a Maurizio, compagno di vita, che mi ha sempre sostenuta nelle scelte di lavoro e che ha offerto il suo tocco poetico alla realizzazione di questo libro.

List of abbreviations

2-D	two dimensional gel electrophoresis, i.e. peptide separation by charge on isoelectric focusing followed by size fractionation at right angle in presence of SDS
2,4-D	2,4-dichlorophenoxyacetic acid
AcP	acid phosphatase
ADH	alcohol dehydrogenase
BA	6-benzyladenine
C%	relative percentage of the cross-linker in a polyacrylamide gel
CA	carrier ampholytes for isoelectric focusing
EST	esterase
G6PDH	gluconate-6-phosphate dehydrogenase
GA_3	gibberellic acid
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
IEF	isoelectric focusing
IPG	immobilized pH gradient
IPG-DALT	2-D with focusing on IPG as the first step according to Gianazza *et al.*, 1985b
MS	culture medium according to Murashige and Skoog (1962)
MTT	methyl thiazolyl blue

NAA	2-naphthaleneacetic acid
NN	culture medium according to Nitsch and Nitsch (1969)
NOA	2-naphthoxyacetic acid
NP40	Nonidet P-40R, octyl phenol ethylene oxide condensate
PAA gradient	polyacrylamide gradient
PGM	phosphoglucomutase
PMS	phenazine methosulfate
PMSF	phenyl-methyl-sulfonyl fluoride
SDS	sodium dodecyl sulfate
T%	total monomer concentration in a polyacrylamide gel

Chapter 1

General introduction

Grape

Grape is one of the fruit crops with the highest "cultural" content, and studying this fascinating plant takes to explore fields far beyond the agricultural practice. Indeed, tradition, habit, art and even religion accompanied the development and the domestication of grape since the beginning of mankind.

This woody perennial plant belongs to the order *Rhamnales* (Cronquist, 1981), family *Vitaceae* (Willis, 1973), genus *Vitis* (Cronquist, 1981).

In the genus *Vitis*, two distinct subgenera have been classified: *Euvitis*, the true grape, and *Muscadinia*, with diploid chromosome sets of 38 and 40, respectively. Such a large chromosome number suggests that secondary polyploidization occurred (Olmo, 1995). Palaeobotanical studies suggest that the separation of the subgenera took place about two million years ago, before the onset of the Quaternary era. *Euvitis* fossils have been recovered in Tertiary deposits of Eurasia and North America, while *Muscadinia* has been found only in North America (Unwin, 1991). *Muscadinia rotundifolia* is the most ancient native species cultivated in North America.

The systematics of *Vitis* is difficult and artificial, and the groups that are distinguished have been considered eco-species rather than species since experimental crossings yield fully fertile hybrids. Therefore, the number of species within the subgenus *Euvitis* is disputed (Galet, 1968; Barrett *et al.*, 1969; Rogers and Rogers, 1978) and a number as high as 60, including Eurasian and North American, has been estimated.

American *Vitis* species are cultivated as rootstocks (*V. berlandieri*, *V. rupestris*, *V. riparia*, *V. champini*) or for industrial food processing (*V. labrusca*) (Colby and Meredith, 1993). The Eurasian *Vitis vinifera* is, however, the primary cultivated one.

The origins of grape vine are still uncertain (Unwin, 1991); since fossilized vine leaves and seeds from the Palaeocene and Eocene (Winkler, 1962; Galet, 1979; Mai, 1987) have been found, it can be concluded that this plant is truly ancient (Unwin, 1991). In Western Europe and in the Mediterranean areas the

culture of this crop has been dated back to the Tertiary-Quaternary transitions (Levadoux, 1954; Jelaska, 1956; Negrul, 1938).

According to legend and tradition, Armenia is the home of the first grape and wine culture (Olmo, 1995).

The systematics of grapevines is still the object of discussion and of contradictory reports. Five thousand (Levadoux, 1954; Bouquet, 1982; Dry and Gregory, 1988) to 24,000 (Viala and Vermorel, 1909) cultivars have been described, but recent studies tend to greatly reduce these numbers. Most of the material indicated as cultivars and named under different local names, are now considered identical genotypes (Alleweldt and Possingham, 1988).
Information on the taxonomic relationships is necessary for legislation, viticulture and breeding and, consequently, the development of techniques to describe the germplasm genetically has been strongly pursued.

Ampelography manuals, based on botanical and morphological observations, have for a long time been the only tools for the description of *Vitis* cultivars. Recently, biochemical and molecular markers have been introduced for grapevine identification. Isozyme analysis (Weeden *et al.*, 1988; Parfitt and Arulsekar, 1989; Walters *et al.*, 1989; Scienza *et al.*, 1994) and DNA typing (Thomas *et al.*, 1994; Lodhi *et al.*, 1995; Bowers and Meredith, 1996; Bowers *et al.*, 1996) have greatly enhanced the possibility of genotype characterization. Since the limited number of available isozyme markers, in the near future the cultivar or genotype characterization will certainly be conducted exclusively by DNA markers (RFLP, RAPD, microsatellite).

Ancestral *Vitis* species are thought to have been dioecious, with hermaphroditic forms evolving later (Unwinn, 1991). Today, cultivated grapevines are monoecious. Among wild *Vitis* species and their hybrids, different degrees of atrophy or sterility of male or female structures are known (Galet, 1990).

Grape chromosomes are very small, with an average length of 1.0 μm, (Shetty, 1959). The DNA content of the grape genome is approximately 1.0 pg/2C, and is calculated to be about 475 Mbp (Lodhi and Reisch, 1995).

Vitis vinifera grows mostly in Mediterranean climates; however, due to its easy acclimation, it has been spread through the world by explorers, colonizers and settlers (Krul and Mowbray, 1984). Although a temperate crop, grapes have recently been introduced also into tropical countries and are grown on all continents except Antarctica (Colby and Meredith, 1993).

Grape is a versatile fruit crop, being cultivated for wine, table grape, raisin and juice production.

According to annual statistics by the Office International de la Vigne et du Vin (Paris), in 1995 the cultivated area was 7,768,000 ha., grape harvest reached 54,056,700 tonnes and wine production was 246,423,000 hl; these figures are impressive, though not at the levels of the period 1950 - 1980, when grape cultivation reached its highest quantitative levels (Tinlot and Rousseau, 1996).

Genetic improvement of grape

The programmes for genetic improvement of grapes are mostly related to the geographical areas of culture and involve traits of productivity, biotic and abiotic stress resistance. In particular, *Vitis* species are affected by fungal and viral diseases, and by insect pests; diseases caused by bacteria and intracellular procaryotes (mycoplasma- and rickettsia-like microorganisms) are known as well (Galet, 1995). Many of the more disastrous diseases and pests were common to North America but unknown in Europe until the nineteenth century; of these, powdery mildew (caused by *Uncinula necator*), downy mildew (caused by *Plasmopara viticola*) and *Phylloxera vastatrix*, have historically been the most important (Unwin, 1991). Besides, grey mould (caused by *Botryotinia fuckeliana*) produces heavy losses of yield, and, in the last years, two wood diseases (*Eutypa lata* and *Esca*) have been responsible for serious damages to vineyards (Grenan, 1992). Viruses transmitted by nematodes or insects are the most difficult to control. Of this latter group, fanleaf (GFLV), leafroll (GLRV), fleck (GF), stem pitting (GSP), Kober stem grooving (KSG) and corky bark

and technological value of grapevine. In this context, anthocyanin genes (involved in the determination of taste and quality of the wines), as well as a polyphenol oxidase gene (responsible for the enzymatic activity leading to browning in white wines and raisins) have been recently cloned (Dry and Robinson, 1994; Sparvoli *et al.*, 1994).

State of the art in tissue culture and genetic transformation of grape

Even tough grape was one of the first plants employed in tissue culture studies (Morel, 1944), the story of genetic transformation in *Vitis* is very recent since the first positive result was reported only in the 1990's (Mullins *et al.*, 1990). Further advances have been made, but this technology is still far from routine.

This delay is related to the low regeneration competence of grapevine which is the principal requirement for transformation strategies. However, recently, in the European Union two field tests with transgenic rootstocks have been performed by the Institut National de la Recherche Agronomique of Colmar (France) (notification number B/FR/94/11/04) and by the same institution with the French Moët et Chandon Company (notification number B/FR/96/03/14).

Micropropagation is the most well established tissue culture methodology in grapes, since suitable cultural conditions have been already defined for the different *Vitis* genotypes (Chee and Pool, 1983; Reisch, 1986; Monette, 1988; Grenan, 1992; Galzy *et al.*, 1990).

In vitro propagation is generally obtained by axillary shoot development from nodal cuttings (Fournioux and Bessis, 1986), shoot tip culture (Chee and Pool, 1982; Harris and Stevenson, 1982; Stevenson and Monette, 1983) as well as by adventitious shoot formation from fragmented shoot apices (Barlass and Skene, 1979 and 1980). Meristem cultures have been specially developed for the production of virus free plants (Gifford and Hewitt, 1961; Galzy, 1962) and is still used for this purpose with or without heat- or drug-therapy (Duran-Vila *et al.*,

1988; Grenan, 1992; Staudt and Kassemeyer, 1994). This proved effective also for elimination of bacterial diseases (Robacker and Chang, 1992). Besides, the propagation of special hybrids (Monette, 1988) as well as germplasm conservation (Harris and Stevenson, 1982; Harst-Langenbucher and Alleweldt, 1990; Gray and Compton, 1993) are the most common practical applications of micropropagation.

When poor embryo development and/or abortion occur, such as in the case of postzygotic barriers in interspecific hybrids (Gray *et al.*, 1990; Ramming, 1990) or of stenospermocarpy in seedless grapes (Stout, 1936), *in ovulo* embryo rescue proved to be an important strategy for the recovery of seedlings. In particular, this technique provides an unique alternative to conventional methods of breeding for seedless grapes (Gray *et al.*, 1987; Gribaudo *et al.*, 1993; Bouquet and Danglot, 1996) when the production of table and raisin varieties are pursued, according to the consumer demand (Cain *et al.*, 1983; Spiegel-Roy *et al.*, 1985; Pommer *et al.*, 1990). Of these latter, the most appreciated are the *V. vinifera* cultivars Thompon Seedless, Flame Seedless and Perlette (Ramming *et al.*, 1991).

Protoplast technology is less developed in grape tissue culture, despite to the efforts made during the last years (Lee and Wetzstein, 1988; Barbier and Bessis, 1990; Reustle and Alleweldt, 1990; Ui *et al.*, 1990; Mii *et al.*, 1991; Siminis *et al.*, 1993 and 1994). Only in one case (Reustle *et al.*, 1994) plant regeneration from protoplast cultures has been reported so far (*Vitis vinifera* cv. Seyval blanc).

Even tough far from routine, research has been considerably increased in developing protocols for regeneration *via* organogenesis and somatic embryogenesis, in the last years. However, while organogenesis has been reported with agreable efficiencies, also for several important genotypes (Cheng and Reisch, 1989; Clog *et al.*, 1990; Stamp *et al.*, 1990a and 1990b; Reisch *et al.*, 1990; Colby *et al.*, 1991; Martinelli *et al.*, 1996a; Torregosa and Bouquet, 1996), somatic embryogenesis protocols for grapevines have not been very successful, although the first report on somatic embryogenesis was in the *V. vinifera* cv. Cabernet Sauvignon (Mullins and Srinivasan, 1976).

Somatic embryogenesis has been induced from anthers (Hirabayashi *et al.*, 1976; Bouquet *et al.*, 1982; Mauro *et al.*, 1986; Gray and Mortensen, 1987; Rajasekaran and Mullins, 1983; Stamp and Meredith, 1988a; Newton and Goussard, 1990; Harst-Langenbucher and Alleweldt, 1993; Mozsár and Süle, 1994; Perl *et al.*, 1995; Mozsár and Viczián, 1996), ovules (Mullins and Srinivasan, 1976; Srinivasan and Mullins, 1980; Gray and Mortensen, 1987; Vallania *et al.*, 1994), leaves (Hirabayashi, 1985; Stamp and Meredith, 1988a; Matsuta, 1992; Martinelli *et al.*, 1993a; Robacker, 1993; Harst, 1995), petioles (Reisch and Roberts, 1985; Martinelli *et al.*, 1993a), and zygotic (Stamp and Meredith, 1988b; Gray, 1992; Emershad and Ramming, 1994) as well as somatic embryos (Matsuta and Hirabayashi, 1989; Vilaplana *et al.*, 1989; Martinelli *et al.*, 1993a).

The most adapted explant for somatic embryogenesis induction of *Vitis* proved to be anther. From this tissue somatic embryogenesis has been principally pursued with the aim of recovering dihaploid plants (Gresshoff and Doy, 1974; Zou and Li, 1982) for genetic improvement programmes. However, this strategy needs to be improved since regenerated plants proved in most cases to be derived from somatic tissues of the anther rather than from microspores (Rajasearan and Mullins, 1983; Reisch *et al.*, 1996).

When obtaining haploids is not pursued, we believe that to obtain somatic embryogenesis from tissues such as leaves and petioles which are commonly available throughout the year, would be particularly useful (Martinelli *et al.*, 1993a). This explant source could then also be used for genetic transformation.

A high genotype-dependency restricts the occurrence of somatic embryogenesis to a few species of *Vitis* genus. In particular, via somatic embryogenesis, plantlet regeneration has been obtained in *V. riparia*, *V. rotundifolia*, *V. rupestris*, and interspecific hybrids with both *V. vinifera* and *V. rupestris* parentages. As for *V. vinifera*, Cabernet Sauvignon and some seedless cultivars proved the most suited genotypes for somatic embryogenesis.

Since the efficiency was found to be strongly genotype dependent in grape regeneration (Bouquet *et al.*, 1982; Gray, 1995; Martinelli *et al.*, 1996a; Mozsár and Viczián, 1996), transgenic plants have been obtained in only a few

responding wild species (*V. rupestris*), interspecific hybrids (Chancellor) and rootstocks (41B, SO4 and 110 Richter) (Mullins *et al.*, 1990; Le Gall *et al.*, 1994; Martinelli and Mandolino, 1994; Mauro *et al.*, 1995; Krastanova *et al.*, 1995; Kikkert *et al.*, 1996) as well as in some V. vinifera cultivars (Chardonnay, Koshusanjaku, Superior seedless and Thompson seedless).

Depending on the *in vitro* plasticity of the genotypes adopted, most experiments have focused on the definition of transformation strategies where the insertion of marker genes has been studied (Mullins *et al.*, 1990; Nakano *et al.*, 1994; Martinelli and Mandolino, 1994; Scorza *et al.*, 1995; Kikkert *et al.*, 1996; Perl *et al.*, 1996; Martinelli and Mandolino, 1996). The preferred marker for evaluating the fate of a foreign gene in transgenics has been β-glucuronidase gene expression (Jefferson *et al.*, 1987).

Genes involved in biotic stress resistance, such as the lytic peptide Shiva-1 and osmotine genes, have been introduced respectively into dessert (Scorza *et al.*, 1996) and wild (Martinelli *et al.*, 1996b) grapes, and the expression of the viral coat protein genes in transgenic rootstocks (Le Gall *et al.*, 1994; Krastanova *et al.*, 1995; Mauro *et al.*, 1995) as well as in the *Vitis vinifera* cultivar 'Chardonnay' (Mauro *et al.*, 1995) have proved to confer resistance to grapevine chrome mosaic and fanleaf nepoviruses respectively.

In the past years, further progress in grape transformation has been also achieved by private companies such as the French Moët Hennessy Company interested in virus resistance (Mauro *et al.*, 1995) and the Californian Dry Creek Laboratories carrying out a project for the rootstock protection against nematodes (Coghlan, 1997); furthermore, the United States Tobacco Company is founding research on transgenic grapes (personal communication). In most cases, however, the real research advancements are obscure since company secrecy does not allows the publication of protocols and results in scientific papers.

Agrobacterium tumefaciens has been the most common vector employed for gene transfer, either alone or in combination with biolistic strategies (Scorza *et al.*, 1995 and 1996), while *A. rhizogenes* has been used less. As for this latter,

shoot-regeneration from transformed root explants has not been reported so far (Hemstad and Reisch, 1985; Gribaudo and Schubert, 1990; Guellec *et al.*, 1990); however, transgenic plants of *V. vinifera* (cv. Koshusanjaku) have been recently obtained when embryogenic calli were employed for the co-culture with this bacterium (Nakano *et al.*, 1994).

Generally, the discrimination of transgenic cells during regeneration has been conducted in the presence of kanamycin, but herbicide and hygromycin resistance genes have been used as well (Perl *et al.*, 1996).

In transformation experiments, embryonic tissues (both zygotic and somatic) have proved to be the best cell source for transgenic plant regeneration.

Somatic embryos at varying stages, i.e. whole (Martinelli and Mandolino, 1994; Scorza *et al.*, 1996), or embryo sections (Mullins *et al.*, 1990), embryogenic callus or cell suspensions (Le Gall *et al.*, 1994; Krastanova *et al.*, 1995; Mauro *et al.*, 1995; Kikkert *et al.*, 1996; Perl *et al.*, 1996) and zygotic embryos (Scorza *et al.*, 1995) give raise to homogeneous and stable gene insertion.

Meristems have proved to be less amenable because of the formation of chimeric tissues following adventitious bud formation in the presence of kanamycin as selective agent; furthermore, the high sensitivity of shoots during micropropagation to this antibiotics does not allow a further discrimination of non-chimeric tissues (Baribault *et al.*, 1990; Colby and Meredith, 1993).

This has created a limitation for the routine production of transgenic grapes; while somatic embryogenesis is confined to a few genotypes and occurs only at low efficiencies, direct organogenesis can be induced at suitable frequencies in several important genotypes within *Vitis* genus (Martinelli *et al.*, 1996a).

Therefore, further research in this field is expected to address the enhancement of somatic embryogenesis among grape genotypes and the definition of proper transformation and selection strategies during direct organogenesis.

Outline of the present work

The present research has been carried out in the framework of a programme aiming to develop recombinant DNA techniques for the genetic improvement of grape for biotic stress resistance. The most important problems related with grape tissue culture and genetic transformation have been studied.

At the start of this research, no detailed reports of somatic embryogenesis from established callus cultures of leaf and petiole explants, neither studies on biochemical markers for morphogenesis were available in grape. Also suitable and reproducible protocols for genetic transformation were not defined.

Since *in vitro* manipulation of tissues is one of the most crucial steps of genetic transformation, at first we assayed the *in vitro* behaviour of *Vitis* genus, and we identified the genotype with the most suitable morphogenic competence. *Vitis rupestris* Scheele proved the best material for this purpose and became our model for studying somatic embryogenesis and genetic transformation. There, we considered other agronomically more important grape genotypes.

Vitis rupestris Scheele is an American species which is regarded as valuable rootstock used for its phylloxera resistance (Gray and Compton, 1993). It is also used for the indexing of viral diseases (Galet, 1977).

For this genotype, we developed a protocol from somatic embryogenesis induction to plant regeneration from leaves and petioles ***(Chapter 2)***

Based on our own observations we found that even under strictly controlled conditions the response of a grape genotype to *in vitro* culture is often non-predictable. Since the events leading to somatic embryogenesis are poorly understood, the selection of the most suitable developmental stages leading to embryogenesis often relies on subjective evaluation. Assuming that a better knowledge of the biochemical events during morphogenesis could help the selection of the tissues and enhance the efficiency, we carried out a study aiming to defining suitable markers for the characterization of precise morphogenic events during somatic embryogenesis. First, a two-dimensional electrophoretic analysis of the total proteins during the different developmental stages, from

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Chapter 2

Somatic embryogenesis from leaf- and petiole-derived callus of *Vitis rupestris*.

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Abstract *Somatic embryogenesis from leaf- and petiole-derived calli of Vitis rupestris was obtained with an efficiency of 3.2% and 4.2% of plated explants, respectively on two combinations of 6-benzyladenine and 2,4-dichlorophenoxyacetic acid (1/0.1 and 1/1 mgl^{-1}) added to Murashige and Skoog (MS) medium. Embryogenic callus, embryo subcultures and somatic embryogenesis from somatic embryos were obtained either in the presence of 1 mgl^{-1} indole-3-acetic acid or 0.1 mgl^{-1} indole-3-butyric acid added to MS or Nitsch and Nitsch (NN) media. Within a 4-month culture, embryo germination occurred at a frequency of 13% of explanted embryos when chilling at 4°C was provided for two weeks and a combination of 6-benzyladenine (1 mgl^{-1}) with indole-3-butyric acid (0.01 mgl^{-1}) was added to NN medium supplemented with casein hydrolysate (250 mgl^{-1}). A higher frequency (51%) was obtained in a longer culture time (9 months) when only indole-3-butyric acid was present in the medium and in absence of chilling.*

Introduction

Genetic improvement of grape by the adoption of molecular techniques is hindered by the low frequency of regeneration from *in vitro* cultured explants.

However, the recent report of Mullins on a successful genetic transformation of *Vitis rupestris* somatic embryos (Mullins *et al.*, 1990) stresses the importance of improving the *in vitro* performance of grapevine.

In grape somatic embryogenesis is usually induced from anthers (Hirabayashi *et al.*, 1976; Bouquet *et al.*, 1982; Gray and Mortensen, 1987; Mauro *et al.*, 1986; Rajasekaran and Mullins, 1983; Stamp and Meredith, 1988a) and ovules (Gray and Mortensen, 1987; Mullins and Srinivasan, 1976), but rarely from leaves (Hirabayashi, 1985; Stamp and Meredith, 1988a) and petioles (Reisch and Roberts, 1985). In which explants of the latter, on the other hand, regeneration *via* organogenesis has been successfully reported (Cheng and Reisch, 1989; Clog *et al.*, 1990 and Stamp *et al.*, 1990a, and 1990b).

To obtain somatic embryogenesis with a high frequency from tissues such as leaves and petioles which are commonly available throughout the year, would be particularly useful.

This paper reports a detailed study of somatic embryogenesis from such explants on *Vitis rupestris* (Martinelli *et al.*, 1991).

In our protocol somatic embryogenesis has been successfully obtained in established leaf- and petiole-derived callus cultures which have been described as unsuitable material for the induction of regeneration (Krul and Worley, 1977).

Materials and Methods

Explant source and preparation

Forty *Vitis rupestris* Scheele leaves with their petioles were harvested from locally cultivated plants in early spring, choosing the first three starting from the shoot tip. They were sterilized for 15 min in a 0.8% v/v sodium hypochlorite solution, and rinsed 3 times in sterile water. Leaves were cut in 40 mm^2 square pieces, and petioles in 2 mm long segments. Samples of 261 and 116 fragments were obtained from 40 leaves and petioles, respectively.

Media preparation and culture vessels

The pH of the media was adjusted with NaOH at 5.7 before autoclaving for 20 min at 121°C and 1 atm. When needed, filter-sterilized (0.2 μm pore size) indole-3-acetic acid (IAA) was added to the media after autoclaving.
For solid cultures, 30 ml medium were dispensed on plastic Petri dishes of 9 cm diameter. For liquid cultures, 50 ml medium were poured in 125 ml flasks closed with a cotton-wool plug covered with aluminium foil.

Callusing, embryogenesis induction and culture

Leaf explants were placed with their lower surface in contact with MS-based medium (Murashige and Skoog, 1962) supplemented with 3% sucrose and 0.6% Difco Bacto-agar, and petiole cuttings were plated horizontally.

Explants were incubated at 25±1°C in the dark, and, after callusing, in a 16 light photoperiod (60 µmol $m^{-2}s^{-1}$ cool white). Eight combinations of the growth regulators 6-benzyladenine (BA) and 2,4-dichlorophenoxyacetic acid (2,4-D) were adopted to optimize culture conditions for callus and somatic embryogenesis induction (the BA/2,4-D combinations were: 0.2/0, 1/0, 0/0.1, 0.2/0.1, 1/0.1, 0/1, 0.2/1 and 1/1 mgl^{-1}).
Cultures were transferred every four weeks to fresh media.

Embryonic cultures

Embryogenic calli were propagated on solid medium containing 0.8% Difco Bacto-agar, while liquid medium was used to induce multiplication and elongation of single embryos. MS- versus NN- (Nitsch and Nitsch, 1969) formulations, either full- or half-strength, containing 3% sucrose, were compared, and the requirement of casein hydrolysate (250 mgl^{-1}) tested.
The effect of two auxins was also tested, by adding IAA (1 mgl^{-1}) or indole-3-butyric acid (IBA) (0.1 mgl^{-1}) separately to the basic media.
Approximately 2700 and 6800 mg of leaf- and petiole-derived embryogenic callus were used to start solid cultures.
Two 125-ml flasks with 50 ml of each medium were prepared for liquid cultures. Groups (0.135 g) of small somatic embryos (length 1 - 2 mm) were poured into every flasks.
Cultures were incubated at 25°C in dim light (15 µmol $m^{-2}s^{-1}$) and media renewed every three weeks. Each 50-ml liquid culture was divided into two flasks; to each of them 25 ml of fresh medium were added. They were shaken continuously at 90 rpm.

Secondary somatic embryogenesis

Single somatic embryos were isolated from liquid cultures and placed horizontally on solid half strength MS- or on full strength NN-based media, both containing 0.8% Difco Bacto-agar and supplemented with IAA (1 mgl^{-1}) or IBA (0.1 mgl^{-1}) respectively.
The cultures were incubated at 25°C in dim light (15 µmol $m^{-2}s^{-1}$) and moved to a fresh medium every four weeks.

Somatic embryo maturation and germination

A population of 1481 somatic embryos (214 from leaf- and 1267 from petiole-derived callus, respectively) was employed to define the optimal cultural conditions for embryo germination.

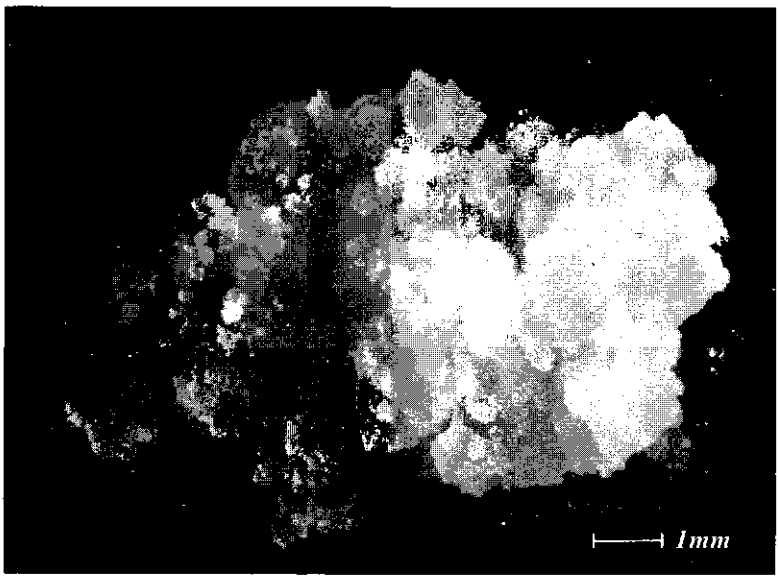

Figure 1 - *Callus induced from petiole explant.*

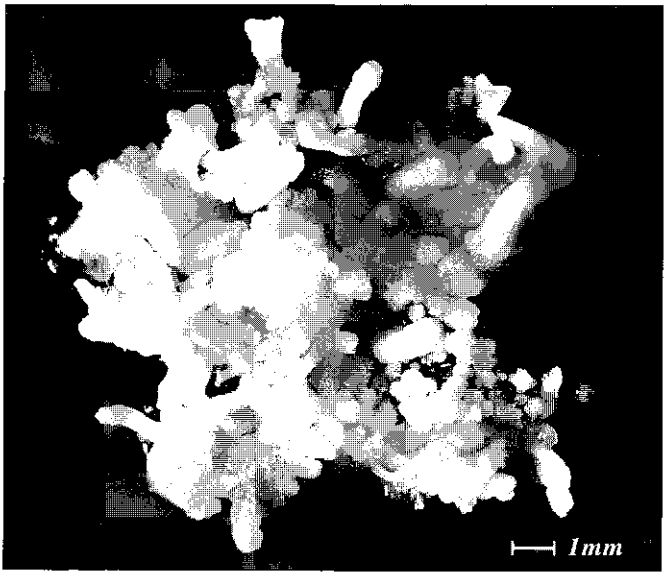

Figure 2 - *Embryogenic callus.*

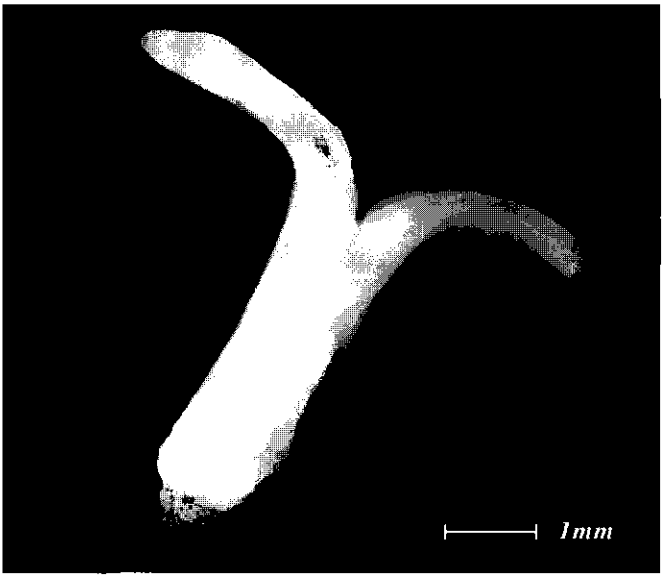

Figure 3 - *Isolated somatic embryo.*

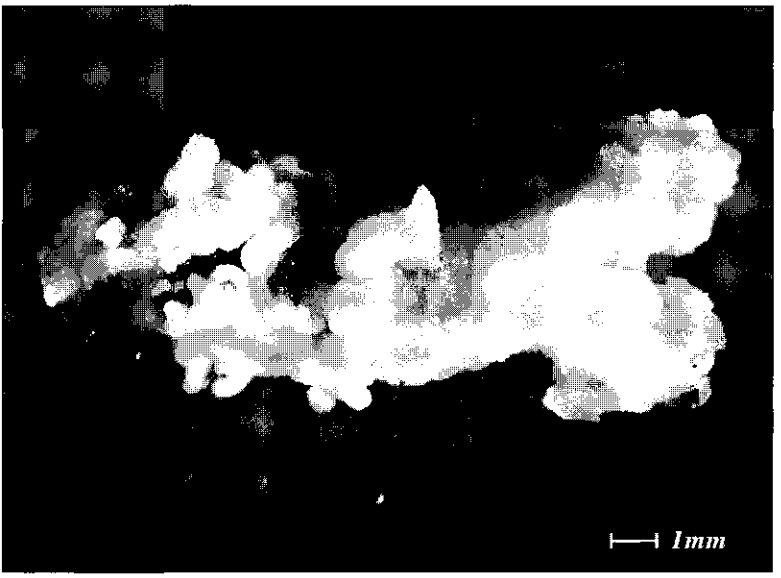

Figure 4 - *Induction of secondary embryogenesis on a single somatic embryo.*

In our protocol, secondary somatic embryogenesis was induced on single somatic embryos isolated from the liquid cultures. Somatic embryogenesis began on the root/shoot transition zone (Figure 4), confirming a gradient of embryogenic competence along the embryo tissues (Krul and Worley, 1977). Both media supplemented with 1 mgl^{-1} IAA or 0.1 mgl^{-1} IBA proved suitable.

Somatic embryo maturation and germination

Germination of embryos is a limiting step during somatic embryogenesis of grapevine, while germination efficiency is reduced by embryo dormancy (Rajasekaran *et al.*, 1982; Mauro *et al.*, 1986; Gray, 1989).

Figure 5 - *Germination of somatic embryo: influence of chilling and four growth regulator combinations; from the left: no growth regulators, BA, IBA and BA with IBA.*

In spite of these drawbacks, we obtained a great number of plantlets from petiole callus embryos. On the contrary, a low frequency of germinating embryos was obtained from leaf-derived callus (6 plants from 214 somatic embryos). Worth stressing, the careful choice of the embryos appeared to be a crucial factor supporting germination, because this occurred only in white well shaped, well polarized embryos, with root and shoot axes, a hypocotyl and two cotyledons (Figure 3) (Gianazza *et al.*, 1992).

Figure 5 shows the effect of four growth regulator compositions during a 2-month induction of germination in the presence of chilling, and Table 1 reports the efficiency of embryo germination obtained from petiole-derived callus, during a 9-month culture.

Growth regulators (mgl^{-1})				Germination efficiency during a 9-month culture							
BA	IBA	Chilling	Number of embryos tested	2	3	4	5	6	7	8	9
0.0	0.0	+	164	2	6	6	6	6	7	7	7
		-	60	0	0	0	7	10	12	12	12
1.0	0.0	+	174	0	4	5	5	5	5	5	5
		-	101	0	5	5	5	5	5	5	5
0.0	0.01	+	84	0	1	1	2	6	7	7	7
		-	–	–	–	–	–	–	–	–	–
0.0	0.1	+	133	0	4	5	6	6	6	6	7
		-	70	5	5	5	8	12	24	42	51
1.0	0.01	+	303	9	12	13	13	13	13	13	13
		-	47	0	0	0	0	0	0	0	0
1.0	0.1	+	88	1	7	8	9	11	14	14	14
		-	43	0	0	0	0	0	0	0	0

Table 1 - *Efficiency (percentage) of embryo germination in chilled and unchilled somatic embryos derived from petiole calli cultured on 6 different growth regulator combinations, during a 9-month culture.*

For both chilled and unchilled embryos, the lowest efficiencies were in the presence of BA alone. When chilling was provided in the presence of BA and IBA combined - at both 1/0.1 and 1/0.01 mgl^{-1} ratios - a large number of plantlets germinated. Within 2 to 4 months, germination efficiency reached desirable levels (8 to 13% respectively) and stabilized within 7 months (14 and 13% respectively).

When embryos were incubated at a constant temperature of 25°C, no plantlets were obtained with the same combination of growth regulators. When either BA, or IBA, or no growth regulators were added to the medium, in the presence of chilling, germination efficiency decreased (5 to 7%) and germination occurred between 2 and 6 months. In the absence of chilling, on the other hand, very high efficiencies (from 12 to 51%) were observed, but germination was dramatically delayed (from 6 to 9 months). Thus, chilling increased the speed of embryo germination. Furthermore, without chilling, embryos frequently produced a green disorganised callusing tissue, and from this tissue, plant regeneration occurred *via* organogenesis rather than embryo germination. Due to this, and in view of the length of the germination process in unchilled cultures, chilling appeared the preferable alternative. However, this treatment, commonly used as a dormancy-breaking strategy on zygotic embryos, already proved effective on somatic embryos (Rajasekaran and Mullins, 1979; Rajasekaran *et al.*, 1982; Takeno *et al.*, 1983; Gray, 1989; Mauro *et al.*, 1986).

Either in presence and absence of chilling, deformations, vitrification and inhibition of germination of somatic embryos were observed in presence of GA_3 (Stamp and Meredith, 1988b), a growth regulator which has been reported as a stimulator of embryo germination in the absence of chilling (Mullins and Srinivasan, 1976; Rajasekaran and Mullins, 1979; Stamp and Meredith, 1988a).

Plantlet culture

The plants germinated from somatic embryos showed a normal phenotype, and in two plantlets only, out of the many obtained in our laboratory, *in vitro* flowering has been observed.

Conclusions

Vitis rupestris proved a good system for establishing a procedure for somatic embryogenesis procedure going from explant to plantlet.

This paper constitutes the first detailed report of somatic embryogenesis from established callus culture of leaf and petiole explants. However, embryogenic callus induction from callus cultures appeared the limiting step of the overall process, and reported levels are susceptible of improvement.
Efforts are required to increase the efficiency of embryogenetic induction, and to eliminate the lag time before plant germination.

The culture conditions used in this experiment proved, on the other hand, suitable for embryo culture and propagation over long periods of time, and for plant germination which occurred with satisfactory efficiency.

Induction of secondary embryogenesis appears as a suitable strategy for *Agrobacterium*-mediated genetic transformation of grapevine.

Chapter 3

Monitoring by two-dimensional electrophoresis somatic embryogenesis in leaf and petiole explants from *Vitis*.

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Abstract *A two-dimensional electrophoretic analysis of the total proteins was carried out in Vitis rupestris as model system in order to characterize the different developmental stages - from callus to plantlets - of somatic embryogenesis events in the grapevine. The patterns of callus, embryogenetic callus, somatic embryos and plantlets derived from leaf and petiole explants were compared. Each differentiation step was characterized by specific peptide spots.*

Introduction

In vitro culture techniques in plant molecular biology

Totipotency is the property - peculiar to plants - for somatic tissues to differentiate into unorganized cells and to develop back, in a suitable environment, into an entire new plant (Gauthered, 1983; Steward, 1983; Salibury and Ross, 1985).

This feature offers the molecular biologist a wealth of strategies for the production, selection, and propagation of agronomically interesting biotypes (Evans *et al.*, 1983; James, 1988; Cocking, 1990).

As a first possibility, *in vitro* culture may support the normal growth pattern. A fast, large-scale production of virtually homogeneous populations is obtained through micropropagation, *i. e.* the shaping of new plants from a cluster of meristem (undifferentiated) cells (Hu and Wang, 1983; Pierik, 1987a; Zatyko, 1988). The technique of *in ovulo* embryo rescue allows the recovery of progenies from abortive ovules of incompatible crosses, including seedless x seedless fruit varieties (Goldy *et al.*, 1988; Bouquet and Davis, 1989; Ramming, 1990).

Contrary to the above instances, regeneration processes imply the reversion of the development path, with dedifferentiation into a callus (Collin and Dix, 1990), followed by a new differentiation step (Salibury and Ross, 1985; Steward, 1983). Explants from different organs of the plant may be directly put into culture, or tissue organization released and cell walls removed by enzyme treatment, so as to give protoplasts (Wright, 1985; Collin and Dix, 1990).

Direct organogenesis (Favre, 1977), where shoot and root are formed at a different time by a heterogeneous cell population, results in a chimeric plant (Gauthered, 1966; Thorpe, 1980). On the contrary, possibly a single cell is at the origin of somatic embryogenesis events (Steward *et al.*, 1958; Reinert, 1959; Steward *et al.*, 1964; Haccius, 1978; Vasil and Vasil, 1982). Embryo development is then characterized by its polarity, *i. e.* the growth of stem and root at once (see below).

When the explant tissues are immature anthers and ovaries, androgenetic or gynogenetic embryogenesis (Bajaj, 1983) may be obtained, resulting in haploid organisms suitable for the detection of mutations, the recovery of unique recombinants and for the rapid production of homozygous plants (Pierik, 1987b); this approach is meant to obtain inbred lines for hybrid production.

In order to recognize the optimal conditions for *in vitro* culture during the different stages of the regeneration process, a number of variables must be tested by trial and error. These include the genotype of the donor plants (different varieties are more or less amenable to *in vitro* culture) (Koornneef *et al.*, 1987), physiological situation of the explant (e. g. age: Croes *et al.*, 1985; gradients of regeneration efficiency are observed in a number of organs: Wernicke and Milkovits, 1984; Hanning and Conger, 1986; Mohanty and Gosh, 1988) and environmental factors, *i. e.* temperature, light, and culture medium.

Of the utmost importance is the presence and concentration of growth regulators such as auxins and cytokinins (Tanimoto and Harada, 1980; Wenck *et al.*, 1988).

The above procedures may be applied within selection programs since genetic variability - somaclonal variation - is often associated with *in vitro* culture

(Larkin and Scowcroft, 1981), possibly resulting from various types of DNA rearrangements (Bajaj, 1990).

Also useful for crop improvement is genetic transformation, which may be direct (through macro- or microinjection, gun, electroporation, polyethylene glycol treatment) or mediated, by viral or bacterial (*Agrobacterium tumefaciens* or *rhizogenes*) infection (Draper *et al.*, 1988; Davey *et al.*, 1989).

Although the technical, not to mention the ethical, problems that make it problematic to apply the advances in basic biological knowledge to current medical therapy have no equivalent with plants, the details of the successful protocols must be labourious worked out for every sample and for every step.

Somatic embryogenesis in Vitis

In most Mediterranean countries, grape-growing ranks as one of the major farming activities. However, biochemical and genetic knowledge on the genus *Vitis* lags behind the advances with staple crops - cereals, legumes, and potatoes (Monette, 1988).

Just as for the other wooden plants, restraints for investigations on the grapevine come from the interference by, *e. g.*, polyphenols on protein and DNA extraction, from a long reproductive cycle, and from low efficiency of regeneration. As for the latter point, *Vitis vinifera* cultivars are less amenable than wild genotypes to *in vitro* culture techniques.

Plant regeneration, somatic embryogenesis and adventitious shoot formation from different grapevine tissues has been reported (Rajasekaran and Mullins, 1979; Reisch and Roberts, 1985; Mauro *et al.*, 1986; Gray and Mortensen, 1987; Stamp and Meredith, 1988a; Clog *et al.*, 1990; Stamp *et al.*, 1990b).

The Biotechnology group at the Istituto Agrario San Michele all'Adige was recently able to define a protocol for high efficiency plant regeneration from somatic embryos on leaf- and petiole-derived callus in *Vitis rupestris* (Martinelli *et al.*, 1993a, Chapter 2). This advance prompted an investigation on the

biochemical make-up along the different steps of the somatic embryogenesis process in order to better define the most favourable culture conditions.

In the present report, we compare the total protein patterns resolved by two-dimensional (2-D) electrophoresis, while a related paper (Chapter 4) deals with the isozyme shifts between callus and plantlet (Martinelli *et al.*, 1993b).

As the sampling for these biochemical investigations is destructive, analysis will then be attempted on the byproducts, released in the culture medium, as a possible predictive index for the onset of the callus-embryo transition.

Materials and Methods

Tissue culture

Young leaves were collected in the spring from field-grown *Vitis rupestris*. Sterilized blade disks and petiole cuttings were slightly sunk in soft Murashige and Skoog (MS)-based medium (Murashige and Skoog, 1962) supplemented with 6-benzylamino purine (BA) and 2,4-dicholorophenoxyacetic acid (2,4-D) (at 1/0.1 mgl^{-1}, in a conventionally called V7 formulation, and at 1/1 mgl^{-1}, in its V8 variant). The induction to proliferate with rapid cell division resulted in the formation of a disorganized homogeneous mass, the callus (Figure 1, Chapter 2) that could be serially propagated in the above media.
Somatic embryogenesis induction required BA/2,4-D concentration of 1/0.1 mgl^{-1} for leaves and 1/1 mgl^{-1} for petioles. Globular, white embryogenetic structures (Figure 2, Chapter 2) appeared on the callus surface after 7 months from the explant induction, with 3% efficiency from leaves and a 4% yield for petioles. Transfer to liquid culture, removal of 2,4-D and BA, and addition of either indole-3-acetic acid (IAA, 1 mgl^{-1}) or indole-3-butyric acid (IBA, 0.1 mgl^{-1}) allowed embryo separation and elongation into a typical polar structure (Figure 3, Chapter 2). On Nitsch and Nitsch (NN) (Nitsch and Nitsch, 1969) solid media, single embryos could either give rise to secondary somatic embryogenesis (Figure 4, Chapter 2) or to maturation and germination into plantlets (Figure 5, Chapter 2). The former possibility was favoured by the presence if IAA or IBA as growth regulators, while the latter required the gradual reduction of the hormonal component. Best germination efficiency was obtained with 1 mgl^{-1} BA and 0.01 mgl^{-1} IBA and two weeks chilling at 4 °C, or with 0.1 mgl^{-1} IBA when no thermal shock was provided. The plantlets could then elongate and root on hormone-free NN medium. All incubation in the growth chamber was at ± 1°C, with a 16h photoperiod at 70 $\mu mol\ m^{-2}s^{-1}$ cool white light. Direct light exposure was avoided during callus induction and embryo culture.

Two-dimensional electrophoresis by immobilized pH gradient/sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Gianazza et al., 1985a)

Tissue samples were supplemented with quartz powder and homogenized in 5 volumes of 9 M urea, 4% w/v Nonidet P-40 (NP-40), 2% w/v Ampholine carrier ampholytes (CA), pH range 9-11, and 2% v/v ß-mercaptoethanol, according to Anderson, 1988. After centrifugation, 15 µl (for the embryo extract) or 40 µl (for the other samples) were applied at the anode on non-linear 4-10 immobilized pH gradient (IPG) (Gianazza *et al.*, 1985b) reswollen in 8 M urea, 0.5% w/v NP-40 and 0.5% w/v CA (0.25% in pH range 3-10, 0.15% in range 4-6 and 0.10% in range 5-7). The gel, 14 cm long, was run overnight at 50 V/cm, then 1 h at 150 V/cm. The equilibration between the first and second dimension was according to Görg *et al.*, 1988. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) step was run on a 7.5-17.5% T polyacrylamide gradient, 14 x 14 cm^2, with the discontinuous buffer system of Laemmli (Laemmli, 1970). Silver staining was according to Heukeshoven and Dernick, 1988. The results in Figure 6 are representative of three independent replicas.

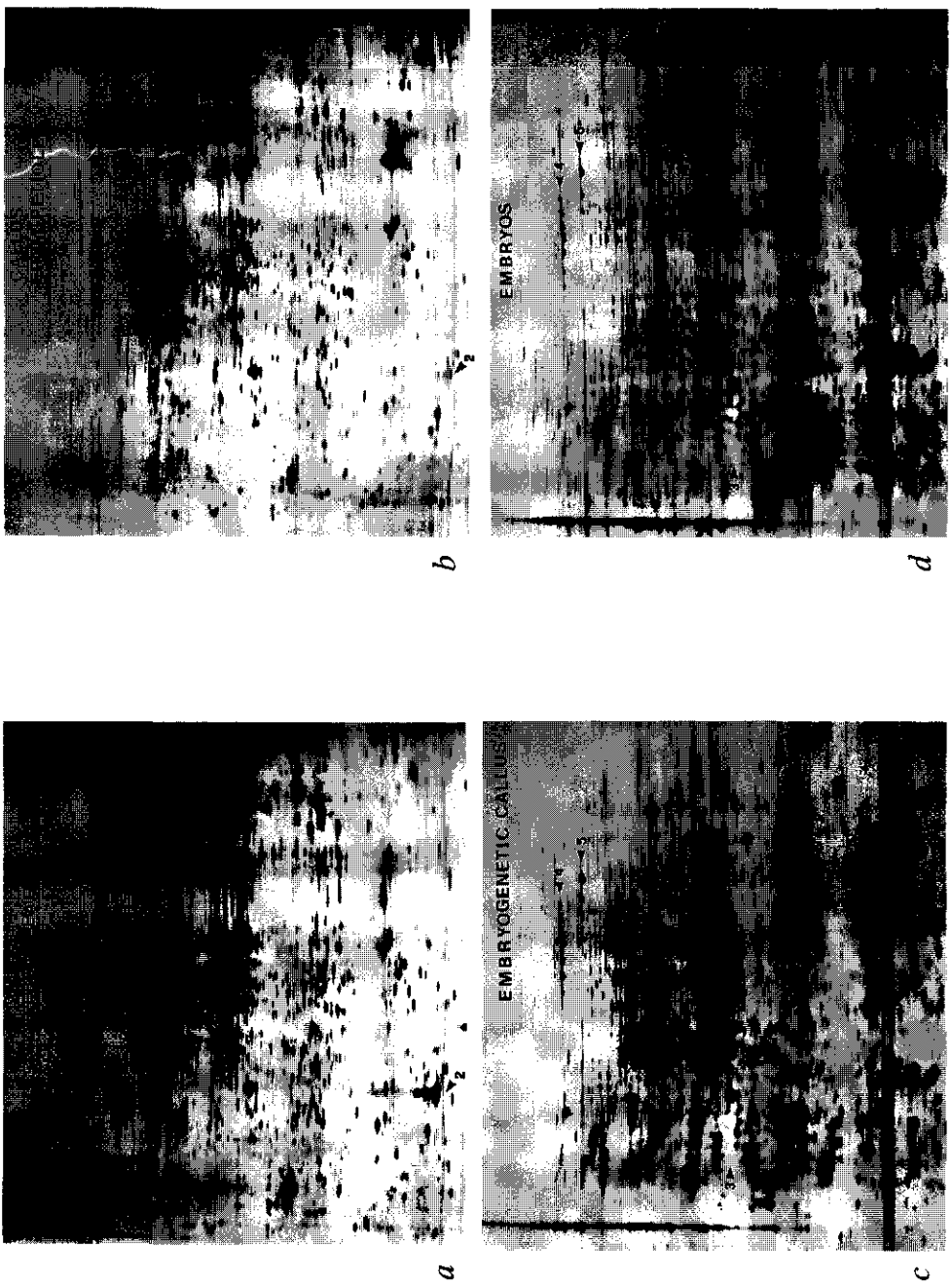
Results

The protein pattern of tissue specimens sampled along the regeneration path from callus to plantlet was analysed in *Vitis rupestris* by 2-D electrophoresis.

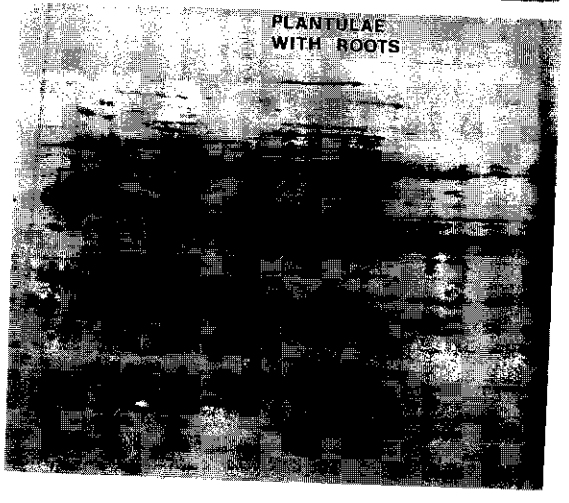
Callus samples from the leaf and petiole, both induced in V7 medium, have the highest water, and hence the lowest protein content. Their pattern shows an unusual prevalence of basic proteins in virtually every range of molecular masses.

Spots 1 and 2, with their peculiar shape, are characteristic of this developmental stage (Figure 6). When a slightly different culture medium, V8, is used, the relative abundance is found to be much lower for spot 1 and much higher for spot 2. As a rule, the patterns for the various primary calli differ more in quantitative than qualitative terms. No protein can be stained from brown callus samples, and this material can thus be identified as non-viable, autolytic tissue (not shown).

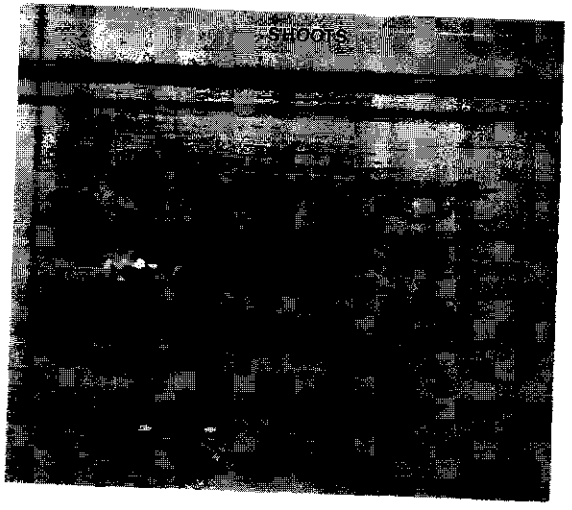
Virtually no resemblance is observed between primary and secondary callus from developing somatic embryos (Figure 6). The latter contains a limited spot subset from the embryo repertoire, with an increased relative concentration of slightly acidic, low M_r components.


EMBRYOS
EMBRYOGENETIC CALLUS
a
b
c
d

e



g



f

Figure 6 - *Steps of somatic embryogenesis in Vitis rupestris: 2-D protein maps.*
Extracts in urea/mercaptoethanol/detergent were run on a 4-10 nonlinear IPG and then at right angle on a 7.5-17.5% polyacrylamide gradient gel in presence of SDS; the protein pattern was stained with silver nitrate.
Samples: (a) callus from leaf and (b) from petiole, (c) embryogenetic callus, (d) early embryos, (e) embryo deformed into callus, (f) shoot, (g) plantlet.

The comparison of the 2-D maps (Figure 6e versus Figures 6d, 6f, 6g) suggests at which stage the normal development path was diverted from maturation into a plantlet and the embryo dedifferentiated into a callus. In the example of Figure 6, the largest number of common spots is found between secondary callus and the "shoot" stage of panel "f".

The highest protein concentration is observed with embryonic callus (Figure 6c) and early embryos (Figure 6d). Their protein pattern is virtually identical in qualitative terms; it corresponds to the most complex situation, with a large number of spots of similar intensity. Markers for this developmental step are some acid components that stain negatively with silver (spot 3), together with a number of spot rows, most likely charge isomers, with constant M_r (refer for instance to spot 4, or to 5, expressed throughout the vegetative cycle).

As the embryo begins to mature into a plantlet, the pattern simplifies and specializes, *i. e.*, a limited number of components tends to prevail (Figures 6f and 6g).

Discussion

Figure 6 shows that the various steps along the path from callus to embryo (somatic embryogenesis, a to d) as well as from embryo to plantlet (embryo maturation and germination, d to g) are characterized by a specific set of peptides, as extracted and resolved by the procedures detailed in Material and Methods.

Doubts on sampling accuracy might actually be cast for the latest stage: tissues begin to harden in a rooted plantlet and the extraction procedure - albeit brisk - could fail to get in solution a representative assortment of all specimen components.

In keeping with this fear is the finding of a lower instead a higher number of individual peptide spots in the 2-D map for plantlet than for embryo extracts. This complication cannot be ruled out, however, we favour a different

explanation to this finding.

Diversity - both morphological and biochemical - among plant cells is much less noticeable then for animal organs. The occurrence of meristem cells and primary metabolism enzymes at the macroscopic and microscopic level, respectively, would then "dilute" the contribution of specific protein by every differentiated tissue to the overall pattern. The complex protein make-up of early embryos (about 900 spots in 2-D) may be explained in terms of a bustling metabolism, with massive protein and DNA synthesis (cell division is most active at this stage), which later subsides as embryo maturation carries on.

Any analysis of our results with reference to data in the literature is made difficult by inconsistencies both in the sample source and in the technical approach. As for the latter, zymograms are most often applied for the detection of specific enzymes, whose expression varies across the vegetative cycle.

For instance, alcohol dehydrogenase activity is expressed in *Arabidopsis* seeds and tissue cultures but it is no longer detectable in 9-to-10-day-old seedlings (Dolferus *et al.*, 1985).

In *Nicotiana tabacum* the isozyme patterns of peroxidase and esterase differ in leaf and pith tissues in comparison with callus (Maršálek and Provazniková, 1984).

For *Phaseolus vulgaris*, peroxidase in callus cultures retains genotype-specific bands while tissue-specific isozymes (as observed in seedlings) are no longer expressed (Del Grosso *et al.*, 1987).

In *Zea mays* the zymograms of peroxidase and glutamate dehydrogenase differ between non-embryogenetic and embryogenetic callus (Fransz *et al.*, 1989), whereas, for *Hordeum vulgare* and *Triticum sativum*, new bands appear during morphogenesis for esterase and acid phosphatase, and some disappear for peroxidase (but only after shoot or root development), which makes them unsuitable as differentiation markers (Chawla, 1988).

In *Oryza sativa*, the selection of callus with 90% regeneration in presence of 2 mgl^{-1} 2,4-D correlates with the finding of a distinctive peroxidases pattern (Abe and Futsuhara, 1989).

These latter few examples testify to the variability of the gene expression pattern even within a botanical family. As a result, tedious preliminary work is required to test a panel of likely targets before enzyme activity is assessed by spectrophotometric techniques or isozyme pattern compared by zymogram.

Studies on the protein complement as a whole often require a more complex technical approach but in turn allow for a comprehensive view on tissue metabolism.

Elements most closely involved in transcriptional events, hence in protein synthesis, are non-histone chromosomal proteins; in fact, during dedifferentiation (pith explants to callus) and differentiation in *Nicotiana tabacum*, some high molecular weight bands (in SDS-PAGE) increase during an induction period to later decrease as cell division declines, while the opposite is true for a number of low M_r components (Guerri *et al.*, 1982).

In *Hordeum*, several proteins are associated with dedifferentiation into callus of both embryo and root tissues, 24 independent of, and 8 relative to genotype or species (Ramagopal, 1989).

In *Solanum carolinense*, stem explants producing callus (with 2,4-D) or giving rise to adventitious shoot formation (in presence of BA) differ both qualitatively and quantitatively in their 2-D pattern; two polypeptides may be classified as putatively specific for callusing growth and two for organogenesis (Reynolds, 1990).

In *Trifolium*, two peptides seem to be associated with embryogenesis in regenerating lines but are also expressed, with a different timing, by a non-regenerating genotype (McGee *et al.*, 1989).

Consistency of results has so far been found only between *Daucus carota* (Sung and Okimoto, 1981) and *Pisum sativum* (Stirn and Jacobsen, 1987). In both cases, with a first step separation by charge under native conditions and a second fractionation by SDS-PAGE, two proteins characteristic of the embryogenetic callus lines can be identified; with a slightly different approach, however - typical 2-D mapping according to O'Farrell, 1975 - three spots specific of the embryonic and two of the non-embryonic cells can be observed in the

carrot (Choi and Sung, 1984). Finally, after raising an antiserum against non-synchronized somatic embryos and absorbing antibodies to unorganized cell cluster components, four major and a number of minor embryo-specific antigens can be identified, none of which corresponds to a visible band upon general protein staining (Choi *et al.*, 1987).

The diversified (and confusing) evidence at the level of protein expression, as stated above, parallels an assorted body of protocols for embryogenesis induction in different species, resulting in widely different yields (in percent) from the quantitative transformation in *D. carota*, *N. tabacum*, or *Petunia* - acknowledged model systems for *in vitro* culture - to the 2-4% embryogenesis we obtain in *V. rupestris* (Martinelli *et al.*, 1993a and 1993b). In the latter system, the 2-D map of callus proteins differs extensively from embro-specific patterns, both in qualitative and in quantitative terms, which seems to imply a larger metabolic gap between the two sampled stages in this plant than in the species referred to above.

Within the framework of our research program, aimed at establishing optimal conditions for plant regeneration on *Vitis* specimen, the recognition of a distinct polypeptide make-up for an amorphous callus, in comparison with a potentially embryogenetic one, may allow early recognition, through biochemical markers, of a shift towards the differentiation path.

Studies are in progress to establish the delay between the onset of embryo-specific protein synthesis and the morphological changes to become apparent.

Abstract *A protocol has been detailed for somatic embryogenesis from leaf- and petiole-derived callus of Vitis rupestris. Isozymic pattern of acid phosphatase, alcohol dehydrogenase, esterase, gluconate-6-phosphate dehydrogenase and phosphoglucomutase were analyzed during precise steps of somatic embryogenesis from callus to plantlet. Among the five enzymes investigated, alcohol dehydrogenase, acid phosphatase and esterase gave different results, either in their specific activity, as in the case of alcohol dehydrogenase, or in the number and isoelectric point of the isoforms expressed, as in that of acid phosphatase and esterase. Non-morphogenic calli were clearly different from embryogenic calli, and from embryos. Moreover, the calli dedifferentiated from somatic embryos did not retain the level and types of isozymes that characterize the embryo state. Our analysis of the electrophoretic patterns of specific enzymes proves to be an effective approach to the characterization of the main steps of Vitis rupestris somatic embryogenesis.*

Introduction

Within the genus *Vitis*, plant regeneration from *in vitro* cultured calli was obtained only in a few instances *via* organogenesis (Cheng and Reisch, 1989; Clog *et al.*, 1990; Stamp *et al.*, 1990b) or somatic embryogenesis (Rajasekaran and Mullins, 1979; Bouquet *et al.*, 1982; Reisch and Roberts, 1985; Mauro *et al.*, 1986; Gray and Mortensen, 1987; Stamp and Meredith, 1988a). Due to those findings, grape has been considered an *in vitro* recalcitrant species.

Recently, we defined a protocol in *Vitis rupestris* for the induction of somatic embryogenesis from petiole- and leaf-derived calli. The protocol allows embryo germination and a recurrent secondary embryogenesis from somatic embryos (Martinelli *et al.*, 1991 and 1993a, Chapter 2).

Induction of embryogenesis from grapevine callus cultures has been also reported by Krul and Worley (1977), while the formation of somatic embryogenesis on the surface of somatic embryos was first described by

2-D electrophoresis

Callus samples of 50 to 100 mg were homogenized in 5 volumes of 9 M urea after the addition of a small quantity of quartz powder, in the presence of 4% w/v NP40 (Sigma), 2% w/v Ampholine^R (Pharmacia LKB) pH range 9-11, and 2% β-mercaptoethanol (Anderson, 1988). IPG (Bjellqvist *et al.*, 1982) with an exponential gradient course between pH 4 and 10 (Gianazza *et al.*, 1985b) was polymerized according to Righetti (1990), dried and reswollen in 8 M urea, 0.5% w/v NP40 and 0.5% CA (0.14% Pharmalyte^R 3-10, 0.1% 4-6.5, 0.14% Ampholine^R 3.5-10, 0.12% 4-7, 0.1% 4-6, from Pharmacia LKB). Forty µl extract was applied at the anodic edge on 7 mm wide, 140 mm-long, 0.5 mm-thick gel strips. The first step of the IPG-DALT separation (Gianazza *et al.*, 1985a) was run overnight at 15°C at 50 V/cm, then 1 h at 150 V/cm. The equilibration between 1st and 2nd dimension was according to Görg *et al.* (1987). SDS-PAGE was then run at right angle on a 7.5-17.5% T polyacrylamide gradient, with the discontinuous buffer system of Laemmli (1970). Silver staining was according to Heukeshoven and Dernick (1988).

Zymograms

We analysed leaf- and petiole-derived calli induced on 1/0.1 and 1/1 mgl^{-1} combination of BA/2,4-D, together with embryogenic calli, single embryos, and calli derived from embryos (all the latter from petiole cultures).

Protein extraction was performed with 0.2 M glycine (isoelectric solution) in the presence of the protease inhibitors leupeptine (0.11 mg ml^{-1}) and phenyl-methyl-sulfonyl fluoride (PMSF) (8.7 µg ml^{-1}), with a tissue to buffer ratio of 1:4. The samples were crushed in a ice-chilled mortar with quartz powder, the slurry suspended in the extraction medium, stirred for 15 min at 4°C and centrifuged 15 min at 12,000 rpm. Protein concentration was evaluated according to Bradford (1976).

Isoelectric focusing was performed on non-linear 4-10 IPG strips, 11 cm in length, reswollen in 0.5% CA; 25 µl per lane was loaded near the cathode. The electric parameters during the run were those used with the urea gels above, while temperature was lowered to 10°C.

Enzyme activity was revealed by incorporating the relevant chromogenic substrates and cofactors into 1% agarose (high electroendoosmosis - low gelling temperature; from Merck) poured on Gel Bond foils (FMC Corp.).

Staining was performed according to Swallow and Harris (1972) for AcP, to Smith *et al.* (1971) for ADH, to Coates *et al.* (1975) for EST, to Stuber and Goodman (1980) for G6PDH, and to Spencer *et al.* (1964) for PGM. The sandwiches between IPG slab and agarose overlay were incubated in a 37°C oven for 2h.

Results

Figure 6, Chapter 3 compares the 2-D peptide maps following IPG-DALT under dissociating conditions of primary non-embryogenic calli (a and b) and of embryogenic calli (c).

Both types of calli were derived from the same petiole cutting in the presence of 1 mgl^{-1} BA and 1 mgl^{-1} 2,4-D.

The differences between the two sets of protein spots are striking, both in qualitative and in quantitative terms, and most of the embryo specific spots can not be found in the callus patterns.

Zymograms were then compared for four types of primary calli, calli from embryo, embryogenic calli and early embryos after protein resolution under native conditions on IPG spanning the pH range 4-10. The banding patterns of G6PDH and of PGM revealed qualitatively similar patterns for all tested tissues and also their relative intensities were constant (not shown).

Figure 7 compares the enzyme activities for ADH, AcP and EST.

For enzymes, we found very similar patterns both in calli that had dedifferentiated from single embryos (sample 6) and in primary calli obtained either from leaf or from petiole, induced in the presence of different ratios of the two growth regulators BA and 2,4-D (1/1 and 1/0.1 mgl^{-1}) (samples 1-4, see Figure 1, Chapter 2). Their zymograms were however, clearly distinct from those of embryogenic calli (sample 5, see Figure 2, Chapter 2) and early embryos (sample 7, see Figure 3, Chapter 2); in both cases the number of isozymes resolved was higher in regenerating tissues than in unorganized cell clusters.

For all specimens, sample load was made with reference to fresh weight, with no adjustment to match varying protein concentrations. As for ADH, the increase in banding intensity between primary and embryogenic calli was, however, much larger than the increase in protein concentration between the two sets of samples. At least for this enzyme it can thus be concluded that ADH specific activity rises as soon as the embryogenic path is undertaken.

In fact, among the five enzymes investigated, ADH, AcP and EST gave different results, either in their specific activity, as in the case of ADH, or in the number and pI of the isoforms expressed, as in that of AcP and EST.

On the contrary, as shown by Gianazza *et al.*, (1992, Chapter 3), the IPG-DALT patterns of primary calli induced from different explants (leaf versus petiole) and under various hormonal regimes (Martinelli *et al.*, 1991 and 1993a, Chapter 2) reveal the same electrophoretic components, with only minor variations in their relative intensities. Complete overlap of spotting patterns is also observed when embryogenic calli and early embryos are compared.

In the well-characterized model system *Daucus carota*, embryogenic and non-embryogenic calli differed by 2 to 4 peptids (IEF was run under native or dissociating conditions: Sung and Okimoto, 1981; Choi and Sung, 1984). In this system, regeneration is easily achieved.

On the contrary, in *Vitis rupestris* we noticed a massive change, suggesting a radical shift in gene expression. This may justify both the low frequency and the slow progression from callus to embryogenesis.

All the following developmental steps leading to embryo, mature embryo, and seedling, respectively, took place at relatively high frequencies (Martinelli *et al.*, 1993a, Chapter 2). Accordingly, the electrophoretic analysis of total proteins performed during embryo maturation and germination shows less pronounced and more gradual changes.

The possibility to identify markers of tissue development might lead to the prediction of the morphogenetic potential of a given culture. Since the time required by embryogenesis induction from callus was rather long - about 7 months (Martinelli *et al.*, 1991 and 1993a, Chapter 2) - there might be some doubt on whether the differences between primary and embryogenic callus, detected by 2-D electrophoresis and zymograms, may depend upon the accumulation of mutations, a corollary of *in vitro* culture techniques (somaclonal variation; Larkin and Scowcroft, 1981). It should be noticed, however, that the likelihood of three such mutations occurring recurrently together in a callus line is negligible, and that the differences we detected are always related with the

subsequent steps of embryogenesis, i.e. the enzymatic changes observed in embryogenic callus were always retained by developing embryos.

Moreover, it was noted that the callus dedifferentiated from somatic embryos does not retain level and types of isozymes that characterize the embryo state.

It is worth mentioning the variability among different species in their behaviour during tissue culture: single enzyme markers may play a different role in different biotypes.

As an example, Fransz *et al.* (1989) found AcP not suitable for a discrimination between embryogenic and non-embryogenic callus in *Zea mays*; Chawla (1988) reports that AcP and EST are non informative in *Hordeum vulgare* and *Triticum sativum*, while Freier and Simon (1991) found AcP discriminating but EST and ADH invariant in *Daucus carota*.

In conclusion, the 2-D peptide mapping analysis (Chapter 3) and some of the isoenzymatic systems tested describe accurately the embryogenetic development from callus to plant in *Vitis rupestris*.

Efforts are needed to understand the somatic embryogenesis of grapevine, still recalcitrant to *in vitro* culture.

With our study, nevertheless, we hope to have contributed to future success in monitoring such mechanisms, also in *Vitis vinifera*.

Abstract *Isolated somatic embryos from petiole- derived callus cultures of Vitis rupestris Scheele have been employed in experiments of genetic transformation. Co-cultivation of somatic embryos during embryogenesis induction with Agrobacterium tumefaciens strain LBA4404, which contains the plasmid pBI121 carrying the neomycin-phosphotranspherase and the β-glucuronidase genes, produced transformed cellular lines capable of recurrent somatic embryogenesis. Precocious selection on high levels of kanamycin (100 mgl^{-1}) was an important part of our transformation protocol. Transformed lines still have strong β-glucuronidase expression as well as stable insertion of the marker genes after three years of in vitro culture, during which they have maintained their capacity to organize secondary embryos and to regenerate transgenic plants with an agreeable efficiency (13%).*

Introduction

Species of the genus *Vitis* are particularly difficult to transform: stable transgenic grapevine genotypes, for example, have not yet been reported.

The potentiality of biolistic transformation on grapevine cell cultures is currently under investigation (Hébert *et al.*, 1993).

Regeneration from transformed *Agrobacterium rhyzogenes* root explants has not been described so far (Hemstad and Reisch, 1985; Gribaudo and Schubert, 1990; Guellec *et al.*, 1990).

Agrobacterium tumefaciens seems a more amenable vector, since plant regeneration has already been reported for several genotypes starting from tissues adopted for transformation (Hirabayashi *et al.*, 1976; Mullins and Srinivasan, 1976; Krul and Worley, 1977; Barlass and Skene, 1979 and 1980; Rajasekaran and Mullins, 1979; Bouquet *et al.*, 1982; Mauro *et al.*, 1986; Stamp and Meredith, 1988a and 1988b; Cheng and Reisch, 1989; Clog *et al.*, 1990;

Kanamycin sensitivity

To optimize the level of kanamycin for the selection of the transformed tissues, a kanamycin response curve was evaluated during a 1-month selection.
Based on this, somatic embryos during embryogenesis induction on solid media, as well as maturation in liquid cultures and germination, were cultured with 15, 25, 50, 100 and 150 mgl^{-1} of the antibiotic added to the media after autoclaving.
To verify the kanamycin effect on the meristems, buds were tested at the same concentrations.

Transformation

Bacteria were grown in a solution of 2.85 mM K_2HPO_4, 0.82 mM $MgSO_4$, 0.01% NaCl, 0.04% yeast extract, 1% mannitol and 100 mgl^{-1} of kanamycin.
Infection was accomplished by a 5-min submersion of selected somatic embryos into an overnight culture of *Agrobacterium tumefaciens* strain LBA4404 containing the plasmid pBI121 (Jefferson *et al.*, 1987). This plasmid is a derivative of pBin19 (Bevan, 1984) and contains both the neomycin-phosphotranspherase (NPT) gene under control of the nopaline-synthase (NOS) promoter and terminator, and the *E. coli* β-glucuronidase gene fused to the Cauliflower Mosaic virus (CaMV) 35*s* promoter.
Embryos were blotted on filter paper and then placed horizontally on solid induction medium, with or without scratching with a needle, to prepare them for co-culture. The cultures were incubated at 28°C in the dark. After 3 days the embryos were moved to fresh medium containing 300 mgl^{-1} of cefotaxime and incubated at 26°C in the dark.

Culture maintenance on selective medium

Twenty-day-old cultures were moved to fresh medium containing 100 mgl^{-1} of kanamycin and 300 mgl^{-1} of cefotaxime, and incubated at 25°C in dim light (15 $\mu mol\ m^{-2}s^{-1}$).
The selection was carried out over a period of 9 months during which only white and embryogenic tissues were kept in culture.
After 1 year the concentrations of kanamycin and cefotaxime were reduced to 50 and 100 mgl^{-1}, respectively. Four months later, these levels were scaled down to 50 mgl^{-1} for both chemicals, and after a further 4 months kanamycin was reduced to 25 mgl^{-1}.
During callus subcultures, clusters of small embryos were periodically sampled to induce multiplication and elongation of isolated embryos in liquid cultures. Such clusters were isolated for the first time after a 7-month subculture of the embryogenic callus. They were cultured on a liquid NN-based medium containing 0.1 mgl^{-1} of IBA, 50 mgl^{-1} of kanamycin and 100 mgl^{-1} of cefotaxime. Two months later the levels of kanamycin and cefotaxime were reduced to 25 and 50 mgl^{-1} and four months later to 15 and 50 mgl^{-1}, respectively.

The overall process was repeated starting with 496 somatic embryos selected from liquid cultures and induced to produce secondary embryos on 50 mgl^{-1} of kanamycin and 100 mgl^{-1} of cefotaxime. During the subsequent liquid culture phase, 25 mgl^{-1} of kanamycin and 50 mgl^{-1} of cefotaxime were used.

Plant regeneration and culture

Single somatic embryos were isolated from liquid cultures and placed with the radicals downward on NN-based medium containing 1.5% sucrose, gelled with 0.8% Difco Bacto-agar, and supplemented with BA (1 mgl^{-1}) and IBA (0.1 mgl^{-1}). The medium was renewed monthly.

A sample of 52 somatic embryos was inseminated on this medium containing 50 mgl^{-1} of kanamycin and 100 mgl^{-1} of cefotaxime, and subjected to a chilling treatment (15 days in the dark at 4°C). Additionally, 73 somatic embryos were tested in the absence of kanamycin but in the presence of chilling. Finally, a population of 285 somatic embryos in four distinct replications were induced to regenerate plants in the absence of both kanamycin and chilling.

The embryo-derived plantlets were separated from the embryo tissues and cultured on growth-regulator-free NN-based medium containing 1.5% sucrose and 0.8 % Difco Bacto-agar, in order to promote rooting and elongation. The same medium was employed for micropropagation of grown plants. Both regenerating embryos and plant cultures were incubated in a climate room at 25 C with a 16 h photoperiod (70 mol $m^{-2}s^{-1}$ cool white light).

Media preparation and culture vessels

The pH of the media was adjusted with NaOH at 5.7 before autoclaving for 20 min at 121°C and 1 atmosphere. When needed, filtersterilized IAA was added after autoclaving. For solid cultures of both somatic embryos and regenerating embryos, 30 ml of medium were dispensed on plastic Petri dishes of 9 cm diameter.

For plant cultures, 70 ml of medium were dispensed in magenta boxes.

Marker gene evaluation

Periodically, embryos were randomly sampled from liquid cultures to screen for evidence of the insertion of the marker genes.

Assays have also been performed at the end of a complete cycle of recurrent somatic embryogenesis after 12, 18 and 36 months of selection on kanamycin. GUS expression was analyzed according to Jefferson (1987).

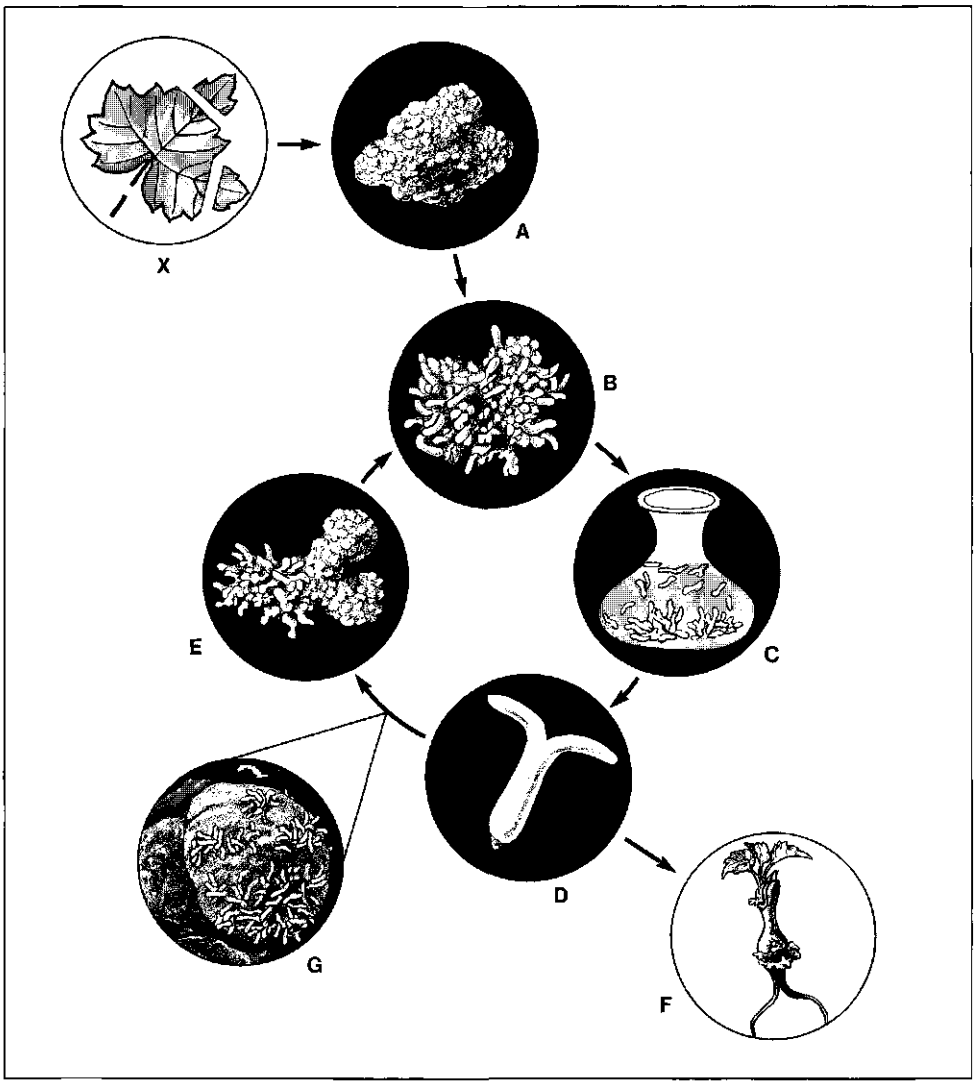


Figure 8 - *Schematic representation of the main steps of somatic embryogenesis and genetic transformation in Vitis rupestris. From petiole- and leaf- (X) derived callus (A), embryogenic callus (B) has been induced. Further liquid culture (C) matured somatic embryos which were isolated (D) for respectively secondary embryogenesis (E) or plant germination (F) induction. Steps from (D) to (C) can be cyclically repeated over and over, and coculture with Agrobacterium (G) occurs during secondary embryogenesis induction.*

The attachment of the bacteria to the cell surface was seen through the scanning electron microscope by observing how the bacterial clumps are tightly anchored to plant tissues by synthesized fibrils (Figure 9) (Mooney and Goodwin, 1991).

Wounding of embryo tissue proved unnecessary for achieving DNA delivery and insertion, since both scratched and intact embryos gave rise to transformed somatic embryos.

Figure 9 - *Somatic embryo co-cultured with Agrobacterium tumefaciens during secondary embryogenesis induction as seen with the scanning electron microscope. Particulars of a bacterial clump: fibrils allow strong contact of the bacteria with the embryo surface.*

All selected cell clumps originated embryogenic calli which differentiated clusters of somatic embryos (58% and 33% during the first and the second cycle, respectively). The selective conditions were modified during the subculture progress, as described under Materials and Methods. Nevertheless, the decrease in antibiotic concentration did not alter the level of expression of the GUS gene.

In agreement with the high level of GUS activity showed by transformed embryos (Figure 10), the integration of the GUS gene within the DNA of *V. rupestris* was evident when analyzed at the molecular level.

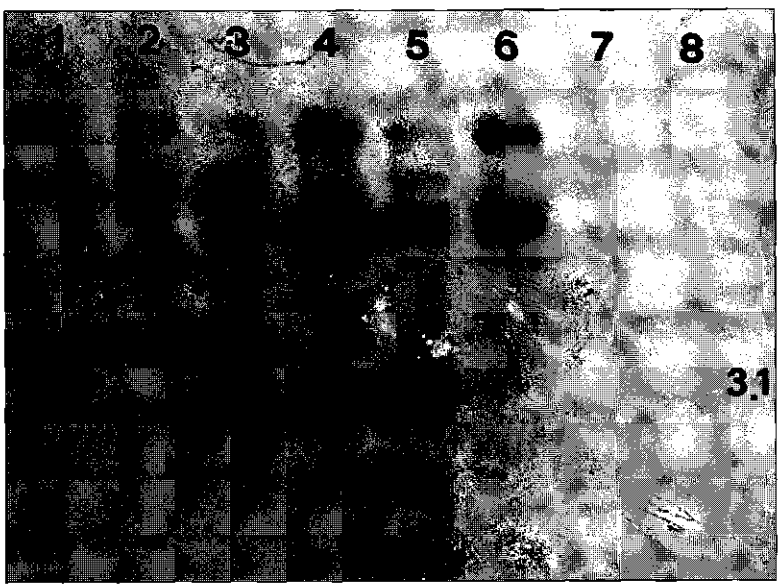


Figure 11 - *Autoradiograph of a Southern Blot of DNA extracted from embryo cultures transformed at different times. Lane 1, 12-month-old transformed culture, EcoRI and HindIII; lane 2, 12-month-old, EcoRI; lane 3, 18-month-old, EcoRI and HindIII; lane 4, 18-month-old, EcoRI; lane 5, 36-month-old, EcoRI and HindIII; lane 6, 36-month-old, EcoRI; lane 7, untransformed, EcoRI and HindIII; lane 8, untransformed, EcoRI.*

In three assays carried out 12, 18 and 36 months after the transformation, a sharp 3.1-kb band appeared consistently in Southern blottings of *Eco*RI-*Hind*III digested DNA when hybridized to the GUS fragment of pBI221 (Figure 11, lanes 1, 3 and 5); in *Eco*RI digested samples this band is not present, but is substituted by two heavier bands (Figure 11, lanes 2, 4 and 6).

In untransformed embryos, by contrast, no hybridization occurred at all (Figure 11, lanes 7 and 8).

Comparable results were observed when regenerated plantlets were tested. In Figure 12, in fact, the same pattern of hybridization occurred (lane 2 and 3). Additionally, when undigested genomic DNA was blotted, the lower-molecular-weight bands are substituted by a unique band corresponding to undigested DNA (lane 1).

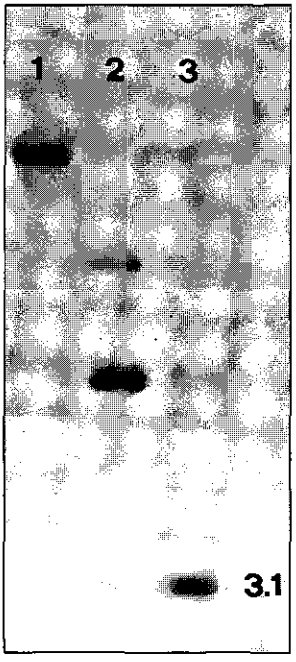


Figure 12 - *Autoradiograph of a Southern Blot of genomic DNA extracted from a transgenic plantlet. Lane 1, undigested DNA; lane 2, EcoRI-digested DNA; lane 3, EcoRI- and HindIII-digested DNA.*

These data, along with histochemical assays and stable antibiotic resistance, showed that a highly-stable integration of foreign genes in *Vitis rupestris* somatic embryos was achieved.

Only 4 weeks were required for inducing maturation and multiplication of somatic embryos in liquid culture. The abundant embryo production at this stage compensated for the low efficiency of the initial process.

Transgenic plant regeneration

Regenerative capacity has been described as a major problem for transgenic grapevines (Colby *et al.*, 1991b). In spite of this drawbacks, we obtained plant regeneration with a relevant efficiency from distinct transformation events: in fact, in the absence of kanamycin, both chilled and unchilled embryos regenerated plants at good levels (respectively 10% and 13%) within a 7-month culture.

Figure 13 - *Plant regeneration from a transgenic somatic embryo: regeneration occurs via organogenesis and many individual shoots are often produced from each embryo.*

As a result, respectively 11 and 36 different clones were obtained from distinct transformation events. Since similar values were obtained for untransformed *V. rupestris* somatic embryos during germination induction (Martinelli *et al.*, 1993a, Chapter 2), we assume that the presence of foreign DNA in the genome does not affect the regeneration capability.

The embryos produced a green disorganised callusing tissue, and from this tissue plant regeneration occurred *via* organogenesis, with many individual shoots regenerated from each embryo (Figure 13).

***Figure 14** - Plantlet regenerated from a transgenic somatic embryo for the β-glucuronidase gene, showing a strong GUS activity after the X-GLUC histochemical assay (Chapter 5).*

Shoots were dissected from the embryogenic tissues for growing, rooting and micropropagating.

Histochemical assays showed a strong GUS activity in the deformed embryogenic tissues and in the regenerating shoots, as well as in the grown plantlets (Figure 14) and in the roots (data not shown).

Because of the presence of the epicuticolar wax, leaf wounding is necessary for a proper incubation with X-Gluc.
In contrast, undamaged root tissues are very sensitive to this substrate since a strong blue coloration appears after few hours of incubation.

The molecular analysis of both transformed embryo cultures (Figure 11) and regenerated plantlets (Figure 12) fully demonstrates the stable insertion of the exogenous gene into the plant genome. Consequently, no loss of the chimeric GUS gene occurs following both the long-term embryo cultures and the plant regeneration events.

These results are a corollary of our transformation strategy.

In other systems, by contrast, difficult regeneration of whole plants and chimerical production and/or expression of the inserted genes have been reported as a disadvantage of the techniques adopted (Baribault *et al.*, 1989; and 1990; Mullins *et al.*, 1990; Colby *et al.*, 1991b). Nevertheless, here we have a system leading to good genome stability of the inserted genes and to a low level of somaclonal variation.

The effect is a suitable overlap between transgenic and regenerating cells.

In contrast, when kanamycin was present in the medium, embryos failed either to turn green and to evolve any morphogenic structures. However, when transferred on kanamycin free medium, embryos gradually developed greenish deformed structures.

The inhibitory effect of kanamycin on the organogenesis of grapevine has already been described (Baribault *et al.*, 1990; Colby and Meredith, 1990; Mullins *et al.*, 1990). However, our results prove the non-requirement for a selection on kanamycin during the plant regeneration stage.

Conclusions

The present work is a contribution to the success of genetic transformation in grapevine.

Our transformation strategy highlights some of the potential advantages of using somatic embryos for inserting foreign genes. In fact, from a single transformed somatic embryo (or a part of a single embryo) it is possible, through secondary embryogenesis, to propagate transformed tissues which give rise to a population of stably-transformed secondary somatic embryos. This population would be a clone originating from a single transformation event, and the data provided show that these transformed clones remain stable for 3 years.

Most remarkable, they are competent to regenerate transgenic plants with a suitable efficiency.

Chapter 6

Stability of the β-glucuronidase gene in a R0 population of grape (*Vitis rupestris* S.).

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Abstract *A study on the stability of the β-glucuronidase gene has been conducted in a population of transgenic plants regenerated from somatic embryos of Vitis rupestris S. Molecular tests demonstrated no loss of the inserted gene following either the long-term embryo culture and the plant regeneration events. Dot-Blot analysis proved to be a useful assay for a simultaneous assay of the exogenous gene in the population, and Southern Blot analysis showed the marker gene in the inserted form with a agreeable efficiency (92%).*

Introduction

Recombinant DNA techniques offer interesting possibilities for the genus *Vitis*.

Recently, promising results have been obtained with various approaches in different laboratories (Le Gall *et al.*, 1994; Martinelli and Mandolino, 1994, Chapter 5; Krastanova *et al.*, 1995; Scorza *et al.*, 1995; Kikkert *et al.*, 1996).

Agrobacterium-mediated transformation has proved to be an applicable technique for grape; however, relevant problems such as unstable gene insertion, chimeric tissue production and difficult plant regeneration (Colby *et al.*, 1991b) have been described as the most important factors affecting transgenic grapevines.

However, we have established a suitable protocol for genetic transformation and transgenic plant regeneration of *Vitis rupestris* S. with the marker gene for β-glucuronidase (GUS) (Martinelli and Mandolino, 1994, Chapter 5).

The present paper is a study of the stability of the marker gene and its insertion effciency in a plant population regenerated from transgenic somatic embryos.

Materials and methods

Transformation of somatic embryos

Genetic transformation of isolated somatic embryos of *Vitis rupestris* Scheele was performed as previously described (Martinelli and Mandolino, 1994, Chapter 5).
The embryos were infected with *Agrobacterium tumefaciens* strain LBA4404 which contained the plasmid pBI121 (Jefferson *et al.*, 1987) carrying the neomycin-phosphotransferase and the β-glucuronidase genes.
Eight transgenic somatic embryos (Figure 15) developed into transformed cellular lines and were labelled A, B, F, J, K, M, N and Z. These lines were competent for recurrent secondary somatic embryogenesis as well as for plant regeneration and as a result, eight families of transgenic plants were obtained. Moreover, many sister plants were yielded within each family since every secondary somatic embryo regenerated numerous shoots *via* organogenesis (Figure 15 and Figure 13, Chapter 5).
The molecular assays for the demonstration of the GUS gene insertion into the genomes were carried out with fully developed micropropagated plants, each derived from a different regeneration event.
The plantlets used for Southern blot experiments were 6-8 cm tall.
Tables 2 and 3 summarize the 8 families used, and the number of the regenerated plants assayed within each family.

Molecular analysis

Dot-Blot analysis

In order to determine the presence of the GUS gene within the genome of a group of 66 transformed and 2 non-transformed plants, genomic DNA was extracted from 0.5-1.0 g of leaf tissue, following the CTAB protocol (Rogers and Bendich, 1985).
Genomic DNAs (5 μg each) were blotted onto nylon membranes (Hybond-N, Amersham) using a Bio-Dot apparatus (BioRad).
Filter treatment, hybridization and washing conditions were similar to those described for the Southern blot analysis.

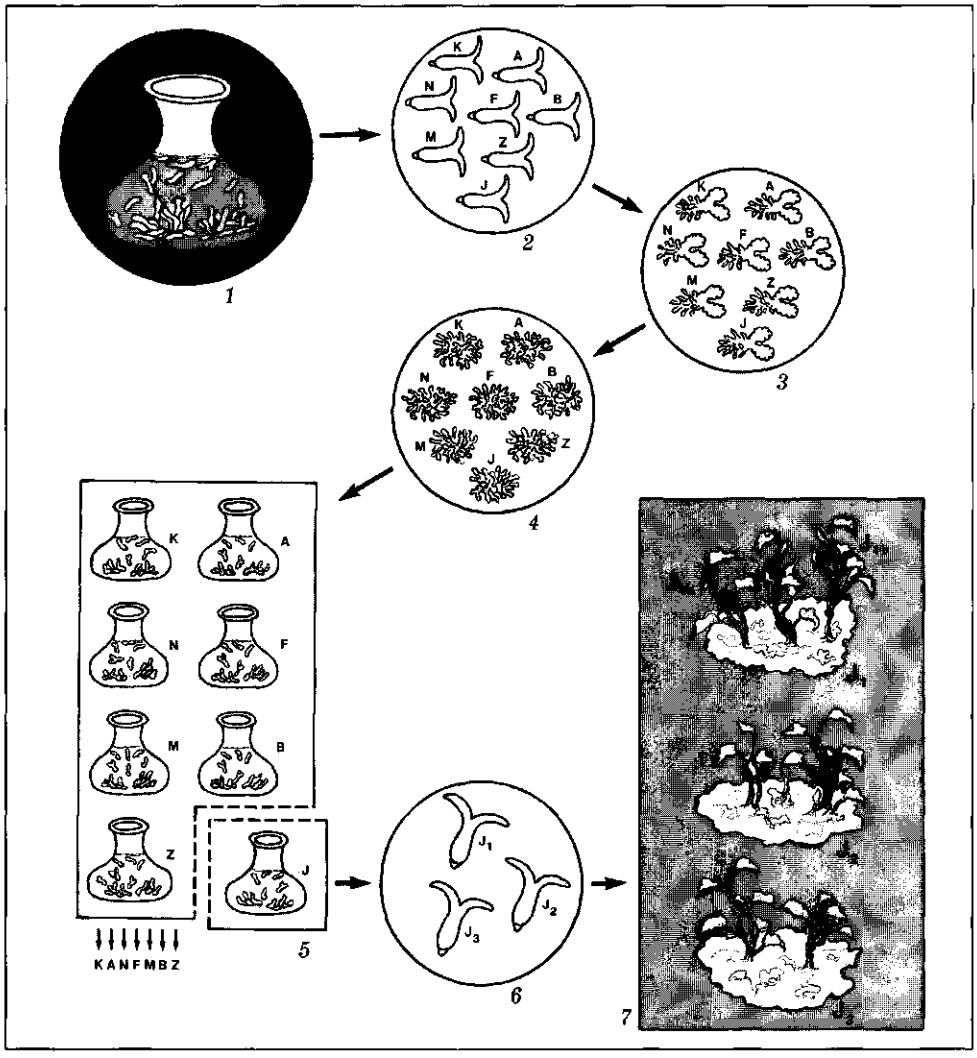

Figure 15 - *Schematic representation of the principal tissue culture steps leading to the establishment of eight lines of transgenic somatic embryos (A, B, F, J, K, M, N and Z) competent for regeneration of transgenic plants. Embryos were isolated from liquid cultures (1) and induced to secondary embryogenesis (2) (the co-culture with Agrobacterium effected during this stage is not shown here: see Figure 8, Chapter 5); from each of the 8 embryos, embryogenic lines were developed (3 and 4) and matured (5); then, induction of plant regeneration (6) produced families of transgenic plants (7).*

Southern Blot analysis

The genomic DNA extracted from 47 transgenic and one non-transformed plant (Rogers and Bendich, 1985) was restricted with *Eco*RI at a concentration of about 5 units/μg of DNA, and the digestion was carried out for 6 h under the conditions specified by the supplier. The restricted DNA was run in 1% agarose gels at a voltage of 5 V/cm. The gels were subsequently stained to check for complete digestion, depurinated for 15 min in HCl 0.25 M, denatured for 45 min in NaOH 0.5 M/NaCl 1.5 M, neutralized for 45 min in Tris-Cl 0.5 M (pH 7.2)/NaCl 1.5 M/EDTA, and capillary blotted overnight onto nylon Hybond-N (Amersham) filters. The filters were dried at 80°C for 10 min followed by cross-linked for 3 min on a 305 nm transilluminator. The filters were prehybridized at 65°C in 5x SSC (NaCl 0.75 M, Sodium citrate 0.51 M, pH 7.0) supplemented with 5x Denhardt's solution and 0.5% SDS (Sodium dodecyl sulphate).
Denatured herring test DNA was added at a concentration of 20 μg/ml. The probe corresponding to the GUS coding region was prepared by digesting 2 μg of the plasmid pBI221 (Clonthech) and gel-purifying the 3.1 kb fragment containing the GUS gene using the GeneClean (Bio 101) procedure. The purified DNA fragment was labelled to high specific activity with γ-^{32}P-dCTP using the random priming protocol (Feinberg and Vogelstein, 1983). The labelled probe was added directly to the prehybridation solution. After a 16-hours hybridization at 65°C, the filters were washed once in 3x SSC at 65°C and twice in 2x SSC at the same temperature. After the final wash the filters were exposed to autoradiographic cassettes with Kodak X-Omat AR 5 type films for the required period.

Results and discussion

Dot-Blot analysis

In order to evaluate a wide population of transformed plants, dot-blot analysis was carried out to assess the presence of the GUS sequence in the genome of 66 individual regenerated plants.

Although in dot-blot evaluation the foreign gene integration into the genome is not assessed, this technique appears to be reliable for a rapid screening of several plants in a simultaneous test.

Table 2 summarizes the results of this assay.

The percentage of GUS sequences found in a group of 66 leaf DNA preparations of individual plants within the eight families reached an average of 86.4% with some differences between distinct families.

Family code	No. of plant tested	No. of plants with exogenous gene presence
A	8	7
N	4	4
F	4	2
K	18	17
M	8	6
B	8	7
Z	9	8
J	7	6
	—	—
tot.:	66	57 (86.4%)
Control	2	—

Table 2 - *Results of Dot-Blot analysis for the presence of the β-glucuronidase gene in a population of 66 transgenic and 2 non-transgenic plants. Letters label the eight transgenic families assayed; within each family, the number of plants tested is proportional to the number of plants regenerated.*

Southern Blot analysis

The insertion of the GUS sequence within the plant genome was assessed in a group of 47 transformed plants by single digestions of total genomic DNA extracted from individual plantlets with *Eco*RI (Figure 16 and Table 3).

Under these conditions the 3.1 kb fragment used as hybridization probe detected one or more DNA fragments above 15 kb in length (Figure 16, lanes A14, N1-4, K1-11, M1-6, B1-6, Z1-7 and J1-5), confirming the integration of the homologous sequence in the plant genome. In comparison, no hybridization of the labelled GUS coding region to non-transformed plant tissue was observed (Figure 16, lane C).

Of 47 transformed plants tested, belonging to eight families derived from eight different transformed embryos, only four gave no detectable hybridization signals in genomic Southern Blots (Figure 16, lanes F1-3, and J1, and Table 3). In the other cases (92%), one to four bands were detectable at dimensions all above 15 kb.

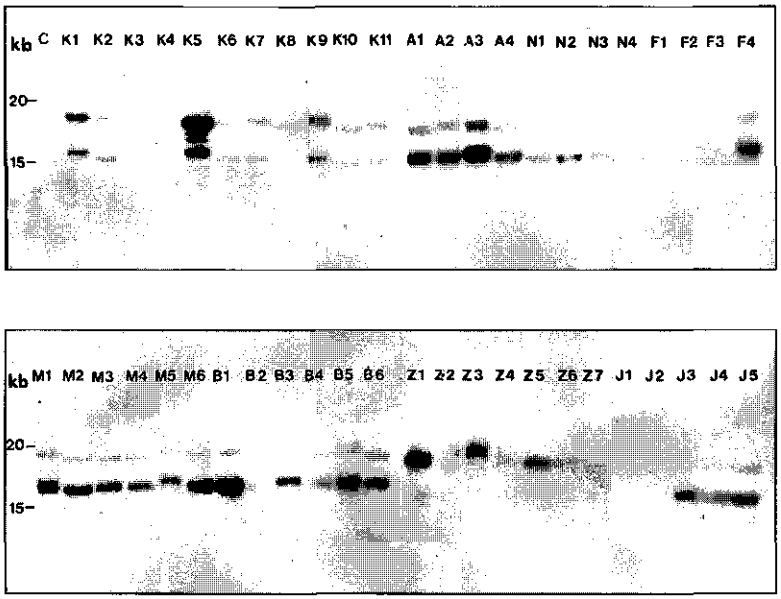

Figure 16 - *Southern Blot analysis of 47 transformed plants (lettered as K, A, N, F, M, B, Z, J) and one non-transformed plant (lane C). The probe used was the 3.1 kb GUS fragment from pBI221 (Clontech).*

These results confirm, at the level of a wide plant population, the gene integration in the genome as previously described by Martinelli and Mandolino (1994, Chapter 5) with research on transformed somatic embryos and young regenerated plantlets.

The occurrence of multiple bands in several cases suggests the presence of more than one copy of the inserted gene *per* genome. Since small polymorphisms, both in the insertion process and in the copy number, are not associated with different transformation events, this variability could have occurred after the GUS cassette insertion, i.e. during regeneration. Besides, the basic homogeneity of the fragments' number and size across all the transgenic lines suggests the possibility of constraints in the insertion site(s) of the exogenous construct.

Family code	No. of plant tested	No. of plants with exogenous gene integration
A	4	4
N	4	4
F	4	1
K	11	11
M	6	6
B	6	6
Z	7	7
J	5	4
	—	—
tot.:	47	43 (92%)
Control	1	—

Table 3 - *Results of Southern Blot analysis for the presence of the integrated β-glucuronidase gene in a population of 47 transgenic and one non-transgenic plants. Letters label the eight transgenic families assayed; within each family the number of plants tested is proportional to the number of plants regenerated.*

Abstract *In order to increase the number of grape genotypes adapted to in vitro manipulation studies, an investigation on the regeneration potential in the Vitis genus was conducted on cultivars of Vitis vinifera L., (Barbera, Cabernet franc, Cabernet Sauvignon, Canner Seedless bianco, Chardonnay, Enantio, Moscato bianco, Riesling, Sultana moscato bianco and Sultanina rouge), V. vinifera silvestris G., V. amurensis R., V. armata D. and G., V. riparia M., V. simpsonii M., as well as the rootstocks 110 Richter and Schwarzmann, and the interspecific variety Staufer. Regeneration was induced via direct organogenesis from young leaves, and a relevant genotypic influence on the morphogenic potentiality was observed. Among the 18 different genotypes tested, regeneration occurred with different efficiencies and precocity. Agreeable efficiencies were obtained for Sultana moscato, Riesling, Chardonnay and Cabernet franc, while Enantio and Cabernet Sauvignon were recalcitrant to shoot regeneration. V. armata was the most favourable to this regeneration system.*

Introduction

The adoption of molecular techniques for genetic improvement of grape results promising: recently, in fact, transgenic plants have been obtained in several laboratories with both *Agrobacterium*- mediated and direct transformation (Le Gall *et al.*, 1994; Martinelli and Mandolino, 1994; Nakano *et al.*, 1994; Krastanova *et al.*, 1995; Scorza *et al.*, 1995; Kikkert *et al.*, 1996). However, results are mostly restricted to the few genotypes with agreeable regenerative potential since genetic transformation is strictly related to *in vitro* plasticity. Thus, the genotype has been proven the most relevant component of morphogenic response within *Vitis* genus as well as other species (Bouquet *et al.*, 1982; Mauro *et al.*, 1986; Koornneef *et al.*, 1987).

From different explants, somatic embryogenesis and organogenesis have been obtained in some *Vitis* species (Mozsár and Süle, 1994; Martinelli *et al.*,

1993a; Robacker, 1993) and hybrids (Clog *et al.*, 1990; Harst, 1995), as well as rare *V. vinifera* cultivars (Mauro *et al.*, 1986; Stamp *et al.*, 1990a and 1990b; Harst and Alleweldt, 1993; Reustle *et al.*, 1994). Among the various regeneration approaches proposed, direct shoot organogenesis from young leaves (Stamp *et al.*, 1990a and 1990b; Colby *et al.*, 1991a) could be considered the most amenable because of its simplicity. Furthermore, this strategy represents a very interesting potential since regenerated leaflets proved to be juvenile tissues adapted to embryogenesis induction in species with difficult regeneration (James *et al.*, 1988; Rugini and Caricato, 1995).

In our laboratory we have already conducted some research on grape tissue culture; however, our best results have been obtained on *V. rupestris* (Gianazza *et al.*, 1992; Martinelli *et al.*, 1993a and 1993b; Martinelli and Mandolino, 1994 and 1996, Chapters 2, 3, 4, 5 and 6).

In order to increase the number of grape genotypes adapted to our *in vitro* manipulation studies, we carried out an investigation on the regeneration potential in the *Vitis* genus, following a protocol derived from the "direct shoot organogenesis from leaves" technique (Stamp *et al.*, 1990b). Cultivars of *V. vinifera*, species of *Vitis* genus and hybrids were analyzed. The results of this study are reported in the present paper.

Materials and Methods

Explant source

The test was conducted on *Vitis vinifera* L. cultivars (Barbera, Cabernet franc, Cabernet Sauvignon, Canner Seedless bianco, Chardonnay, Enantio, Moscato bianco, Riesling, Sultana moscato bianco and Sultanina rouge), *V. vinifera silvestris* G., *V. amurensis* R., *V. armata* D. and G., *V. riparia* M., *V. simpsonii* M., as well as the rootstocks 110 Richter and Schwarzmann, and the interspecific variety Staufer (Bacchus x Villard blanc) - from the collections of our Institute - already established in *in vitro* cultures. *In vitro* plants cultured on NN medium (Nitsch and Nitsch, 1969) with 1.5% sucrose and incubated at

25°C with a 16 h photoperiod (70 µmol $m^{-2}s^{-1}$ cool white light) were sampled as starting material. One-node segments were dissected and induced to adventitious shoot proliferation on a NN (Nitsch and Nitsch, 1969) based medium with iron as in MS (Murashihe and Skoog, 1962) medium, 2% sucrose and 2 mgl^{-1} BA. Cultures were incubated at 25°C in the shade (10 µmol $m^{-2}s^{-1}$). The number of segments cultured for each of the genotypes tested is summarized in Table 4.

Organogenesis induction

Young leaves (3 - 4 mm) were separated by a cut across the petiole, according to Stamp *et al.* (1990b), from 40-day-old nodal cultures, and placed adaxial side down on half strength MS medium supplemented with 2% sucrose, 2.25 mgl^{-1} BA and 0.03 mgl^{-1} NAA. Cultures were incubated at 25°C in the dark for 10 days and then moved to the shade (10 µmol $m^{-2}s^{-1}$). Table 4 reports the number of leaves tested within the genotypes.

Plantlet culture

Regenerated shoots were dissected from regenerating leaf explants and grown at 25°C with a 16 h photoperiod (70 µmol $m^{-2}s^{-1}$ cool white light) in Magenta boxes containing 70 ml growth regulator-free NN-based medium with 1.5% sucrose.

Media completion and culture vessels

Media were solidified with 0.9% bacteriological Agar (Oxoid), and pH was adjusted with NaOH at 5.7 before a 20 min autoclaving at 121°C and 1 bar. Nodal cultures and regeneration from leaves have been conducted in plastic Petri dishes of 9 cm diameter sealed with Parafilm. Plant micropropagation has been carried out in Magenta boxes.

Results and Discussion

Within 40 days of culture, one-node explants produced adventitious bud formation with young leaves for the further regeneration induction. This first

step yielded a homogeneous explant population within the tested genotypes as for age and development of the tissues for the subsequent morphogenic induction.

The number of internode segments and leaves assayed for each genotype are reported in Table 4. Shoot regeneration from young leaves was followed during an 80-day culture period.

Typically, shoot regeneration occurred *via* direct organogenesis without callusing from the cut surface of the petiole, according to Colby *et al.* (1991a) (Figure 17). However, a different organogenic pathway was observed in *V. armata*, *V. simpsonii* and Sultana moscato where regeneration followed callusing (Figure 18). Regeneration occurred also from the central vein of the leaf without callusing in Sultana moscato (Figure 19). Generally, more than one regeneration event occurred from each leaf, according to Stamp *et al.* (1990a and 1990b), and a considerable number of shoots (up to 30) was obtained when regeneration occurred after callusing.

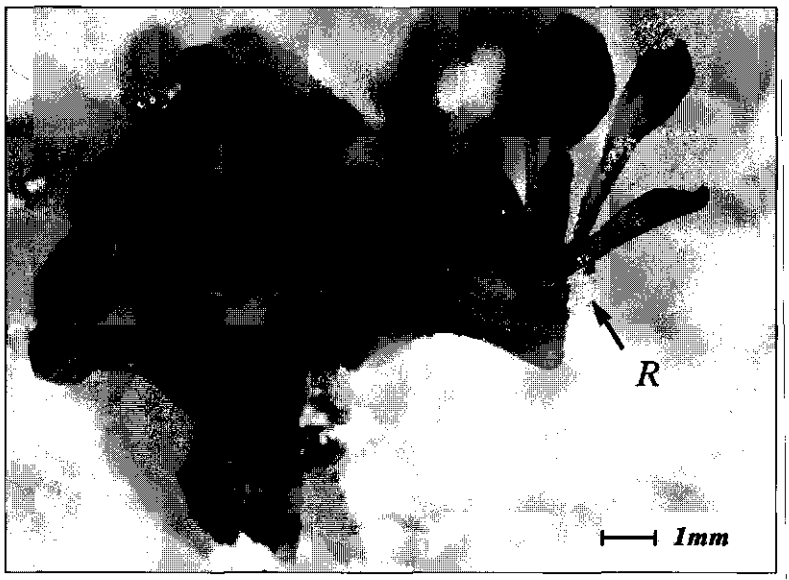

Figure 17 - *Shoot organogenesis from the cut surface of the petiole without callusing. R = regenerating shoots.*

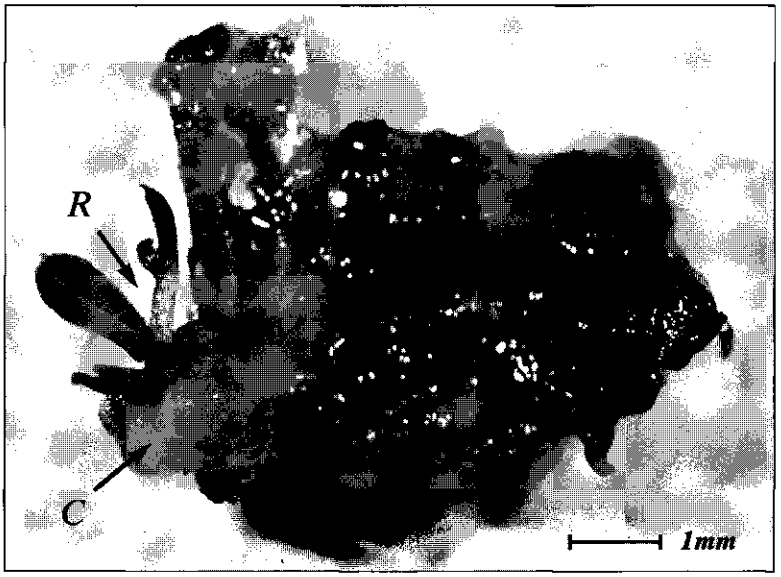

Figure 18 - *Shoot organogenesis from the cut surface of the petiole in the presence of callusing. R = regenerating shoots; C = callusing.*

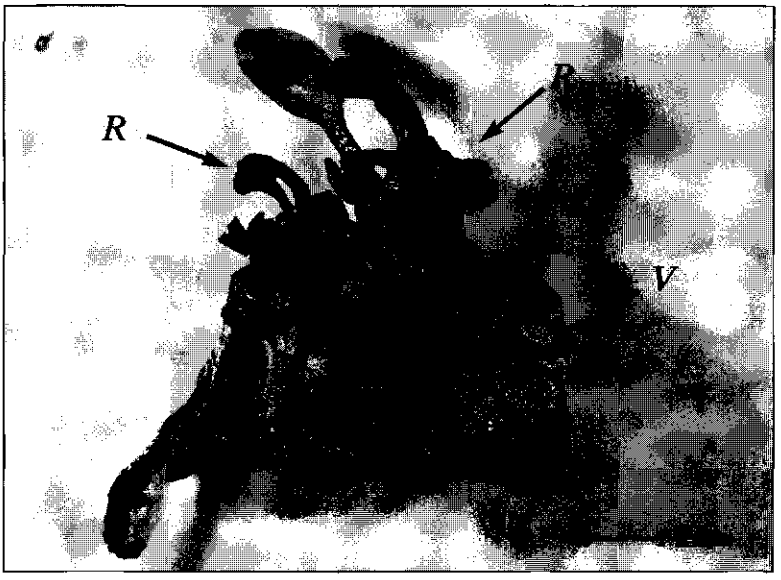

Figure 19 - *Shoot organogenesis from the middle vein of the leaf.*
R = regenerating shoots; V = middle vein of the leaf.

Chapter 8

General discussion and conclusion

In the past years, progress in molecular biology have led to a dramatic increase of the knowledge of structure and function of the plant genomes. This technology has been applied to produce and release commercially important transgenic plants. Promising results have been also obtained for those species considered as "recalcitrant" to biotechnology due to problems with *in vitro* culture; among these, species of the *Vitis* genus.

Recently, transgenic grapes have been produced in several laboratories (Le Gall *et al.*, 1994; Martinelli and Mandolino, 1994 and 1996; Nakano *et al.*, 1994; Mauro *et al.*, 1995; Krastanova *et al.*, 1995; Perl *et al.*, 1996; Scorza *et al.*, 1996; Kikkert *et al.*, 1996) and genes involved in important pathways have been isolated from grapevines (Dray and Robinson, 1994; Sparvoli *et al.*, 1994). However, results are still restricted to the few genotypes with agreeable regenerative potential since genetic transformation is strongly related to *in vitro* culture plasticity also in the *Vitis* genus (Bouquet *et al.*, 1982; Gray, 1995; Martinelli *et al.*, 1996a; Mozsár and Viczián, 1996).

In the "recalcitrant" species, the limited knowledge of morphogenesis restricts tissue culture to an empiric practice, where optimal culture conditions needs to be defined by testing a huge number of variables by trial and error. These include the genotype of the donor plants, physiological situation of the explant, such as age or gradients of regeneration competence within the explants, and environmental factors, such as temperature, light, and culture medium.

In the present research, we highlight some of the most relevant problems related to regeneration in grape (Chapters 2, 3, 4, and 7) with the aim of improving regeneration efficiencies for the adoption of molecular techniques (Chapters 5 and 6). Most of the research has been here conducted on *Vitis rupestris* Scheele, a genotype which proved a useful model for our investigations. In this genotype, a procedure for somatic embryogenesis and subsequent plantlet regeneration has been established ***(Chapter 2)***. This is the first detailed report of somatic embryogenesis from callus culture derived from leaf and petiole explants. The culture conditions proved suitable for embryo culture and

propagation over long periods of time, as well as for the establishment of plantlets from embryos which occurred with satisfactory efficiency. However, additional efforts are required to increase the rate of embryogenetic induction, and to eliminate the time it takes to established plantlets. In particular, embryogenic callus induction from the explants appeared the limiting step of the overall process, occurring with low efficiencies (4%) and only after prolonged culture time (7 months). Furthermore, the interplay between different factors affecting the behaviour of plant cells in *in vitro* culture is poorly understood. Definitions such as "due to the physiological status" and "recalcitrant species" are generally used to describe the unknown behaviour of tissues during regeneration. A better understanding of the events leading to somatic embryogenesis requires the knowledge of the biochemical changes associated with regeneration, from the explant to the plantlet. This should lead to the finding of suitable markers to predict the embryogenic potential of cultured explants and calli, and to allow an objective selection among cultures. Furthermore, the possibility to identify markers of tissue development might lead to the prediction of the morphogenetic potential of a given culture. This point appears crucial in grape tissue culture since the low efficiency of regeneration and the long time requested for embryo induction lead to a time consuming work for subculturing tissues that reveals unproductive only after long time.

In our system, we first focused on embryogenic callus induction since this process represents the limiting step of the overall procedure, then we analysed some biochemical parameters along the different following steps of somatic embryogenesis up to plants. Our analysis of the electrophoretic patterns of total proteins ***(Chapter 3)*** and specific enzymes (acid phosphatase, alcohol dehydrogenase, esterase) ***(Chapter 4)*** proved effective approaches to the characterization of the main steps of *Vitis rupestris* somatic embryogenesis. The developmental stages between primary callus and embryogenic callus as well as between embryo and plantlet have been characterized and appeared different due to the presence of a stage-specific set of peptides.

In the well-characterized model system *Daucus carota*, embryogenic and non-embryogenic calli differed by 2 to 4 peptides (Sung and Okimoto, 1981; Choi and Sung, 1984). In this system, regeneration is easily achieved. On the contrary, in *Vitis rupestris*, we noticed a massive change suggesting a radical shift in gene expression. This may justify both the low frequency and the slow progression from callus to embryogenesis since it suggests that expression of many genes is involved. All the following developmental steps leading to embryo, mature embryo and seedling, respectively, took place at relatively high frequencies. Accordingly, the electrophoretic analysis of total proteins performed during embryo maturation and germination showed less pronounced and more gradual changes.

Since the time required by embryogenesis induction from callus was rather long, there might be some doubt on whether the differences between primary and embryogenic callus, detected by 2-D electrophoresis and zymograms, may depend upon somaclonal variation, a corollary of *in vitro* culture techniques. It should be noticed, however, that the likelihood of such mutations occurring recurrently together in a callus line is negligible, and that the differences we detected were always related with the subsequent steps of embryogenesis, i.e. the enzymatic changes observed in embryogenic callus were always retained by developing embryos. Moreover, it was noted that the callus dedifferentiated from somatic embryos did not retain level and types of isozymes that characterize the embryo state indicating that there are gene expression differences.

The most relevant performance that made *Vitis rupestris* our model also for genetic transformation studies ***(Chapters 5 and 6)***, has been the efficient regeneration of secondary embryogenesis exhibited by this genotype. In our cultures, somatic embryogenesis develops in cycles that can be repeated over and over. This morphogenic process proved to be particularly effective for *Agrobacterium tumefaciens*-mediated genetic transformation and is the basis of our protocol. The interaction between bacteria and our cultures, in fact, neither seriously affect somatic embryos nor inhibits their morphogenic potential.

Besides, the low sensitivity to kanamycin of embryogenic tissues allows an effective discrimination between transgenic and non-transgenic cells that can be achieved on high levels of this antibiotic during the secondary embryogenesis induction. Furthermore, transgenic cells that resulted were competent for embryogenesis regeneration. As a consequence, from a single transformed somatic embryo, it was possible to propagate the transformed tissues which gave rise to a "population" of stably transformed secondary somatic embryos capable of regenerating various stable transgenic plants. Histochemical assays showed a strong expression of the marker gene (β-glucuronidase) both in the embryos and in the regenerated plantlets. Accordingly, molecular analysis of a fairly large group of selected plants confirmed that the exogenous gene was integrated into the genome. The most common pattern of integration observed consisted of one or two bands, and only in one case as an apparent multiple insertion. The size and relative intensity of multiple bands in the same family of transgenic plants showed some variation which was probably due to genomic rearrangements during the regeneration events.

On the basis of our results, and of similar reports of transgenic plant production (Mullins *et al.*, 1990; Le Gall *et al.*, 1994; Martinelli and Mandolino, 1994; Nakano *et al.*, 1994; Krastanova *et al.*, 1995; Mauro *et al.*, 1995; Scorza *et al.*, 1996; Kikkert *et al.*, 1996; Perl *et al.*, 1996), it can be concluded that somatic embryos are a very suitable material in which to obtain stable insertion and high expression levels of exogenous genes, especially if severe criteria of selection are applied in order to restrict chimerism. Even tough an hypersensitive response of embryogenic cells of *Vitis vinifera* (cv. Superior Seedless) to *Agrobacterium* has been pointed out as a drawback of this technique (Perl *et al.*, 1996), both *Agrobacterium* co-culture and biolistic technology proved to be valuable strategies for the exogenous gene delivering in grape cells. *Agrobacterium* resulted more adapted for embryo transformation while particle gun proved more effective on somatic cell cultures.

The knowledge of crucial aspects related to regeneration and genetic transformation learned in *Vitis rupestris* enabled us to address our research to

other agronomically important grape genotypes. So, in order to increase the number of grape genotypes adapted to our *in vitro* manipulation studies, we carried out an investigation on the regenerative potential in the *Vitis* genus, and we detected a great number of genotypes with relevant morphogenic capability for further *in vitro* studies ***(Chapter 7)***. Direct shoot organogenesis from young leaves resulted to be optimal because of its simplicity. Furthermore, this strategy represents a very interesting potential since regenerated leaflets proved to be juvenile tissues adapted to embryogenesis induction in species with difficult regeneration (James *et al.*, 1988; Rugini and Caricato, 1995). In particular, the regeneration of juvenile tissues for somatic embryogenesis induction (James *et al.*, 1988; Rugini and Caricato, 1995) associated to this protocol, represents an interesting perspective since somatic embryos resulted the best system for stable transgenic plants production in *Vitis* genus.

In our assays, among the 18 different genotypes tested, regeneration occurred with varying efficiencies and precocity. Thus, genotype resulted a relevant component of morphogenic response within *Vitis* genus as already proved for other species (Koornneef *et al.*, 1987).

At this point of our research, we started to introduce in our *Vitis rupestris* system, constructs related to biotic stress resistance (Martinelli *et al.*, 1996b; Minafra *et al.*, 1997b) as well as studying suitable strategies for transform the grape genotypes which proved to be the most adapted to *in vitro* manipulation. And these aspects are the topics of our present research.

It is worth to consider, at this point, that public concern might be an obstacle to the release of transgenic grapes and its products.
While rootstock manipulation should have a less impact on the public opinion, this aspect could be relevant, at the opposite, in the table and grape vines since consumers demonstrated to be still aware of transgenic food. Furthermore, innovations might be hardly accepted in the wine market which is very conservative.

We believe, however, that recombinant DNA techniques would be a valuable opportunity for genetic improvement programmes especially in respect

of the environmental issues. This is particularly the case when disease resistance is pursued since intrinsically resistant plants should lead to a reduction of the chemical product use in the agricultural practice. Therefore, in addition to rootstock improvement, the production of transgenic scions could be an interesting goal too.

Furthermore, as for research purposes, since genetic transformation is a fundamental technique for the study of gene function and expression, this strategy could provide new insights in some important diseases affecting grapes (Minafra *et al.*, 1997).

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Summary

Grape (*Vitis vinifera*) is a versatile fruit crop, being cultivated for wine, table grape, raisin and juice production. According to annual statistics by the Office International de la Vigne et du Vin (Paris), in 1995 the cultivated area was 7,768,800 ha., grape harvest reached 54,056,700 tonnes and wine production was 246,423,000 hl.

The programmes for genetic improvement of grapes are mostly related to the geographical areas of culture and involve traits of productivity, biotic and abiotic stress resistance. In particular, *Vitis* species are affected by fungal and viral diseases, and by insect pests.

According to biological constraints as well as market rules, genetic improvement of grapes is carried out by clonal selection or breeding programmes.

Crossings are relatively feasible within *Vitis* genotypes which are outbreeders; however, genetic drawbacks - such as high degree of heterozygosity, heavy load of deleterious recessives and severe inbreeding depression, combined with a long generation time from seed to fruit - discourage the use of breeding.

In this framework, genetic transformation offers unique perspectives for the genetic improvement of *Vitis* genus. The most relevant features of this strategy are concerned with the possibility to overcome disadvantages associated with conventional breeding, and to modify specific characteristics in already selected genotypes. This can be realized with the insertion of genes of specific traits or of antisense sequences. The most ambitious goal is the production of new plants intrinsically resistant to biological stress which will have ecological and economic advantages.

Even though grape was one of the first plants employed in tissue culture studies, this technology is still far from routine: this delay is related to the low

regeneration competence of grapevine which is the principal requirement for transformation strategies.

The aim of the present research is the definition of a valuable protocol based on recombinant DNA techniques for the genetic improvement of grape for biotic stress resistance.

Since *in vitro* manipulation of tissues is one of the most crucial steps of the overall procedure, we first found within the genus *Vitis* the genotype with the most suitable morphogenic competence, then we studied in this genotype (*V. rupestris* S.) the insertion of marker genes, and, finally, we concentrated on agronomically important grape genotypes.

In our work, somatic embryogenesis from leaf- and petiole-derived calli of *V. rupestris* was obtained with an efficiency of 3.2% and 4.2% of plated explants, respectively on two combinations of 6-benzyladenine (BA) and 2,4-dichlorophenoxyacetic acid (2,4-D) (1/0.1 and 1/1 mgl^{-1}) added to Murashige and Skoog (MS) medium ***(Chapter 2)***. Embryogenic callus, embryo subcultures and somatic embryogenesis from somatic embryos were obtained either in the presence of 1 mgl^{-1} indole-3-acetic acid (IAA) or 0.1 mgl^{-1} indole-3-butyric acid (IBA) added to MS or Nitsch and Nitsch (NN) media.

Within a 4-month culture, embryo germination occurred at frequency of 13% of explanted embryos when chilling at 4°C was provided for two weeks on NN medium supplemented with a combination of BA (1 mgl^{-1}) with IBA (0.01 mgl^{-1}) and casein hydrolysate (250 mgl^{-1}).

A higher frequency (51%) was obtained in a longer culture time (9 months) when only IBA was present in the medium and in absence of chilling.

Then, we carried out a study for the definition of suitable markers for the characterization of morphogenic events during somatic embryogenesis.

First, a two-dimensional electrophoretic analysis of the total proteins was carried out in our *V. rupestris* cultures during the different developmental stages ***(Chapter 3)***, such as callus, embryogenetic callus, somatic embryos and plantlets derived from leaf and petiole explants. Each differentiation step was characterized by specific peptide spots. Besides, isozymic pattern of acid

phosphatase, alcohol dehydrogenase, esterase, gluconate-6-phosphate dehydrogenase and phosphoglucomutase were analysed during the same morphogenic steps ***(Chapter 4)***. Among the five enzymes investigated, alcohol dehydrogenase, acid phosphatase and esterase gave different results, either in their specific activity, as in the case of alcohol dehydrogenase, or in the number and isoelectric point of the isoforms expressed, as in that of acid phosphatase and esterase. Non-morphogenic calli were clearly different from embryogenic calli, and from embryos. Moreover, the calli dedifferentiated from somatic embryos did not retain the level and types of isozymes that characterize the embryo state. Our analysis of the electrophoretic patterns of specific enzymes proves to be an effective approach to the characterization of the main steps of *V. rupestris* somatic embryogenesis.

As for genetic transformation, isolated somatic embryos from petiole-derived callus cultures of *V. rupestris* have been employed ***(Chapter 5)***. Co-cultivation of somatic embryos during embryogenesis induction with *Agrobacterium tumefaciens* strain LBA4404, which contains the plasmid pBI121 carrying the neomycin-phosphotranspherase and the β-glucuronidase genes, produced transformed cellular lines capable of recurrent somatic embryogenesis. Precocious selection on high levels of kanamycin (100 mgl^{-1}) was an important part of our transformation protocol. Transformed lines showed strong β-glucuronidase expression as well as stable insertion of the marker genes after long times of *in vitro* culture, and have maintained their capacity of organize secondary embryos and to regenerate transgenic plants with an agreeable efficiency (13%).

Then, the stability of the β-glucuronidase gene in the transgenic plants has been evaluated at population level ***(Chapter 6)***. Southern and Dot Blot analysis demonstrated no loss of the inserted gene following either the long-term embryo culture and the plant regeneration events.

Finally, in order to increase the number of grape genotypes adapted to *in vitro* manipulation studies, an investigation on the regeneration potential in the *Vitis* genus was conducted ***(Chapter 7)*** on cultivars of *V. vinifera* L. (Barbera,

Cabernet franc, Cabernet Sauvignon, Canner Seedless bianco, Chardonnay, Enantio, Moscato bianco and Sultanina rouge), *V. vinifera silvestris* G., *V. amurensis* R., *V. armata* D. and G., *V. riparia* M., *V. simpsonii* M., as well as the rootstocks 110 Richter and Schwarzmann, and the interspecific variety Staufer. Regeneration was induced via direct organogenesis from young leaves, and a relevant genotypic influence on the morphogenic potential was observed.

Among the 18 different genotypes tested, regeneration occurred with different efficiencies and precocity. *V. armata* was the most favourable to this regeneration system. Agreeable efficiencies were obtained for Canner Seedless bianco, Sultana moscato bianco, Cabernet franc, Chardonnay and Riesling, while Cabernet Sauvignon, Enantio and *V. riparia* were recalcitrant to shoot regeneration.

In conclusion, we believe that the present work is a contribution to the success of regeneration and genetic transformation in the *Vitis* genus and opens interesting perspectives to the application of molecular techniques for the further study of resistance to pathogens as well as for the genetic improvement of this fruit crop.

Samenvatting

De druif (*Vitis vinifera*) is een veelzijdige vrucht. Het gewas wordt verbouwd voor de wijnbereiding, voor de verse consumptie (tafeldruif), voor het gebruik van de gedroogde vrucht (rozijn en krent), en voor de bereiding van druivensap. Volgens de statistieken van het "Office International de la Vigne et du Vin" te Parijs, werd er in 1995 op 7.768.000 hectaren land druiven geteeld, waarvan 54.056.700 ton vruchten geoogst werd. De hoeveelheid geproduceerde wijn bedroeg in 1995 246.423.000 hectoliter.

Veredelingsprogramma's voor de druif zijn meestal gericht op specifieke geografische gebieden en hebben betrekking op productiviteit, biotische en abiotische stress-resistentie. *Vitis* soorten zijn vooral vatbaar voor schimmel en virusziekten en voor schade door insecten.

Zowel vanwege biologische beperkingen als vanwege handelseisen, wordt veredeling van de druif vooral uitgevoerd via selectie binnen klonen of via kruisingsprogramma's.

Kruisingen zijn gemakkelijk uit te voeren tussen *Vitis* genotypen, die kruisbevruchters zijn. Het toepassen van kruisingsveredeling wordt echter ontmoedigd door bezwaren, zoals een grote mate van heterozygotie, onder andere voor veel recessieve allelen met letale effecten en een sterke inteelt-depressie, en een lange generatieduur.

In het kader hiervan biedt genetische transformatie unieke mogelijkheden voor de verbetering van het genus *Vitis*. Het belangrijkste voordeel hierbij is dat bestaande elite genotypen verbeterd kunnen worden zonder de bovengenoemde problemen van de conventionele kruisingsveredeling. Dit kan gerealiseerd worden door antisense sequenties of genen die coderen voor specifieke eigenschappen of door in deze genotypen binnen te brengen. Vooral het verkrijgen van rassen met een hogere resistentie tegen biologische stress is een belangrijke uitdaging.

Hoewel de druif één van de eerste planten is geweest die in weefselkweekstudies is gebruikt, is het gebruik van deze technologie nog lang geen routine. Dit komt o.a. doordat de regeneratie-competentie van de wijndruif gering is, wat een probleem is, juist omdat regeneratie vanuit weefselkweek een belangrijke voorwaarde is voor het toepassen van transformatie technieken.

Het doel van het hier beschreven onderzoek is het ontwikkelen van een protocol voor de genetische verbetering van de druif gebaseerd op recombinant-DNA technieken. Deze kunnen vervolgens gericht worden op het verkrijgen van resistentie tegen biotische stress.

Aangezien in-vitro manipulatie van weefsels één van de meest cruciale stappen van de procedure is, is er gezocht naar het *Vitis* genotype met het beste regeneratievermogen. Vervolgens is dit genotype (*V. rupestris* S.) gebruikt voor het inbrengen van merkergenen. Tenslotte is er onderzoek uitgevoerd naar de regeneratie van de landbouwkundig meer belangrijke druifgenotypen.

Hoofdstuk 2 beschrijft somatische embryogenese vanuit blad - en bladsteel - explantaten. Somatische embryogenese werd verkregen met een efficiëntie van 3.2-4.2% van het aantal uitgelegde explantaten, respectievelijk op twee combinaties van 6-benzyladenine (BA) en 2,4-dichlorophenoxy azijnzuur (2,4 D) (1/0.1 en 1/1 mgl^{-1}), dat aan het Murashige-Skoog (MS) medium was toegevoegd. Embryogeen callus, embryo subcultures en embryogenese van somatische embryo's werden verkregen, hetzij in de aanwezigheid van 1 mgl^{-1} indolazijnzuur (IAA), hetzij in 0.1 mgl^{-1} indolboterzuur (IBA) toegevoegd aan MS of Nitsch en Nitsch (NN) media. In een vier maanden oude cultuur vond kieming van embryo's plaats met een frequentie van 13% van de uitgelegde somatische embryo's, wanneer een koudebehandeling van 2 weken werd toegepast en 1 mgl^{-1} BA, 0.01 mgl^{-1} IBA en caseïne hydrolysaat (250 mgl^{-1}) aan het NN medium was toegevoegd. Een kiemingsfrequentie van 51% werd gerealiseerd na een kweektijd van 9 maanden wanneer alleen IBA in het medium aanwezig was en de koudebehandeling werd weggelaten.

In ***hoofdstuk 3*** is onderzoek beschreven, dat gericht was op het vinden van geschikte merkers voor morfogenetische gebeurtenissen tijdens somatische

embryogenese. Hiertoe werd twee-dimensionale eiwitelektroforese van *V. rupestris* in verschillende morfogenetische ontwikkelings stadia zoals callus, embryogeen callus, somatische embryo's en vanuit blad en bladsteel geregenereerde planten uitgevoerd. Iedere regeneratiestap bleek gekenmerkt te zijn door specifieke eiwitvlekken.

Vervolgens (***hoofdstuk 4***) werden isozympatronen van zure fosfatase, alcohol dehydrogenase, esterase, gluconate-6-fosfaatdehydrogenase en fosfoglucomutase geanalyseerd tijdens dezelfde morfogenetische stadia. Van de 5 onderzochte enzymen, gaven alcoholdehydrogenase, zure fosfatase en esterase verschillen te zien in isozym patronen, afhankelijk van het onderzochte stadium. Niet-morfogenetisch callus verschilde duidelijk van embryogene calli en van embryo's. De typen isozymen en de activiteitsniveaus, die de embryogene toestand kenmerken, bleven niet behouden bij calli die op somatische embryo's ontstonden. De analyse van elektroforesepatronen van specifieke isozymen laat zien dat dit een effectieve benadering is om de belangrijkste stappen van de somatische embryogenese van *Vitis rupestris* te karakteriseren.

Hoofdstuk 5 beschrijft de genetische transformatie van somatische embryo's van *V. rupestris*. Co-cultivatie van somatische embryo s met de *Agrobacterium tumefaciens* stam LBA4404, in het bezit van het plasmide pBI121 waarop het neomycine-fosfotransferase -en het β-glucuronidase-gen gelegen zijn, leidde tot getransformeerde cellijnen, die herhaald tot somatische embryogenese in staat waren.

Een vroege behandeling met een hoge concentratie kanamycine (100 mgl^{-1}) was belangrijk voor een efficiënte selectie van transformanten. De getransformeerde cellijnen vertoonden een sterke β-glucuronidase expressie, evenals een stabiele insertie van het merkergen na een langdurige in-vitro kweek. De cellijnen behielden het vermogen om secundaire embryo's te vormen en transgene planten te produceren met een bevredigende frequentie van 13%.

In ***hoofdstuk 6*** is de stabiliteit van het β-glucuronidasegen beschreven op het niveau van celpopulaties. Moleculaire analyses met "Southern blots- en dot blots" lieten zien dat er geen verlies optrad van het ingebrachte gen, noch na

langdurige weefselkweek, noch na plantregeneratie.

Met het doel het aantal druifgenotypen dat toegankelijk is voor in-vitro manipulatie te vergroten, werd het regeneratievermogen van meerdere genotypen in het genus *Vitis* onderzocht in ***hoofdstuk 7***.

Hiertoe werden de *V. vinifera* rassen Barbera, Cabernet franc, Cabernet Sauvignon, Canner Seedless bianco, Chadonnay, Enantio, Moscato bianco, Riesling, Sultana moscato bianco en Sultana rouge, de soorten *V. vinifera silvestris* G., *V. amurensis* R., *V. armata* D. & G., *V. riparia* M., *V. simpsonii* M., evenals de onderstammen 110 Richter en Schwarzmann en de soorthybride cultivar Staufer onderzocht.

Regeneratie werd vanuit jonge bladeren geïnduceerd via directe organogenese en een duidelijk genotypeverschil in morfogenetische competentie werd waargenomen, zowel wat betreft de efficiëntie als de snelheid van regeneratie. Goede regeneratie frequenties werden verkregen met, Cabernet franc and Chadonnay, Riesling, Sultana moscato terwijl Cabernet Sauvignon en Enantio geen scheutregeneratie vertoonden. *V. armata* reageerde het beste in dit regeneratiesysteem.

Verwacht wordt dat het in dit proefschrift beschreven onderzoek interessante perspectieven opent voor de toepassing van moleculaire technieken, gericht op het verhogen van de resistentie van de druif tegen pathogenen en andere stressfactoren en op de verbetering van de kwaliteit van dit gewas.

Riassunto

La vite, uno dei fruttiferi più diffusi, è coltivata per la produzione di vini, uve da tavola e da dessert e succhi. L'Ufficio Internazionale della Vite e del Vino (Parigi) nel 1995 ha stimato che la superficie mondiale coltivata aveva raggiunto i 7.768.00 ha., il raccolto i 540.567.000 q. e la produzione di vino i 246.423.000 hl.

La coltura della vite è basata principalmente su cultivar tradizionali perpetuate da centinaia di anni mediante propagazione vegetativa. I programmi di miglioramento genetico, che riguardano caratteri di produttività e resistenza a stress biotici e abiotici, a seconda dell'area geografica di coltura, sono effettuati con programmi di incrocio o selezione clonale in funzione di fattori biologici, regole di mercato e perfino aspetti culturali ed emotivi.

La realizzazione degli incroci tra i genotipi del genere *Vitis* è relativamente semplice, ma alcune difficoltà ne scoraggiano tuttavia la pratica. I programmi di incrocio sono infatti accompagnati da eterozigosi, depressione da inincrocio ed eredità poligenica dei caratteri di pregio e sono associati ad un ciclo biologico molto lungo; inoltre, mentre le uve da tavola sono un prodotto dinamico, la produzione enologica, al contrario, richiede un confronto con la tradizione, la cultura e le esigenze di un mercato restio a modificare esperienze già affermate. Questa strategia costosa e laboriosa è perciò impiegata solo nei casi in cui particolari esigenze climatiche e condizioni economiche ne giustifichino fortemente l'applicazione. I programmi di miglioramento genetico della vite risultano però necessari, poiché questo fruttifero soffre di malattie virali e fungine, è attaccato da insetti e nematodi e richiede miglioramento per i caratteri coinvolti nella produttività.

La trasformazione genetica, che consente di superare questi inconvenienti associati al miglioramento genetico classico e permette di migliorare - rispettandoli - genotipi già affermati, si pone quindi come uno strumento molto promettente per il miglioramento genetico nel genere *Vitis*. Questa strategia si

basa sull'inserimento nella pianta da migliorare di geni per caratteri specifici o di sequenze che sopprimono l'espressione dei geni endogeni. L'introduzione di costrutti virali, o di loro sequenze antisenso, potrebbe così limitare la diffusione di importanti virosi della vite, mentre la resistenza a patogeni fungini potrebbe essere indotta con il trasferimento di geni per resistenza ad ampio spettro, come quelli codificanti per l'osmotina o i peptidi litici. Il recente clonaggio del gene per la stilbene sintetasi offre inoltre interessanti prospettive per la possibilità di manipolare il grado di produzione e accumulo di stilbene, dimostratosi correlato alla resistenza a *Plasmopara viticola* e *Botrytis cinerea*.

L'approccio biotecnologico può consentire anche il miglioramento della qualità e del valore tecnologico del vino. Infatti, sono stati recentemente isolati i geni per gli enzimi responsabili della biosintesi delle antocianine, coinvolte nella determinazione del gusto e della qualità del vino, e del gene per la polifenolo ossidasi, responsabile dell'imbrunimento nel vino bianco e nelle uve da trasformazione.

Nella storia delle colture *in vitro*, la vite fu una delle piante in cui furono effettuati i primi saggi negli anni '40. Le prime viti transgeniche sono state tuttavia ottenute solo negli anni '90 e la tecnologia ricombinante rimane ancora molto lontana dalla routine. In aggiunta, le prospettive offerte da queste tecnologie sono ancora ristrette a pochi genotipi; ciò è dovuto principalmente alla scarsa competenza rigenerativa nel genere *Vitis*, propria del genotipo. La trasformazione genetica è stata ottenuta perciò solo in poche specie del genere *Vitis*, tra cui genotipi selvatici, portinnesti e ibridi interspecifici e rare cultivar di *vinifera*.

In questo libro vengono riportati i risultati ottenuti dal Laboratorio di Biotecnologie dell'Istituto Agrario di San Michele all'Adige, nell'ambito di un'attività di ricerca che si prefigge di ottenere resistenza agli stress biotici nella vite, con strategie basate sulla trasformazione genetica. Questo lavoro costituisce la base delle attività del laboratorio, che proseguiranno anche nei prossimi anni.

Poiché la coltura *in vitro* dei tessuti è il requisito essenziale per applicare le tecnologie del DNA ricombinante, in un primo tempo è stato necessario

individuare nel genere *Vitis* il genotipo con le migliori attitudini morfogeniche. Su questo genotipo è stato in seguito possibile svolgere i saggi che hanno portato alla definizione della tecnica ottimale per la realizzazione della trasformazione genetica. Dopo questo primo risultato, la ricerca si è infine rivolta ai genotipi agronomicamente più importanti.

Una parte rilevante dell'attività qui descritta ha riguardato la comprensione dei meccanismi genetici e fisiologici che regolano lo sviluppo e la differenziazione dei tessuti nel genere *Vitis*. Nel corso del nostro studio, anche in accordo con l'esperienza di altri Autori, abbiamo convenuto che l'embriogenesi somatica sia il sistema morfogenico più adatto per l'inserimento di geni esogeni nella vite; questo modello rigenerativo è stato quindi affrontato ed esaminato con molta attenzione. Il genotipo con il potenziale rigenerativo più efficiente è risultato essere *Vitis rupestris* S. Per questa ragione, la maggior parte dello studio qui pubblicato è stata svolta su questa specie di origine americana, largamente utilizzata per la produzione di portinnesti (in particolare resistenti alla fillossera) e per i saggi biologici nella diagnostica virale.

In questo genotipo abbiamo ottenuto embriogenesi somatica da callo di foglia e picciolo ***(Capitolo 2)***, con efficienze del 3.2% e 4.2% degli espianti indotti, quando 6-benziladenina e acido 2,4-diclorofenossiacetico (rispettivamente nelle due combinazioni 1/0.1 e 1/1 mgl^{-1}) sono state aggiunte al terreno di coltura Murashige and Skoog (MS). Gli embrioni somatici hanno poi prodotto subcolture embrionali ed embriogenesi somatica secondaria, nei terreni MS o Nitsch and Nitsch (NN) in presenza di acido indolacetico (1 mgl^{-1}) oppure di acido indolbutirrico (0.1 mgl^{-1}). Gli embrioni somatici hanno germinato con buone efficienze (13%, dopo 4 mesi di coltura), in terreno NN contenente 6-benziladenina (1 mgl^{-1}), acido indolbutirrico (0.01 mgl^{-1}) e idrolisato di caseina (250 mgl^{-1}), in seguito ad un trattamento termico di due settimane a 4°C.

Una maggiore frequenza di germinazione (51%) è stata invece ottenuta in presenza di solo acido indolbutirrico nel terreno NN, in assenza del trattamento termico. La germinazione ha richiesto tuttavia, in questo caso, un tempo di coltura molto più lungo (9 mesi).

Nel corso dell'embriogenesi somatica, pur in condizioni controllate, la risposta dei tessuti alla coltura è risultata spesso imprevedibile. Si è dimostrato dunque interessante ricercare dei marcatori specifici per l'embriogenesi somatica, al fine di interpretare il comportamento della vite durante la coltura *in vitro*.

Questo studio è stato effettuato nelle colture di *V. rupestris*, in cui abbiamo caratterizzato i cambiamenti biochimici associati alle principali tappe morfogeniche (callo, callo embriogenico, embrione somatico e plantula) che si susseguono nell'evoluzione da callo a pianta.

L'analisi elettroforetica bidimensionale delle proteine totali ***(Capitolo 3)*** e gli zimogrammi degli enzimi alcol deidrogenasi (ADH), fosfatasi acida (AcP), esterasi (EST), gluconato-6-fosfato deidrogenasi (6GPDH) e fosfoglucomutasi (PGM) ***(Capitolo 4)***, si sono dimostrati dei validi approcci per questa indagine.

Mediante l'analisi bidimensionale delle proteine totali, ciascuna di queste fasi morfogeniche è stata caratterizzata da una serie peptidica specifica e caratteristica per ciascuna tappa. Callo e callo embriogenico ed embrioni somatici hanno prodotto mappe nettamente differenti tra loro. Infatti, mentre i campioni di callo sono risultati essere caratterizzati dal più basso contenuto proteico, la concentrazione proteica più elevata e più complessa è stata osservata nei campioni di callo embriogenico e di giovani embrioni. Nel corso della successiva fase di germinazione, poi, sono state trovate nei tessuti situazioni più semplici e più specializzate.

Per quanto riguarda i cinque enzimi saggiati, per ciascuna fase morfogenica, gli zimogrammi di ADH, AcP e EST sono risultati molto diversi, sia nell'attività specifica (ADH), sia nel numero e nel punto isoelettrico delle isoforme espresse (AcP e EST). Anche in questi saggi, il callo è risultato estremamente diverso dal callo embriogenico e dagli embrioni somatici. In più, è interessante sottolineare che il callo non differenziato indotto dagli embrioni somatici non ha ritenuto i livelli ed i tipi di enzimi che caratterizzano lo stato embrionale.

Conclusa questa prima fase dello studio, ci è stato finalmente possibile occuparsi della trasformazione genetica. La definizione della strategia ottimale

è stata effettuata su embrioni somatici ottenuti da callo di picciolo di *V. rupestris* ***(Capitolo 5)***.

Gli embrioni somatici, nel corso dell'induzione di embriogenesi secondaria, sono stati incubati con *Agrobacterium tumefaciens* ceppo LBA4404, contenente il plasmide pBI121 in cui sono stati inseriti i geni per la neomicina fosfotransferasi e per la β-glucuronidasi. La precoce selezione su alti livelli di canamicina (100 mgl^{-1}), applicata al callo embriogenico prodotto nel corso delle subcolture, si è rivelata essere una parte fondamentale nel processo della trasformazione genetica, poiché ha consentito una corretta discriminazione tra le cellule transgeniche e non. I tessuti embriogenici così ottenuti hanno inoltre dimostrato di mantenere nel tempo (dopo oltre 3 anni di coltura *in vitro*) una stabile inserzione dei geni marcatori, una forte espressione della β-glucuronidasi, e - risultato essenziale - di rigenerare piante transgeniche con buone efficienze (13%).

Nella popolazione di piante transgeniche rigenerate dagli embrioni somatici è stata poi valutata la stabilità del gene per la β-glucuronidasi ***(Capitolo 6)***.

I saggi molecolari (Southern Blot e Dot-Blot) hanno infatti provato la permanenza del gene esogeno anche dopo il lungo periodo di coltura *in vitro* degli embrioni e in seguito all'induzione della rigenerazione di piante.

Dopo questo studio svolto su *V. rupestris*, in cui sono state analizzate importanti problematiche connesse alla manipolazione *in vitro* ***(Capitoli 2, 3 e 4)*** e alla trasformazione genetica della vite ***(Capitoli 5 e 6)***, l'interesse della nostra ricerca si è finalmente rivolto all'applicazione delle conoscenze fin qui acquisite a dei genotipi agronomicamente rilevanti del genere *Vitis* in cui sarà importante trasferire geni per la resistenza agli stress biotici. È stato quindi condotto un saggio volto ad evidenziare l'attitudine alla rigenerazione in diverse cultivar di *Vitis vinifera* (Barbera, Cabernet franc, Cabernet Sauvignon, Canner Seedless bianco, Chardonnay, Enantio, Moscato bianco, Riesling, Sultana moscato bianco e Sultanina rouge), nelle specie *V. vinifera silvestris* G., *V. amurensis* R., *V. armata* D. e G., *V. riparia* M., *V. simpsonii* M., nei portinnesti 110 Richter e Schwarzmann e nella varietà interspecifica Staufer ***(Capitolo 7)***.

La rigenerazione è stata indotta in giovani foglie mediante organogenesi diretta. Questa ricerca ha evidenziato una significativa influenza del genotipo sulla potenzialità morfogenica, poiché tra i 18 genotipi saggiati, la rigenerazione è avvenuta con differenti efficienze e precocità. *V. armata* è risultato il genotipo più adatto a questo sistema rigenerativo. Notevoli efficienze sono state ottenute anche per Canner Seedless bianco, Sultana moscato bianco, Cabernet franc, Chardonnay e Riesling, mentre Enanzio, Cabernet Sauvignon e *V. riparia* si sono dimostrati dei genotipi "recalcitranti" alla rigenerazione.

Riteniamo che il lavoro qui esposto rappresenti un contributo significativo allo studio della rigenerazione e della trasformazione genetica nel genere *Vitis*. I risultati ottenuti pongono le basi per introdurre le tecniche molecolari nel miglioramento genetico della vite. La possibilità di produrre piante transgeniche consentirà inoltre di sviluppare strategie per studiare importanti malattie della vite.

Ci sembra interessante considerare, a questo punto, l'impatto sull'opinione pubblica di un eventuale rilascio di viti transgeniche. I consumatori hanno dimostrato, infatti, preoccupazione di fronte ai prodotti alimentari geneticamente manipolati. In più, l'industria vinicola - regolata da leggi di mercato e scelte talvolta emotive da parte del consumatore - potrebbe non essere pronta ad accettare questa innovazione. Così, mentre la manipolazione genetica dei portinnesti potrebbe essere forse accettata più facilmente, il rilascio di viti da tavola o da vinificazione potrebbe sortire un effetto negativo.

Noi crediamo, però, che le tecnologie del DNA ricombinante rappresentino una valida opportunità nei programmi di miglioramento genetico ed offrano prospettive uniche per l'affermazione di una pratica agricola più rispettosa dell'ambiente. Questo è particolarmente vero quando la produzione di piante intrinsecamente resistenti ai patogeni potrebbe portare ad una drastica riduzione dell'utilizzo delle sostanze chimiche in agricoltura. Noi riteniamo perciò che, accanto ai portainnesti, la trasformazione genetica potrebbe risultare uno strumento molto interessante per il miglioramento genetico di ogni genotipo agronomicamente importante del genere *Vitis*.

Curriculum vitae

Lucia Martinelli was born in Trento (Italy) on Nov. 5, 1957.
After the high school, at the Ginnasio-Liceo "G. Prati" in Trento, she studied at the University of Bologna, where she received the Master Degree (Laurea) in Biological Sciences, on 26/3/1982, with a thesis realized at the Institute of Genetics.

Thereafter, research has been carried out at the Genetics Institute of the Faculty of Science of the Bologna University (Italy) (1/4/82 - 30/3/85), where she worked in the tissue culture group also in the frame of a fellowship of ERSO (Cesena, Italy) in the projects "Genetic improvement of eggplant" and "Genetic and agronomic evaluation of the micropropagated crops"; at the Department of Genetics of the Wageningen Agricultural University (The Netherlands) (1/4/85 - 30/9/86), in the group of Prof. Maarten Koornneef. Here, she could do research on the basis of a scholarship of the Utrecht University in the frame of a cultural exchange between this University and the Istituto Trentino di Cultura, and on the basis of a junior grant by the Commission of the European Union, DGXII, frame project "Biotechnology"; at the Company "Agrimont R&S" (Montedison group) (1/4/87 - 7/10/88), where she was engaged in the newly developing project on biotechnology. After a period as visiting scientist at the Plant Cell Research Institute of Dublin (California) (1/6/87 - 31/3/88) she came back to the Italian Centre for the "Agrobiotecnologie" of Massa-Carrara as project leader in the tissue culture section. During the stay at Dublin, she took the opportunity to attend the class "Genetics 200 - gene regulation" taught by Prof. M. Freeling at the University of California at Berkeley and several English courses at the San Francisco University. Since 10/10/88 she is employed at the Agricultural Institute of San Michele all'Adige (TN) (Italy), where she set up the Biotechnology Laboratory and developed the research of grape genetic transformation, and is still working as head of the laboratory.

During the activity in the field of agro-biotechnology, she has been studying the potentiality of in vitro cultures and molecular biology for the comprehension of the genetical and physiological aspects of tissue morphogenesis, and developed systems for transgenic plant production. She carried out research on different herbaceous (potato, eggplant, strawberry, tobacco, tomato, sunflower, alfalfa) and woody plants (cashew and grape), each with a peculiar answer to in vitro manipulation. The results of this activity have been published in a number of scientific and popular papers.

The most important result - which has been appreciated by the scientific community with the 1994 prize by the "Rudolf Hermanns" Foundation of Geisenheim (Germany) - is the demonstration of the possibility to obtain transgenic plants with agreeable efficiencies also in the Vitis genus.

She is regularly invited to give lectures on the topics related to her activity for students, scientists and workers in the field of agro-industry, and from 1995 she joined the Editorial Review Board of the journal "Vitis".

She is the provincial delegate of the National Association of the Biologists and member of the Commission for the Equal Opportunities of the Autonomous Province of Trento.

Curriculum vitae

Lucia Martinelli è nata a Trento il 5 novembre 1957.
Dopo la Maturità Classica presso il Ginnasio-Liceo "G. Prati" di Trento, si è laureata in Scienze Biologiche all'Istituto di Genetica della Facoltà di Scienze Matematiche, Fisiche e Naturali dell'Università degli Studi di Bologna (26/3/82).

Poi, l'attività di ricerca è stata effettuata presso l'Istituto di Genetica della Facoltà di Scienze dell'Università degli Studi di Bologna (1/4/82 - 30/3/85), presso il gruppo di colture in vitro, anche grazie ad una borsa di Studio dell'Ente ERSO di Cesena, per i progetti "Miglioramento genetico della melanzana" e "Valutazione genetica ed agronomica del materiale vivaistico micropropagato"; presso il Dipartimento di genetica dell'Università di Agraria di Wageningen (Olanda) (1/4/85 - 30/9/86), nel gruppo del prof. Maarten Koornneef, prima nell'ambito di uno scambio culturale tra l'Istituto Trentino di Cultura e l'Università di Utrecht, e poi di un contratto di ricerca della Commissione dell'Unione Europea, DGXII, progetto quadro "Biotecnologie"; presso l'industria "Agrimont R&S" (gruppo Montedison) (1/4/87 - 7/10/88), dove fu assunta in relazione al nuovo progetto "Biotecnologie". Dopo un periodo come ricercatore al "The Plant Cell Research Institute" di Dublin (California) (1/6/87 - 31/3/88), venne richiamata in Italia al nuovo Centro per le Agrobiotecnologie di Massa-Carrara, in veste di capo progetto nel settore delle colture in vitro. Nel corso del soggiorno a Dublin, ha frequentato il semestre autunnale del corso "Genetics 200 - gene regulation" all'Università di Berkeley, tenuto dal Prof. M. Freeling, oltre a vari corsi di lingua inglese presso l'Università di San Francisco. Dal 10/10/88 lavora all'Istituto Agrario di San Michele all'Adige (TN), dove ha fondato il Laboratorio "Biotecnologie" di cui è responsabile e sviluppa ricerca nella trasformazione genetica della vite.

Nel corso dell'attività nelle Agrobiotecnologie, ha studiato le potenzialità delle colture in vitro e della biologia molecolare per la comprensione degli aspetti

genetici e fisiologici che regolano la morfogenesi e ha sviluppato strategie per la produzione di piante transgeniche. La ricerca è stata condotta in diverse erbacee (patata, melanzana, fragola, tabacco, pomodoro, girasole ed erba medica) e piante arboree (anacardio e vite), ciascuna specie con attitudini peculiari alla manipolazione in vitro. I risultati di questa attività sono stati pubblicati in numerosi articoli scientifici e divulgativi.

Il risultato più importante, apprezzato dalla comunità scientifica con il premio della Fondazione "Rudolf Hermanns" di Geisenheim (Germania) nel 1994, è la dimostrazione della possibilità di ottenere piante transgeniche con buone efficienze anche nel genere Vitis.

È spesso invitata a tenere conferenze su argomenti correlati alla propria attività di ricerca per studenti, ricercatori ed operatori dell'agro-industria e dal 1995 fa parte del gruppo editoriale della rivista "Vitis".

È delegata provinciale dell'Ordine Nazionale dei Biologi e componente della Commissione per le Pari Opportunità della Provincia Autonoma di Trento.

Front cover: plantlet regenerated from a transgenic somatic embryo for the β-glucuronidase gene, showing a strong GUS activity after the X-GLUC histochemical assay (Chapter 5).

In copertina: piantina rigenerata da un embrione transgenico per il gene della β-glucuronidasi, fortemente positiva al saggio istochimico con X-GLUC. La forte colorazione blu, evidente in tutti i tessuti, dimostra l'avvenuto inserimento e l'espressione del gene esogeno nella pianta (Capitolo 5).

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