การปรับปรุงพันธุ์องุ่นโดยวิธีทางพันธุวิศวกรรมและการคัดเลือกเซลล์บนอาหารเพาะเลี้ยงเนื้อเยื่อ

นายยิ่งยศ จิตตะยโศธร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
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ปีการศึกษา 2550

GENETIC ENGINEERING AND *IN VITRO* SELECTION FOR GRAPE CULTIVAR IMPROVEMENT

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A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy in Biotechnology

Suranaree University of Technology

Academic Year 2007

GENETIC ENGINEERING AND *IN VITRO* SELECTION FOR GRAPE CULTIVAR IMPROVEMENT

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ยิ่งยศ จิตตะยโศธร : การปรับปรุงพันธุ์องุ่นโดยวิธีทางพันธุวิศวกรรมและการคัดเลือกเซลล์บนอาหารเพาะเลี้ยงเนื้อเยื่อ (GENETIC ENGINEERING AND *IN VITRO* SELECTION FOR GRAPE CULTIVAR IMPROVEMENT) อาจารย์ที่ปรึกษา : ศาสตราจารย์ ดร.นันทกร บุญเกิด, 154 หน้า.

การวิจัยนี้มีเป้าหมายเพื่อสร้างกระบวนการปรับปรุงพันธุ์องุ่นด้วยวิธีการทางเทคโนโลยีชีวภาพในประเทศไทย โดยสามารถพัฒนาระบบการเกิดโซมาติกเอ็มบริโอ (somatic embryogenesis) และการชักนำให้เกิดเป็นต้นใหม่ (plant regeneration) จากการเลี้ยงกลุ่มเซลล์ต้นกำเนิดเอ็มบริโอแขวนลอย (PEM suspension culture) ขององุ่นรับประทานผลสดพันธุ์ออทัมรอยัลซีดเลส (*Vitis vinifera* L. cv. Autumn Royal Seedless) ในอาหารเหลวสูตร MSGGN ซึ่งพัฒนาขึ้นใหม่ จากการทดลองพบว่าแสงมีผลกระทบในทางลบอย่างมากต่อการเกิดโซมาติกเอ็มบริโอ ในขณะที่ผงถ่านช่วยให้การเกิดโซมาติกเอ็มบริโอดีขึ้น และอาหาร MS สูตรเข้มข้นปกติดีกว่าสูตรเจือจางหนึ่งเท่า โซมาติกเอ็มบริโอที่ทำการทดลองทั้งหมดสามารถงอกได้บนอาหารแข็งสูตร FMSC โดยร้อยละ 95 ของโซมาติกเอ็มบริโอที่งอกสามารถพัฒนาไปเป็นต้นปกติได้ ด้วยวิธีการเพาะเลี้ยงเดียวกันนี้สามารถสร้างกลุ่มเซลล์ต้นกำเนิดเอ็มบริโอแขวนลอยขององุ่นทำไวน์พันธุ์ชาร์โดเนย์ (*V. vinifera* L. cv. Chardonnay) และองุ่นรับประทานผลสดพันธุ์ทารา (*V. rotundifolia* cv. Tara) จากกลุ่มเซลล์ต้นกำเนิดเอ็มบริโอแขวนลอยขององุ่นทั้งสามพันธุ์ พบว่าออทัมรอยัลซีดเลสมีการเจริญเติบโตและมีความสามารถต่อการชักนำให้เกิดเป็นต้นใหม่สูงที่สุด ในขณะที่ชาร์โดเนย์มีขนาดที่สม่ำเสมอมากที่สุด และทาราให้เซลล์แขวนลอยที่มีคุณภาพต่ำที่สุด

การทดลองที่สองเป็นการพัฒนากระบวนการถ่ายยีนด้วยวิธี *Agrobacterium*-mediated transformation โดยใช้กลุ่มเซลล์ต้นกำเนิดเอ็มบริโอแขวนลอยขนาดเล็ก (SPEM suspension cell) และโซมาติกเอ็มบริโอวัยอ่อน (primary somatic embryo) ขององุ่นพันธุ์ออทัมรอยัลซีดเลสเป็นเนื้อเยื่อเป้าหมาย และใช้ยีน EGFP ซึ่งเป็นยีนต้นแบบการสังเคราะห์โปรตีนเรืองแสงสีเขียวเป็นตัวรายงานผลเพื่อการหาวิธีการที่เหมาะสมที่สุดของการถ่ายยีน จากการทดลองพบว่ากลุ่มเซลล์ต้นกำเนิดเอ็มบริโอแขวนลอยขนาดเล็กไม่เหมาะสมต่อการเป็นเนื้อเยื่อเป้าหมาย เนื่องจากไม่พบเซลล์ที่ได้รับการถ่ายยีน แต่เมื่อทำการถ่ายยีนกับโซมาติกเอ็มบริโอวัยอ่อน สามารถผลิตแคลลัสและโซมาติกเอ็มบริโอระยะกลม (globular somatic embryo) ที่เรืองแสงสีเขียวได้หลังจากการเลี้ยงเป็นระยะเวลา 2 เดือนบนอาหารคัดเลือก อย่างไรก็ตามโซมาติกเอ็มบริโอที่เรืองแสงดังกล่าวไม่สามารถพัฒนาไปได้มากกว่าระยะใบเลี้ยง (cotyledonous stage)

ในการผลิตองุ่นทนเค็ม ได้ทำการคัดเลือกเอ็มบริโอจินิกแคลลัส (embryogenic callus)

ขององุ่นพันธุ์ทาราและกลุ่มเซลล์ต้นกำเนิดเอ็มบริโอแขวนลอยขนาดเล็กขององุ่นพันธุ์ชาร์โดเนย์ ที่สามารถทนต่อความเค็มบนอาหารคัดเลือกซึ่งมีเกลือโซเดียมคลอไรด์เป็นองค์ประกอบ ที่ความเข้มข้นร้อยละ 0.5, 1.0, 1.5, 2.0 และ 2.5 จากคัดเลือกเป็นระยะเวลา 6 เดือน สามารถผลิตเอ็มบริโอจินิกแคลลัสทนเค็มขององุ่นพันธุ์ทาราได้ 10 สายพันธุ์บนอาหารที่ประกอบด้วยเกลือโซเดียมคลอไรด์ความเข้มข้นร้อยละ 1.0 แต่มีเพียงสายพันธุ์เดียว (ST1) เท่านั้นที่มีการเจริญเติบโตต่อไปหลังจากการแยกขยาย (subculture) 2 ครั้ง ประมาณร้อยละ 10 ของโซมาติกเอ็มบริโอที่พัฒนามาจาก ST1 สามารถงอกได้บนอาหารคัดเลือกซ้ำ FMSC ที่ประกอบด้วยเกลือโซเดียมคลอไรด์ความเข้มข้นร้อยละ 1.0 จากการวิเคราะห์รูปแบบโพลิเพปไทด์ด้วยวิธี SDS-PAGE พบการเปลี่ยนแปลงอย่างชัดเจนคือ (ก) การเพิ่มระดับของโพลิเพปไทด์ขนาด 51 และ 24 กิโลดาลตัน และ (ข) การลดระดับของโพลิเพปไทด์ขนาด 48 และ 27 กิโลดาลตัน ทั้งยังพบการเพิ่มระดับของโพลิเพปไทด์ขนาด 26 กิโลดาลตัน ในช่วงแรกและลดลงในช่วงหลังของการทนเค็ม สำหรับชาร์โดเนย์นั้น แม้ว่าสามารถผลิตโซมาติกเอ็มบริโอได้จากอาหารที่ประกอบด้วยเกลือโซเดียมคลอไรด์ความเข้มข้นร้อยละ 0.5 แต่ทั้งหมดเปลี่ยนเป็นสีน้ำตาล ยอดเกิดการแคระแกร็น และตายในที่สุดหลังจากการเลี้ยงบนอาหาร FMSC ที่ประกอบด้วยเกลือโซเดียมคลอไรด์ความเข้มข้นร้อยละ 1.0

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2550 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

YINGYOS JITTAYASOTHORN : GENETIC ENGINEERING AND *IN VITRO* SELECTION FOR GRAPE CULTIVAR IMPROVEMENT. THESIS ADVISOR : PROF. NANTAKORN BOONKERD, Ph.D. 154 PP.

SOMATIC EMBRYOGENESIS/PLANT REGENERATION/GENETIC ENGINEERING/*IN VITRO* SELECTION/GRAPE

This research was attempted at establishing biotechnological approach for grape improvement in Thailand. The system of somatic embryogenesis and plant regeneration was developed via proembryonic mass (PEM) suspension culture of a table grape 'Autumn Royal Seedless' (*Vitis vinifera* L.) in the newly-developed MSGGN liquid medium. The result strongly indicated that light had an extremely negative effect while activated charcoal facilitated somatic embryogenesis, and full-strength MS medium was superior to half-strength. The somatic embryos tested were easily germinated (100%) on FMSC solid medium and up to 95% of them developed into normal plantlets. PEM suspension cells of 'Chardonnay' (*V. vinifera* L.) and 'Tara' (*V. rotundifolia*) were also established using the same protocol. Among the three, 'Autumn Royal Seedless' exhibited the highest growth and regeneratability while highly synchronous suspension cells were obtained from 'Chardonnay'. 'Tara' gave the poorest suspension culture.

In the following experiment, *Agrobaterium*-mediated transformation was developed for 'Autumn Royal Seedless' with small cluster of PEM (SPEM) suspension cell and primary somatic embryo as target tissues. The transformation system was optimized using EGFP reporter gene encoding enhanced green fluorescent protein. The result indicated that SPEM was not suitable for target tissue as no

transformed cell was obtained. On the other hand, fluorescent embryogenic calli and globular somatic embryos were produced after 2 months on selective medium when transformation was performed with primary somatic embryos. The somatic embryos, however, did not grow beyond cotyledonous stage.

To produce salt tolerant grapes, 'Tara' embryogenic calli and 'Chardonnay' PEM suspension cells were *in vitro* selected for tolerant cells on solid media supplemented with 5 concentrations of sodium chloride (NaCl) including 0.5, 1.0, 1.5, 2.0, and 2.5%. Ten salt tolerant (ST) lines of 'Tara' embryogenic callus were initially obtained from 6-month selection on 1.0% NaCl-containing medium; however, only ST1 showed further growth after 2 subcultures. Approximately 10% of somatic embryos developed from ST1 could survive and germinate on FMSC medium containing 1.0% NaCl for double selection. SDS-PAGE revealed distinct changes of polypeptide patterns: (a) increasing levels of 51 and 24-kDa polypeptides and (b) reducing levels of 48 and 27-kDa polypeptides. High level of 26-kDa polypeptide was detected in early stage and became lower at late stage of salt tolerance. For 'Chardonnay', although somatic embryos were obtained from 0.5% NaCl-containing medium, their roots turned brown, shoots stunted, and they died after culturing on FMSC medium containing 1.0% NaCl.

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Student's Signature_____

Advisor's Signature_____

Co-advisor's Signature_____

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor Prof. Dr. Nantakorn Boonkerd for kindly providing me the huge opportunity to study in plant biotechnology. I would to also express my deepest gratitude to my co-advisor Asst. Prof. Dr. Piyada Thipyapong who gave me the first inspiring being a scientist and valuable technical skills and my foreign co-advisor Prof. Dr. Jiang Lu for giving me suggestions and encouragement during my 3 years research in the US, and supporting me to attend international conferences.

This research would have never happened if without financial support. I would like express my gratitude to the Thailand Research Fund (TRF) for providing me the Royal Golden Jubilee (RGJ) Ph.D Scholarship for my study.

I would like to make a special thank to the Center for Viticulture and Small Fruit Research, Florida A&M University, USA for providing me laboratory facilities until this research was successfully accomplished. Dr. Xia Xu is acknowledged for sharing tissue culture materials and techniques. I would like to thank my friends in Genetic Lab; Dr. Clifford Louime, Miss Danae Dalling, Miss Diana Zieglerd, Miss Julian Bourne, Miss Oulimathe Paraiso, Mr. Zhongbo Ren for their friendly encouragement and helps.

I am very grateful to Dr. Subramanian Jayasankar (Department of Plant Agriculture, University of Guelph, Canada) and Dr. Lucia Martinelli (Laboratorio Biotecnologie, Instituto Agrario, Italy) for their valuable suggestions in grape tissue

culture technique and transformation, and also critiques of my manuscripts. I am very grateful to Dr. Stewart Neal, Jr. (Department of Plant Science, University of Tennessee, USA) for his expert opinion of EGFP gene integration and Dr. Julie Kikkert (Department of Horticultural Science, New York State Experiment Station, Cornell University, USA) for generously contributing a biolistic transformation protocol.

My appreciation is also to Asst. Prof. Dr. Mariena Ketudat-Cairns for her contribution of chemicals, materials and the valuable guidance during research in Thailand and Assoc. Prof. Dr. James R. Ketudat-Cairns for kindly contributing a chitinase gene.

Finally, I would like to thank my parents and Miss Watchalee Chuenchor for the love, support, understanding, and encouragement.

Yingyos Jittayasothorn

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LIST OF ABBREVIATIONS

AC	=	activated charcoal
BA	=	6-benzylaminopurine
CaMV 35S	=	35S gene of cauliflower mosaic virus
DNA	=	deoxyribonucleic acid
EGFP	=	enhanced green fluorescent protein
FMSC	=	Full-strength Murashige and Skoog solid medium with activated charcoal
GFP	=	green fluorescent protein
GUS	=	β-glucuronidase gene
kDa	=	kilodalton
LPEM	=	large cluster of proembryonic mass
LUC	=	luciferase gene
MS	=	Murashige and Skoog medium
MSGGN	=	Murashige and Skoog liquid medium with maltose glycerol, glutamine, and β-naphthoxyacetic acid
NaCl	=	sodium chlorite
NOA	=	β-naphthoxyacetic acid
NRA	=	Nitrate reductase enzyme
PEM	=	proembryonic mass
PSII	=	photosynthetic system II

LIST OF ABBREVIATIONS (Continued)

QTL	=	qualitative trait loci
SC0%	=	salt tolerant callus culture in 0% NaCl
SC1%	=	salt tolerant callus cultured in 1% NaCl
SDS-PAGE	=	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SPEM	=	small cluster of proembryonic mass
ST	=	salt tolerant line
TC0%	=	typical callus cultured in 0% NaCl
TC1%	=	typical callus cultured in 1% NaCl
UV	=	ultraviolet ray
V	=	voltage

CHAPTER I

INTRODCUTION

Grape is one of the most broadly distributed fruit crops in the world which cover an area of approximately 10 million hectares. The most intensive grape growing is in European countries and USA. World grape production has ranged between 61-66 million tons over the last few years (Hajar, www, 2006). In Thailand, small number of grape cultivars was first brought from USA to test as potential fruit crop and found to have good adaptation and growth in Thailand. Later on, numerous cultivars were introduced to the country, making grape growing (termed as viticulture) has been commercially established. Data available in 2001 illustrated that grape production in Thailand was ranked number 59th in the world and number 5th in Eastern Asia behind China, Korea, Japan, and Taiwan (world grape production, www, 2003). However, grape especially the most important commercial species *Vitis vinifera* is highly susceptible to diseases, making its culture in tropical regions difficult (Torregrosa et al., 2002). Grape improvement has then become necessary in order to establish disease resistant trait into existing grape cultivars.

Conventional breeding for genetic improvement of grape is rather limited due to several factors such as a long generation cycle, inbreeding depression, polyploidy, and the highly heterozygous nature of existing cultivars (Gray and Meredith, 1992; Nakano et al., 1994; Das et al., 2002). Genetic engineering is then become an alternative method to improve grape because genes encoding agricultural desirable

traits can be directly introduced into pre-existing and desirable cultivars (Nakano et al., 1994). Although many foreign genes have been successfully transferred into grape, the transformation system seems to be limited in their laboratory origin. Among plant transformation techniques, *Agrobacterium*-mediated transformation is the most commonly used for grape genetic engineering. Torregrosa et al. (2002), one of the most well-known groups in grape tissue culture and genetic engineering, suggested three prerequisites in achieving of efficient transformation system; (1) production of highly regenerative transformable tissue, (2) optimal cocultivation conditions for grape tissue and *Agrobacterium*, and (3) an efficient selection regime for transgenic plant regeneration.

Among the recombinant strains, EHA105 has been shown as highly effective in transformation of several *vinifera* grape cultivars. Torregrosa et al. (2002) conducted the experiment using the largest number of *Agrobacterium* strains, to evaluate an influence of *Agrobacteirum* strains on transformation efficiency of *V. vinifera*. They found that EHA105 showed an increasing transformation efficiency compared to the widely used LBA4404, and was the most effective strains among *Agrobacterium tumefaciens* tested.

It is almost impossible to select small fraction of transformed cells without a reporting element. The β-glucuronidase (GUS) gene (Jefferson et al., 1987) and luciferase (LUC) gene (Ow et al., 1986) have been widely used as visual reporter gene. Although these two genes have proven useful in many applications, they require additional substrates that are toxic to plant cells (Zhu et al., 2004), especially assay of GUS is destructive. Green fluorescent protein (GFP) is a newest visual reporter that shares none of these problems. In contrast, fluorescence emission of GFP only requires

excitation by UV light and its observation is easily performed in living cells without disruption. GFP has been expressed in several plant species including dicotyledous and monocotyledous plants (Ghorbel et al., 1999). In grape, many publications have been reported using GFP as a reporter gene.

Somatic embryo is the most widely used plant material for grape transformation. More than 60 experiments of grape somatic embryogenesis have been published in many *Vitis* species. It can simply say that every tissue part above root of grape plant has been *in vitro* cultured for somatic induction, but the most suitable is anther according to high frequency of publications. Somatic embryo has been initially produced in solid medium. When grape tissue culture-based biotechnologies such as genetic transformation and *in vitro* selection are growing intensively, solid medium alone is incapable of producing somatic embryo to serve the need of researchers. Besides, somatic embryos grow highly asynchronous on solid medium but much more synchronous in liquid medium. This makes somatic embryo production in liquid medium more desirable in providing uniform experimental units. Quality of somatic embryos is the main factor affecting regeneration frequency, which varies in each subculture on solid medium (Bornhoff and Harst, 2000; Wang et al., 2004). Furthermore, somatic embryos on solid medium showed exhibitive dormancy, whereas in liquid medium they were not dormant and showed higher plant regeneration efficiency (Jayasankar et al., 2003).

Besides grape diseases which seem to be the most unfriendly for grape cultivation, abiotic stress has also shown its negative influence in viticulture. Soil salinity is a major problem of abiotic stress affecting plant growth. Suppression of growth is generally observed in all plants, however, their tolerant levels and growth

reducing rate under salt stress vary due to defense mechanism of different plant species. In plants, salt stress affects major processes such as growth, photosynthesis, protein synthesis, and energy metabolism.

Flowers (2004) suggested that developing of salt tolerant crops is possible through seven approaches: (1) genetic engineering, (2) conventional plant breeding program, (3) use of *in vitro* selection, (4) pooling physiological traits, (5) interspecific hybridization, (6) developing halophytes as alternative crops, and (7) use of marker-aided selection. Although pooling physiological traits, interspecific hybridization, and marker-aided selection do not require long cycles of recurrent selection, they also deal with many of processes in hybrid or progeny production and conventional screening in experimental field. Genetic engineering had been employed in order to produce transgenic plants tolerant to salinity, however, none of transgenic plants of any crop species has been established or even tested in the field.

In vitro selection using somaclonal variation has become an attractive alternative approach to produce salt tolerant plants. The technique does not require deep knowledge of genetic basic yet much less expensive than conventional plant breeding that needs to produce and carry many hybrids and genetic engineering that is limited by high technology and financial investment. Somaclonal variation is the variation observed among plants regenerated through *in vitro* culture (Larkin and Scowcroft, 1981), and has been proven useful in crops improvement (Skirvin et al., 1993; Jain et al., 1998a; Jain et al., 1998b; Jain and De Klerk, 1998).

1.1 Research objectives

To make biotechnoloigcal grape breeding possible in Thailand, this research was done with three subsequential objectives.

(i) To establish a complete system of grape suspension culture; somatic embryogenesis and plant regeneration.

(ii) To develop a transformation system in grape using GFP gene as a reporter.

(iii) To produce salt tolerant grape using *in vitro* selection technique.

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CHAPTER II

REVIEW OF LITERATURES

2.1 Grape (*Vitis* spp.)

Grapes belong to the widely distributed *Vitaceae* family. There are two subgenera of the *Vitaceae* family.

2.1.1 *Euvitis* (True grapes)

These *Vitis* species have most elongated flowers clusters, berries adhere to stems at maturity (Figure 2.1), tendrils are forked, loose bark detaches in long strips, pith is interrupted in nodes by a diaphram, and seeds are pyriform with a long or short beak. They are believed to have originated in Asia Minor, from where it has been widely disseminated. Chromosome number of these species is 2n = 38.

2.1.2 *Muscadinia* (Muscadine grapes)

V. rotundifolia Michx. is only grape species in this subgenus. Flowers clusters of muscadine grape are short and small, berries detach one by one as they mature (Figure 2.1), smooth bark with prominent lenticels, lack diaphragm in pith at nodes, tendrils are simple, and seeds are oblong without a beak. Chromosome number of these species is 2n = 40.

Figure 2.1 Morphological differences of berry clusters from two subgenera: top row are *Euvitis* and bottom row are *Muscadinia* grapes.

2.2 Grape species and cultivars classified by important species

There are three important species and one hybrid group that takes most grape production worldwide.

2.2.1 *V. rotundifolia* Michx, muscadine grape

Muscadine grape is extremely vigorous and highly disease tolerant comparing to *Vinifera* grapes. This grape specie is native to the southeastern United States. It is well

adapted to warm and humid conditions of the southeastern U.S., whereas American and European grapes do not prosper. Muscaidine grape is divided into two classes based on their flower types: 1) pistillate or female, and 2) perfect flower or hermaphroditic. ‘Cowart’, ‘Hunt’, ‘Noble’, ‘Jumbo’, ‘Nesbitt’, and ‘Southland’ are popular black cultivars, and ‘Carlos’, ‘Higgins’, ‘Fry’, ‘Dixieland’, ‘Summit’, and ‘Tara’ are popular bronze-skinned cultivars.

2.2.2 *V. labrusca* L., American bunch grape or Fox grape

The species is primarily used for sweet grape juice and associated products such as: jelly, jam, and preserves. Their fruits have characteristic of foxy flavor and slip skin. The major cultivar in these species is ‘Concord’ which responsible for 80% of total production. Other important cultivars include 'Niagara' and 'Catawba'.

2.2.3 *V. vinifera* L., European grape

V. vinifera is sometime called European grape since most production occurs in Europe. This species accounts for over 90% of world grape production. Most of the production is used for wine making, however, it is also produced for table and raisin grape production. There are at least 5,000 cultivars of *Vinifera* grapes grown worldwide. These species have been the major world production of wine and table grapes cultivars such as ‘Pinot Noir’, ‘Cabernet Sauvignon’, ‘Merlot’, ‘Shiraz’, ‘Thompson Seedless’, ‘Flame Seedless’, ‘White Riesling’, and ‘Chardonnay’.

2.2.4 French-American Hybrid

These hybrids are created for a need of rootstocks that are resistant to phylloxera

causing grape root louse, in Europe. *V. labrusca* and other native species to host range of the phylloxera were hybridized with *Vinifera* grapes in order to produce a range of resistance in rootstocks. To be used as rootstocks, some of the hybrids had both *phylloxera* resistance and good wine quality attributes. The hybrids include 'Marechal Foch', 'Vidal Blanc', 'Chambourcin', and 'Seyval'.

2.3 Grape species and cultivars classified by food usage

2.3.1 Table grapes

These grape varieties are consumed as fresh fruit. They have a nice combination of fine visual characters (skin color, berry shape, and size) and taste which is sweetness and juicy. 'Thompson Seedless', 'Autumn Royal', 'Flame Seedless' and 'Ruby Seedless' are major commercial cultivars of table grapes.

2.3.2 Raisin grapes

Raisins are traditionally made by dehydrating grapes in a process using heat from the sun or a mechanical process of drying in oven. Among the most popular types of raisins are 'Sultana', 'Fiesta', 'Malaga', 'Monukka', 'Black Corinth' (or known as 'Zante Currant'), 'Muscat', and 'Thompson seedless'. USDA reported in 2005 that 'Thompson Seedless' alone took 95% (222,096 from 238,161 acres total area) of California raisin grapes production (USDA, National Agricultural Statistics Service, and California Field Office, www, 2006).

2.3.3 Sweet juice grapes

This class was truly dominated by ‘Concord’ which the major American bunch grape. Concord grapes have higher yields, higher sugar-acid ratios, and milder *labrusca* flavors than other grape varieties. In Europe, *vinifera* or wine grape varieties are used to make grape juice, but giving a lower sugar-acid ratio as well as a less sweet taste than the juice made from ‘Concord’ and ‘Niagara’ (Amanor-Boadu et al., 2003).

2.3.4 Wine grapes

Wine grape varieties have their own unique combination of characteristics including color, size, skin thickness, acidity, yield per vine and flavors. Not a lot of grape varieties are suited to produce fine quality wine. Some wine can be produced from all grape species, but the commercial production is dominated by *V. vinifera* cultivars such as ‘Chardonnay’, ‘Cabernet Sauvignon’, ‘Shiraz’, ‘Pinot Noir’, and ‘Riesling’. Several French-American hybrids also produce good quality wine.

2.4 Grape production

2.4.1 World production

With wide adaptability, grapes are one of the most broadly distributed fruit crops in the world which cover an area of approximately 10 million hectares. The most concentrated viticulture is in European countries and USA. World grape production has ranged between 61-66 million tons over the last few years. There has been a trend to reduce or stabilize production in leading countries except USA, though other areas of the world continue to extend grape growing. In 2004, Italy produced 7.9, France

6.8, and Spain 5.6 million tons. The top 4 producers, which include Italy, France, United States, and Spain, have been constant for several years, but China has risen to the 5th place, when it was well outside the top 10 twenty years ago (Hajar, www, 2006).

2.4.2. Grape production in Thailand

Viticulture in Thailand started before 1960 when table grapes were imported from the United States and Australia. More than one hundred varieties were tested at that time as potential fruit crop and found that grapes showed good adaptation and growth in Thailand (Surasak Nilnond, www, 1998). In 1963, Professor Pavin Poonnasee and his colleagues from Department of Horticulture, Kasetsart University, Bangkok, was able to solve the problems of grape cultivation and leading grape industry in Thailand has been successfully established since then. The commercial table grape production areas were inicially located in the Central Plain region at Nakhon Pathom, Ratchaburi, Samut Sakhon, and Samut Songkhram (Nantakorn Boonkerd, 2000). Later it was introduced and expanded through out the country including Northern, Northeastern and Western regions. Only Southern region that viticulture and grape industrial could not reach. This is because of high amount of rainfall and humidity, causing diseases which limit grape growing.

2.5 Grape conventional breeding

Conventional plant breeding is generally accomplished by identifying parental plants having desirable traits that complement each other, combining those into individual offsprings through cross-pollination. The offspring plants were then

selected for the desirable traits and established as new cultivars. The sentence to define its definition but to get one cultivar released, it is laborious and time consuming. The art of conventional plant breeding was developed long before Mendel (1822-1884) and other plant hybridizers had produced improved strains of crop plants (Jauhar, 2006). Most conventional plant breeding programs have focused on cereals, field crops, and vegetables since they are main source of food products of mankind. Fruit crops, apple, citrus, kiwi, cherry, and grape for example, are also attractive to plant breeders as many new culivars have been successfully released to fruit markets.

Conventional grape breeding was systematically initiated with grapevines in France in the last half of 19th century when a soilborn aphid (*Phylloxera vastatrix*) from North America began to decimate the vineyards of France and later to all grape growing area in Europe. The breeding strategies were to produce interspecific hybrids between European grapevines of *V. vinifera* with American phylloxera tolerant species and produce *vinifera* cultivars grafted on to phylloxera tolerant rootstocks (Alleweldt and Possingham, 1988). There are two ultimate goals of grape breeding programs throughout the world: (1) to develop new cultivars resistant to broadrange of diseases and (2) to develop new cultivars for high quality of wine and table grape. Since grape improvement through conventional plant breeding requires high financial investment, its breeding projects are intensively located in certain Universities and Institutes with large budget such as Cornell and UC Davis in USA. Unlike annual species, grape is a fruit crop with 3-5 years to complete one generation and yet highly heterozygonous. Therefore, grape conventional breeding therefore is limited in few possible techniques.

2.5.1 Breeding techniques

There are many techniques in conventional plant breeding, but not all of them are suited for grape breeding. Some commonly used techniques in grape breeding are listed below.

2.5.1.1 Clonal selection

This technique is the most ancient and basic procedure of grape conventional breeding. Clonal selection relies on genetic variation as gene mutation can occur spontaneously in nature over time during vegetative growth of grape. Several clones of important grape cultivars, such as 'Chardonnay', 'Cabernat Sauvignon', '', and 'Thompson Seedless', have been developed through clonal selection and each of the clones showed relatively different in degree of disease resistance, stress tolerance, and fruit quality performance. Many attempts had been spent to increase genetic variation of existing grape cultivars by artificially appling some chemical mutagens and radiation to create source of selection. To date, except tetraploids induced by colchicine (Gargiulo, 1960), newly improved cultivars have not yet been produced commercially.

2.5.1.2 Interspecific hybridization

The most frequently employed grape breeding technique is interspecific hybridization. The aim of this technique is to bring together desirable traits from two different cultivar or varieties, with theoretically 50% genetic background each, into one plant of hybrid offspring via cross-pollination. The hybrid offsprings are grown in vineyard and further propagated for evaluation. Interspecific hybridization of table

hybridization of table grape in subgenus *Euvitis* requires up to 10 years to get a new cultivar released and approximately additional 10 years for evaluation of wine characteristics of wine grape. 'Noiret' is one of the newest released *vinifera* grape cultivars from New York State Agricultural Experiment Station at Cornell University (Reisch et al., 2006). It is a red wine grape resulting from cross made in 1973 between NY65.0467.08 and 'Steuben' (Figure 2.2). 'Noiret' has been available since 1994. 'Summit' is a good example of grape cultivar in subgenus *Muscadinia*. The cultivar is released from Georgia Station, University of Georgia (Lane, 1977). This muscadine grape cultivar originated from a cross of 'Fry' and Ga. 29-49 made in 1965 and was distributed in 1974 (Figure 2.3). By crossing of numerous unrelated hybrid cultivars from generation to generation makes grape the most highly heterozygous fruit crop on the planet.

Although interspecific hybridization constantly generates a number of grape cultivars, it is limited for introducing one or few specific traits into existing cultivars such as disease resistance and stress tolerance. The undesirable traits of hybrid plant receiving from donor parent have to be removed by backcrossing to recipient parent. More detail is described below.

2.5.1.3 Backcross

The technique is commonly used in field crops. The first hybrid (F_1 hybrid) is repeatedly crossed back to recipient parent for 6-12 backcrossing cycles (BC). The genetic background of donor parent in the progeny is 50% removed in each BC. Theoretically, BC_6 plant remains 0.78% genetic trait of its donor parent and could be established as a new variety after being self-pollinated for 2-3 cycles. Backcross is

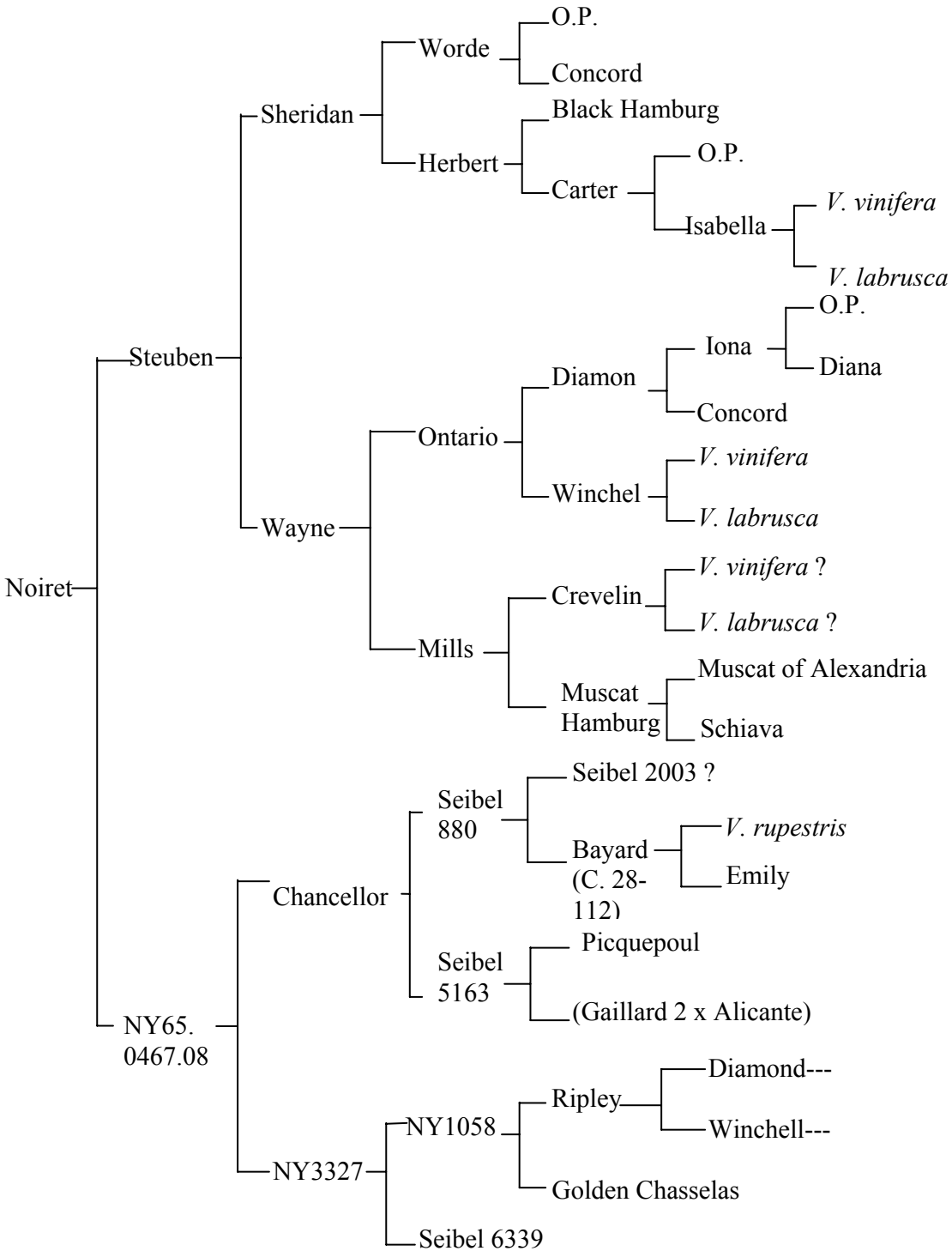


Figure 2.2 Pedigree of *vinifera* grape 'Noiret' (Reisch et al., 2006).

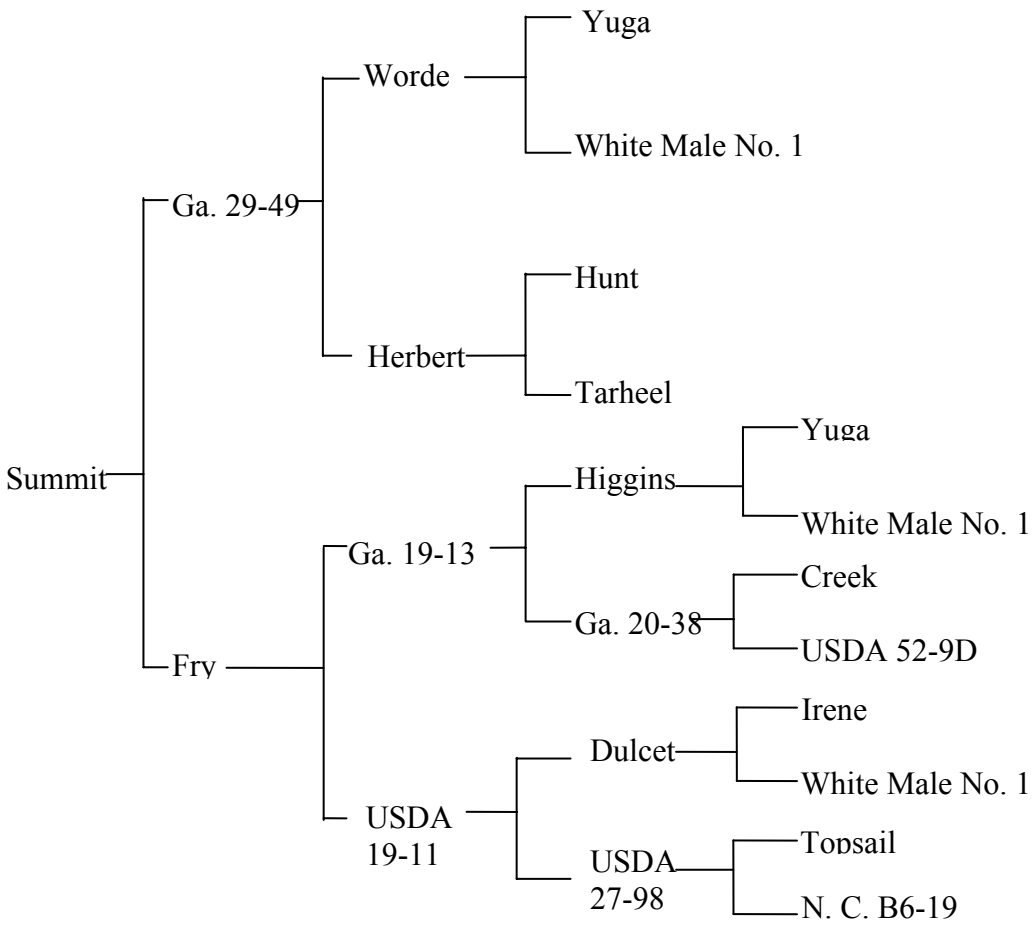


Figure 2.3 Pedigree of muscadine grape 'Summit' (*V. rotundifolia*) (Lane, 1977).

then likely impossible for grape, however, few breeding programs have run for long term project.

2.5.1 Limitation

The most unarguing limitation of conventional plant breeding is availability of genetic traits resource. Since gene controlling desirable trait is sexually transferred to offspring by cross-pollination of the parents, genetic trait from different species then becomes unavailable known as genetic incompatibility. As a fruit crop, time is another major limitation. Grape conventional breeding is laborious and easily takes up to 20 years to get new cultivars released. Genetic engineering is a breakthrough of those problems and becomes an alternative approach to produce new grape cultivars. Advantages and disadvantages of conventional grape breeding compared to grape transformation are further described in 2.7.

2.6 Grape genetic engineering

2.6.1 Somatic embryogenesis

History of grape somatic embryogenesis has started 30 years ago when Mullins and Srinivasan (1976) reported their experiment on *V. vinifera* 'Cabernet Sauvignon' somatic embryogenesis using unfertilized ovules as explant. Although it was the use of one type of explant from one grape cultivar, the experiment has leaded grape tissue culture to next level and made grape genetic transformation possible. The development of grape somatic embryogenesis has run on very long way and yet stopped. Since 1976 to 2006, more than 60 experiments of grape somatic embryos were published and

some of selected literatures are listed in Table 2.1. Not only in *V. vinifera*, which is well-known as the most important commercial species, but also many species have showed their somatic embryo inducibility including, *V. labrusca*, V. *latifolia*, *V. longii*, *V. riparia*, *V. rotundifolia*, *V. rupestris*, and *Vitis* hybrid. Above soil or medium level, every tissue part has been used as explant for somatic induction, and the most suitable is anther according to high frequency of publications. This has made somatic embryo induction in grape cultivars becomes routine in many laboratories. Organogenesis is another regeneration approach to produce new plant (Figure 2.4). However, this approach is unappreciable for grape transformation because of difficulty to obtain fully transgenic plant. Few publications reported usage of organogenesis approach to produce transgenic plant (Mezzetti et al., 2002). Martinelli and Gribaudo (2001) suggested that "In model plant, somatic embryogenesis is the most utilized model system for plant cell totipotency and development studies, while it is one of the most powerful techniques offered for genetic improvement of plant species important to agriculture".

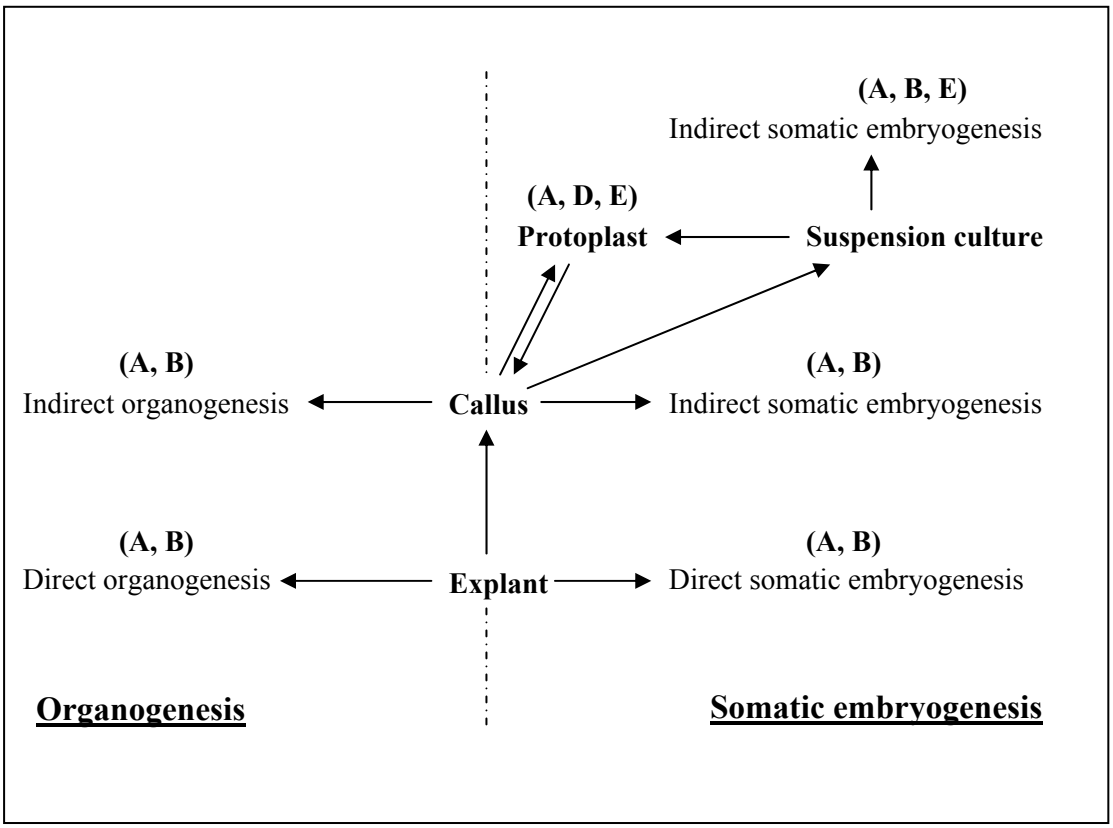


Figure 2.4 A simplified scheme for an integration of plant tissue culture and plant transformation approaches. An explant can be a variety of tissues, depending on particular plant species. There are two ways for plant regeneration: organogenesis and somatic embryogenesis. Different culture types and regeneration methods are amenable to different transformation approaches, including (A) *Agrobacterium*-mediated transformation and (B) biolistic transformation, (D) direct DNA uptake, and (E) electroporation. The scheme is modified from Walden and Wingender (1995).

Somatic embryogenesis itself has two sub-approaches in production which are solid and liquid media. Somatic embryo has been initially produced in solid medium. In the world of research and development when grape transformation and tissue culture-based biotechnologies are growing intensively, solid medium alone is incapable of producing enough somatic embryos to serve the need of researches. Besides, somatic embryos grow highly asynchronous on solid medium but very synchronous in liquid medium. This makes somatic embryo production in liquid medium more desirable in providing uniform experimental unit. Quality of somatic embryos is a main factor affecting regeneration frequency, which varies in each subculture on solid medium (Bornhoff and Harst, 2000; Wang et al., 2004). Furthermore, somatic embryos on solid medium showed exhibitive dormancy, whereas in liquid medium they were not dormant and showed higher plant regeneration efficiency (Jayasankar et al., 2003).

Table 2.1 Chronology of selected literature of somatic embryogenesis in grapes.

Genotypes	**Explants**	**Medium**	**References**
vinifera 'Cabernet Sauvignon'	Unfertilized ovule	NN, NOA, BA	Mullins and Srinivasan (1976)
vinifera x *rupestris*	Anthers	NN, 2,4-D, BA	Rajasekaran and Mullins (1976)
longii, *rupestris* *vinifera* 'Grenache' *vinifera* x rupestris	Anthers	NN, 2,4-D, BA	Mullins and Rajasekaran (1980)
longii, *rupestris* *vinifera* 'Grenache' 'Sumoll' x 'Cabernet Sauvignon' *vinifera* x *rupestris* Vitis hybrid 'Villard Noir' 'Villard Blanc', 'M.G. 60-44'	Anthers	NN, 2,4-D, BA	Rajasekaran and Mullins (1983)
rupestris *vinifera* 'Cabernet Sauvignon' 'Cardinal', 'Grenache' 'Sauvignon Blanc' 'White Riesling' 'Thompson Seedless' *vinifera* x *rupestris*	Anthers	NN, 2,4-D, BA, NOA	Stamp and Meredith (1988a)

Table 2.1 (continued).

Genotypes	Explants	Medium	References
longii *vinifera* 'Cabernet Sauvignon' 'French Colombard' 'Grenache', 'White Riesling'	Zygotic embryos	NN, 2,4-D NOA, BA	Stamp and Meredith (1988b)
vinifera 'Koshusanjaku'	Leaves	NN, MS 2,4-D, KT, BA, TDZ,	Matsuta and Hirabayashi (1989)
rotundifolia 'Dixie', 'Fly' 'Nesbitt', 'Welder'	Zygotic embryos	NN, 2,4-D, BA	Gray (1992)
rupestris	Leaves, Petioles	MS, 2,4-D, BA	Martinelli et al. (1993)
rotundifolia 'Fry', 'Regale'	Leaves	NN, 2,4-D, BA	Robacker (1993)
vinifera 'Seval Blanc' *thunbergii*, Vitis hybrid 'Chancellor'	Leaves	NN, NOA, TDZ	Harst (1995)
vinifera 'Centennial' 'Novornuscat', 'Ruby Seedless', 'Superior Seedless'	Anthers	MS, 2,4-D, BA IASP, ABA	Perl et al. (1995)
vinifera x *rotundifolia*	Leaves	MS, 2,4-D, BA	Torregrosa et al. (1995)

Table 2.1 (continued).

Genotypes	Explants	Medium	References
vinifera 'Mission'	Anthers	MS, 2,4-D, BA	Popescu (1996)
vinifera 'Thompson Seedless' 'Sonaka', 'Tas-e-Ganesh'	Tendrils	NN, NAA, GA, BA	Salunkhe et al. (1997)
vinifera 'Denuta', 'Portan', 'Syrah', 'Ugri Blanc'	Anthers	MS, 2,4-D	Torregrosa (1998)
vinifera 'Sultana' ('Thompson Seedless')	Anthers	MS, B5, 2,4-D, BA, NOA, IAA	Franks et al. (1998)
Vitis hybrid 'Seyve Villard 5276'	Leaves	NN, NOA, TDZ	Passos et al. (1999)
vinifera 'Cabernet Sauvignon', 'Chardonnay', 'Chenin Blanc', 'Muscat Gordo Blanco', 'Pinot Noir', 'Riesling', 'Sauvignon Blanc', 'Semillon', 'Shiraz'	Anthers	MS or NN, 2,4-D, BA, NOA, TDZ, KT	Iocco et al. (2001)
vinifera 'Chardonnay', 'Brachetto'	Anthers, Unfertilized ovaries	NN, 2,4-D	Martinelli et al. (2001)

Table 2.1 (continued).

Genotypes	Explants	Medium	References
'Cabernet Sauvignon', 'Chardonnay', 'Chasselas', 'Gamay', 'Gewurztraminer', 'Grenache', 'Merlot', 'Muscat', 'Pinot Noir', 'Portan', 'Riesling', 'Syrah', '110 Richter', '3309 Couderc', 'SO4'	Anthers	Various media	Perrin et al. (2001)
vinifera 'Chardonnay', 'Shiraz', 'Danuta', 'Portan'	Anthers	MS, B5, 2,4-D, BA, NOA	Torregrosa et al. (2002a)
vinifera 'Muscat of Alexandria', 'Neo Muscat' *labruscana* 'Aki Queen', 'Campbell Early', 'Delaware', 'Kyoho', 'Muscat Bailey A',	Filaments of anthers	MS, 2,4-D, TDZ	Nakajima and Matsuta (2003)
vinifera 'Sugraone'	Stigma-style	NN, NOA, BA	Morgana et al. (2004)
vinifera 'Ugni blanc', 'Cot', 'Morselan', 'Portan'	Anthers	MS, 2,4-D, BA, NOA	Torres-Vinals et al. (2004)

Table 2.1 (continued).

Genotypes	Explants	Medium	References
vinifera 'Bombino', 'Greco di Tufo', 'Merlot', 'Sangiovese'	Stigma-style	NN, NOA, BA	Carimi et al. (2005)
rotundifolia 'Summit', 'Tara', 'Triumph'	Anthers, Leaves, Petioles, Seed integuments	NN, CP, 2,4-D, BA	Lu et al. (2006)
vinifera 'Autunm Royal Seedless', 'Crimson Seedless'	Seed integuments	NN, CP, 2,4-D, BA	Xu et al. (2006)

Modified from Gray et al. (2005)

2.6.2 Transformation

Conventional breeding for genetic improvement of grape is severely limited by several factors such as a long generation cycle, inbreeding depression, polyploidy, and the highly heterozygous nature of existing cultivars (Gray and Meredith, 1992; Nakano et al., 1994; Das et al., 2002). Gene transfer technology became routine in the mid 1980's for easily manipulation of non-woody plant such as tobacco, and it was about time that grape transformation have just started. Genetic engineering is considered one of the most attractive methods to improve grape genetic because genes encoding agricultural desirable traits can be directly introduced into pre-existing and

desirable cultivars (Nakano et al., 1994). Among plant transformation techniques, *Agrobacteium*-mediated transformation is the most commonly used for grape genetic engineering (Table 2.2), as well as other plant species, for example, apple (James et al., 1989; Lambert and Tepfer, 1992; Maheswaran et al., 1992), banana (May et al., 1995), citus (Pena et al., 1997; Bond and Roose, 1998), rice (Datta et al., 2000; Kumar et al., 2005), kiwi fruit (Rugini et al., 1991), and papaya (Yang et al., 1996).

Biolistic transformation is another method to archive grape genetic engineering but it seems to have only been used in few groups. The most famous laboratory is Dr. Bruce I. Reisch's at Department of Horticultural Science, New York State Experiment Station, Cornell University, USA where the first gene gun was created by Dr. John Sanford in 1987. The limitation is caused by two major problems. (1) Particle gun or gene gun and its accessories are expensive investment. It is 18,550 US$ for a gene gun (PDS-1000/He Hepta System, BioRad company), making almost impossible to establish biolistic transformation in general laboratories. (2) Difficulty to develop transformation system due to lack of information from published protocol and several factors involved in this transformation method.

Huang and Mullins (1989) and Mullin et al., (1990) were the first groups who employed *Agrobacterium*-mediated transformation in grape genetic engineering. *Agrobacterium* LBA4404 created by Hoekema, Hirsch, Hooykaas, and Schilperoort, (1983) was the most common strain in 1990's. At this day more than ten *Agrobacterium* strains have been engineered in order to archive high-efficiency transformation. Among of the recombinant strains, EHA105 has been showed as highly effective in transformation of several *vinifera* grape cultivars (Scorza et al., 1995; Franks et al., 1998; Iocco et al., 2001; Li et al., 2001; Torregrosa et al., 2002a;

Li et al., 2006). Torregrosa et al. (2002a) conducted the experiment held the largest number of *Agrobacterium* strains, to evaluate an influence of *Agrobacteirum* strains on transformation efficiency of *V. vinifera*. They found that EHA105 showed increasing transformation efficiency compared to the widely used LBA4404, and was the most effective strains among *Agrobacterium tumefaciens* tested.

Table 2.2 Chronology of selected literature of grape genetic transformation.

Genotype	**Explant**	**Strain**	**Reference**
rupestris	-	-	Mullins et al. (1990)
rupestris	Somatic embryo	LBA4404	Martinelli and Mandolino (1994)
vinifera 'Koshusanjaku'	Leaves	*rhizogenes*	Nakano et al. (1994)
rupestris	Embryogenic	LBA4404	Krastanova et al.
Vitis hybrid '110 Ritcher'	callus		(1995)
vinifera 'Chardonnay'	Embryogenic	LBA4404	Mauro et al. (1995)
Vitis hybrids '41B', 'SO4'	callus		
vinifera	Somatic embryo	EHA101,	Scorza et al. (1995)
open pollinated hybrid		EHA105	
Vitis hybrid 'Chancellor'	Embryogenic	Biolistic	Kikkert et al. (1996)
	callus,		
	suspension		
	culture		
vinifera 'Superior Seedless'	Somatic embryo	LBA4404	Perl et al. (1996)

Table 2.2 (continued).

Genotype	Explant	Strain	Reference
'Thompson Seedless'	Somatic embryo	LBA4404	Scorza et al. (1996)
Vitis hybrids 'Freedom', 'Teleki 5C', '101-14'	Somatic embryo	LBA4404	Viss and Driver (1996)
vinifera 'Sultana' ('Thompson Seedless')	Embryogenic callus, Somatic embryo	EHA101, EHA105	Franks et al. (1998)
vinifera 'Chardonnay', 'Merlot'	Embryogenic callus, Somatic embryo	Biolistic	Kikkert et al. (2000)
Roostocks and hybrids '3309 C', 'MGT 101-14', 'Riparia Gloire', rupestris 'St George', 'Teleki 5C'	Embryogenic callus	C58Z707 LBA4404	Krastanova et al. (2000)
vinifera 'Cabernet Sauvignon', 'Podarok Magaracha', 'Rubinovyi Magaracha', rootstock 'Krona 42'	Primary explant, Organogenic culture	-	Levenko and Rubtsova (2000)
vinifera 'Neo Muscat'	Somatic embryo	LBA4404	Yamamoto et al. (2000)

Table 2.2 (continued).

Genotype	Explant	Strain	Reference
vinifera 'Cabernet Sauvignon', 'Chardonnay', 'Chenin Blanc', 'Riesling' 'Sauvignon Blanc', 'Shiraz', 'Muscat Gordo Blanco'	Embryogenic callus	EHA105	Iocco et al. (2001)
vinifera 'Thompson Seedless'	Somatic embryo	EHA105	Li et al. (2001)
vinifera 'Chardonnay', 'Shiraz', 'Danuta', 'Portan'	Embryogenic callus	EHA105, AGL0, AGL1, LBA4404, K252, A4	Torregrosa et al. (2002a)
vinifera 'Cabernet Sauvignon'	Stem	Biolistic	Torregrosa et al. (2002b)
vinifera 'Chardonnay'	Embryogenic callus, Somatic embryo	Biolistic	Vidal et al., 2003
vinifera 'Chardonnay', 'Thompson Seedless'	Somatic embryo	EHA105	Li et al., 2006

Modified from Gray et al. (2005)

2.7 Green fluorescent protein

2.7.1 History

Green fluorescent protein (GFP) is a protein isolated from a jellyfish *Aequorea victoria* that fluoresces green when exposed to blue or UV light. The *A. victoria* GFP was first extracted, purified, and studied in fluorescent properties by Shimomura et al., (1962). The GFP, comprised of 238 amino acids (27 kDa), has a unique can-like shape consisting of an 11-strand β-barrel with a single alpha helical strand containing the chromophore running through the center (Figure 2.5) (Ormö et al., 1996; Yang et al., 1996). In *A. victoria*, the luminescent protein aequorin interacts with Ca^{+} ions producing blue chemiluminescence or blue light. The blue chemiluminescence of the protein *aequorin* is then transduced into green fluorescent light by energy transfer at GFP. The GFP has become a hot research project when Prasher et al., (1992) reported the cloning and nucleotide sequence of GFP. Although the wild type GFP molecule was able to fold and fluoresce at room temperature without a need of specific exogenous cofactors of the jellyfish, it had poor photostability and poor folding at 37°C. Therefore, many different mutants of GFP have been engineered (Shaner et al., 2005) to serve needs of researchers. The first major improvement of GFP was S65T (Heim et al., 1995). This new mutant GFP was tremendously improved in its spectral characteristics, increasing fluorescence and photostablility. The addition of the 37°C folding efficiency (F64L) mutant to this scaffold yielded enhanced GFP (EGFP). GFPs have been used to make chimeric proteins of GFP by linking GFP to other proteins where it functions as a fluorescent protein tag. Up to date, many bacteria, yeast, fungal cells, plants, fly, drosophila, zebrafish, and in mammalian cells have been created

using GFP gene. This has been proofed that the GFP gene can be expressed throughout given organism.

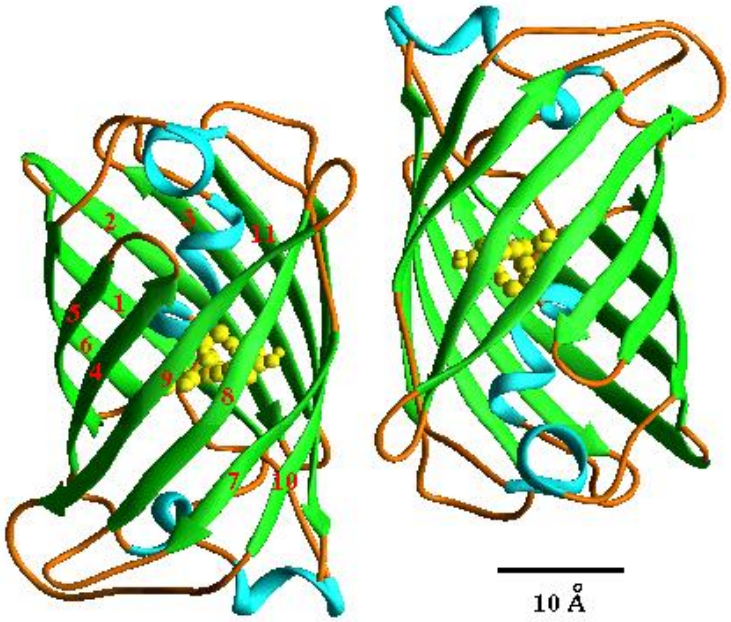


Figure 2.5 Stereoview of the three-dimensional structure of GFP, showing 11 *β*-Strands forming a hollow cylinder through which is threaded a helix bearing the chromophore, showing as yellow balls.

2.7.2 Classification of GFPs

Tsien (1998) divided GFP variants into seven classes based on their distinctive component of chromophores (Table 2.4). Fluorescence excitation and emission of the GFPs, and chromophore structures are shown in Figure 2.6.

Class 1: Wild type mixture of neutral phenol and anionic phenolate The wild type *Aequore*a GFP has the most complex spectra. It has a major excitation peak

at 395 nm which is about three times higher than a minor peak at 475. The major excitation peak gives emission peak at 508 nm, whereas the minor excitation peak gives a maximum at 503 nm (Heim et al., 1994). The wild type GFP folds fairly efficiently when it is expressed at or below room temperature, but folding efficiency declines steeply at higher temperatures. A triple mutant, F99S, M153T, V163A, was produced to have a GFP that works well at 37^{o}C. The GFP folding was improved, aggregation was reduced at high concentration, and diffusibility of the protein inside cells was increased. However, the mutations do not increase brightness of the properly folding protein molecule.

Class 2: Phenolate anion in chromophore The GFPs in this class have become the most widely used in routine cell biological studies. That is because they were the first group combining high brightness with simple excitation and emission wavelengths which are very similar to the most popular small molecule fluorophore orfluorescein. The most oftenly used mutant GFP in class 2 is used to be S65T. Its oxidation was about fourfold faster than wild type (Heim et al., 1995). The S65T folds fairly efficiently like wild type when it was expressed at or below room temperature, however, it tended to fold improperly and produce mostly nonfluorescent aggregates at higher temperatures. Much effort has attempted to find additional mutations in order to produce greater brightness of GFP at warmer temperature because of obvious interest in expression at 37^{o}C. The most commonly use are F64L (EGFP) and V163A.

Class 3: Neutral phenol in chromophore A Thr203 has been mutated to Ile (T203I) (Heim et al., 1994; Kaether and Gerdes, 1995). The mutation greatly

suppresses 475 nm excitation peak, and leave only shorter wavelength peak at 399 and emission peak is still 511 nm. A largest gap in wavelength between the excitation and emission peaks is found in neutral phenol GFPs. The gap has advantage in supporting of laser action.

Class 4: Phenolate anion with stacked π-electron system These GFPs have the longest wavelength of excitation and emission peaks from stacking an aromatic ring next to a phenolate anion of the chromophore by mutation. Mutation of Gln69 to Lys (Q69K) results an emission peak at 529 nm which is the longest wavelength now known. The tail at longer wavelengths gives yellowish fluorescence in overall, though the 529 itself is greenish. Therefore, the GFPs in class 4 have been called yellowish fluorescent proteins or YFPs (Miyawaki, 1997).

Class 5: Indole in chromophore derived from Y66W A Tyr66 in class 5 GFPs is substituted with Trp, producing a new chromophre with an indole instead of a phenol or phenolate (Heim et al., 1994). Excitation and emission peak are 436 and 476 nm, respectively. The wavelengths are intermediate between phenolate anion (class 2) and neutral phenol chromophores (class3). The proteins have been called cyan fluorescent proteins or CFPs because they fluoresce blue-green or cyan.

Class 6: Imidazole in chromophore derived from Y66W A Tyr66 in class 6 GFPs is substituted with His. The substitution puts an imidazole in chromophore and shifts wavelength shorter than Trp66. Excitation and emission wavelengths are 383 and 447, respectively. The emission wavelength gives fluorescence an overall blue

appearance. Therefore, class 4 GFPs has been called blue fluorescent proteins or BFP.

Class 7: Phenyl in chromophore derived from Y66F This mutant has shortest wavelength obtaining by substitution of Phe for Tyr66. It has been little studied.

Table 2.3 Spectral characteristic of major classes of green fluorescent protein (GFP).

Mutation[a]	**Common name**	λ_{exc}[b]	λ_{em}[c]	**Reference**[d]
Class 1, wild-type				
None or Q80R	Wild type	395–397 470-475	504	Patterson et al. (1997), Ward (1997)
F99S, M153T, V163A	Cycle 3	397 475	506	Patterson et al. (1997), Ward (1997)
Class 2, phenolate anion				
S65T		489	509–511	Patterson et al. (1997), Cubitt et al. (1997), Ward (1997)
F64L, S65T	EGFP	488	507–509	Patterson et al. (1997), Cubitt et al. (1997), Ward (1997)
F64L, S65T, V163A		488	511	Cubitt et al. (1997)
S65T, S72A, N149K,	Emerald	487	509	Cubitt et al. (1997)

Table 2.3 (continued).

Mutation[a]	Common name	λ_{exc}[b]	λ_{em}[c]	Reference[d]
M163T, I167T				
Class 3, neutral phenol				
S202F, T203I	H9	399	511	Cubitt et al. (1997)
T203I, S72A,Y145F	H9–40	399	511	Cubitt et al. (1997)
Class 4, phenolate anion with stacked π-electron system (yellow fluorescent proteins)				
S65G, S72A, T203F		512	522	Cubitt et al. (1997)
S65G, S72A, T203H		508	518	Cubitt et al. (1997)
S65G, V68L, Q69K, S72A, T203Y	10C Q69K	516	529	Cubitt et al. (1997)
S65G, V68L, S72A, T203Y	10C	514	527	Cubitt et al. (1997)
S65G, S72A, K79R, T203Y	Topaz	514	527	Cubitt et al. (1997)
Class 5, indole in chromophore (cyan fluorescent proteins)				
Y66W		436	485	Heim et al. (1994)
Y66W, N146I, 153T, V163A	W7	434 452	476 505	Cubitt et al. (1997)

Table 2.4 (continued).

Mutation[a]	Common name	λ_{exc}[b]	λ_{em}[c]	Reference[d]
F64L, S65T, Y66W, N146I, M153T, 163A	W1B or	434	476	Cubitt et al. (1997)
S65A,Y66W, S72A, N146I, M153T, 163A	W1C	435	495	Cubitt et al. (1997)
Class 6, imidazole in chromophore (blue fluorescent proteins)				
Y66H	BFP	384	448	Cubitt et al. (1997)
Y66H, Y145F	P4–3	382	446	Cubitt et al. (1997)
F64L, Y66H, Y145F	EBFP	380–383	440–447	Patterson et al. (1997), Cubitt et al. (1997)
Class 7, phenyl in chromophore				
Y66F		360	442	Cubitt et al. (1995)

Modified from Tsein (1998)

[a]Substitutions from the primary sequence of GFP (Figure 2.6) are given as the single-letter code for the amino acid being replaced, its numerical position in the sequence, and the single-letter code for the replacement.

[b]λ_{exc} is the peak of the excitation spectrum in units of nanometers.

[c]λ_{em} is the peak of the emission spectrum in units of nanometers.

[d]Reference only for the quantitative spectral and brightness data.

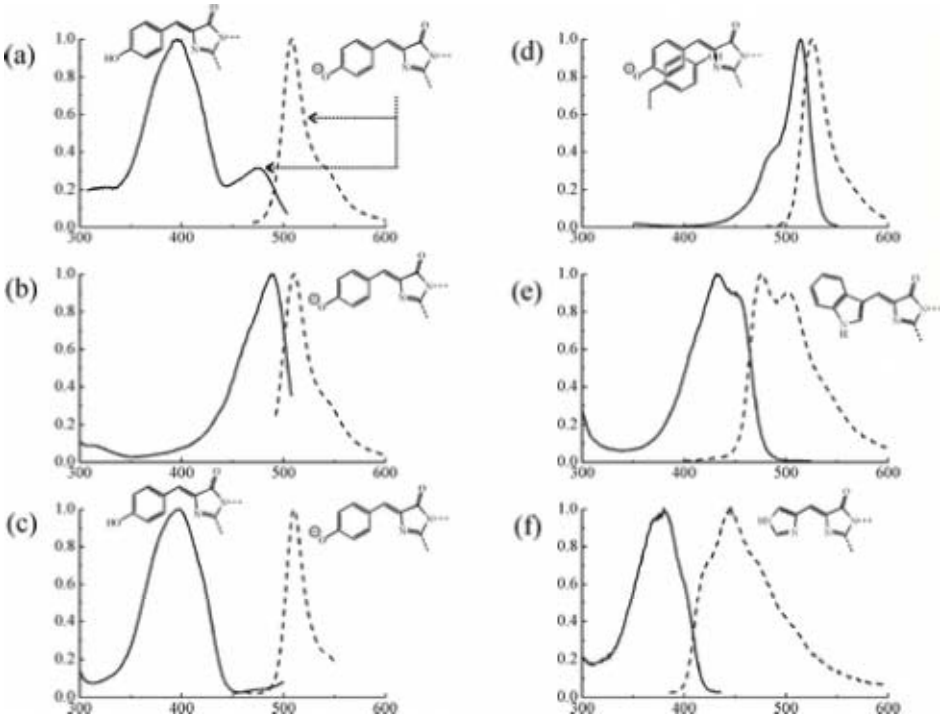


Figure 2.6 Fluorescence excitation and emission spectra (solid and dashed lines, respectively) for typical members of the six major classes of GFP mutants, together with the chromophore structures believed to be responsible for the spectra. Spectra have been normalized to a maximum amplitude of 1. For comparison of absolute brightnesses, see the extinction coefficients and quantum yields in Table 1. When only one structure is drawn, both excitation and emission spectra arise from the same state of chromophore protonation. The actual GFPs depicted are (*a*) wild-type, (*b*) Emerald, (*c*) H9-40, (*d*) Topaz, (*e*) W1B, and (*f*) P4-3 (Tsein, 1998).

2.7.3 Application of GFPs

2.7.3.1 Reporter gene in genetic transformation

GFPs, an enzyme that catalytically processes an indefinite number of substrate molecules, have been mainly used to detect gene expression from genetic transformation. It is very small fraction of transformed cells typically yields from plant genetic transformation. In order to separate those transformed cells from the mass that is not transformed, the use of marker gene that function as reporting element (or called reporter gene) of gene expression is required. The *β*-glucuronidase (GUS) gene (Jefferson et al., 1987) and luciferase (LUC) gene (Ow et al., 1986) are visual reporter gene that have been widely used between late 1980's to early 1990's. Although these two genes have proven useful in many applications, they require additional substrates that toxic to plant cells (Zhu et al., 2004), especially the assay of GUS is destructive. Therefore, GUS and LUC are not appreciable for efficiency selection in plant transformation.

GFP is a newest visual reporter that shares none of these problems. In contrast, fluorescence emission of GFP only requires excitation by UV light and its observation is performed easily in living cells without disruption. Since Chalfie et al., (1994) demonstrated advantage of GFP as marker gene in bacteria and invertebrate, it has been later on applied in numerous transformation-based experiments and recovered in several advantages over other visual reporter genes. GFP has been expressed in several plant species including dicotyledous and monocotyledous plants (Ghorbel et al., 1999). In grape, several publications have been reported using GFP as a reporter gene (Iocco et al., 2001; Li et al., 2001; Torregrosa et al., 2002b).

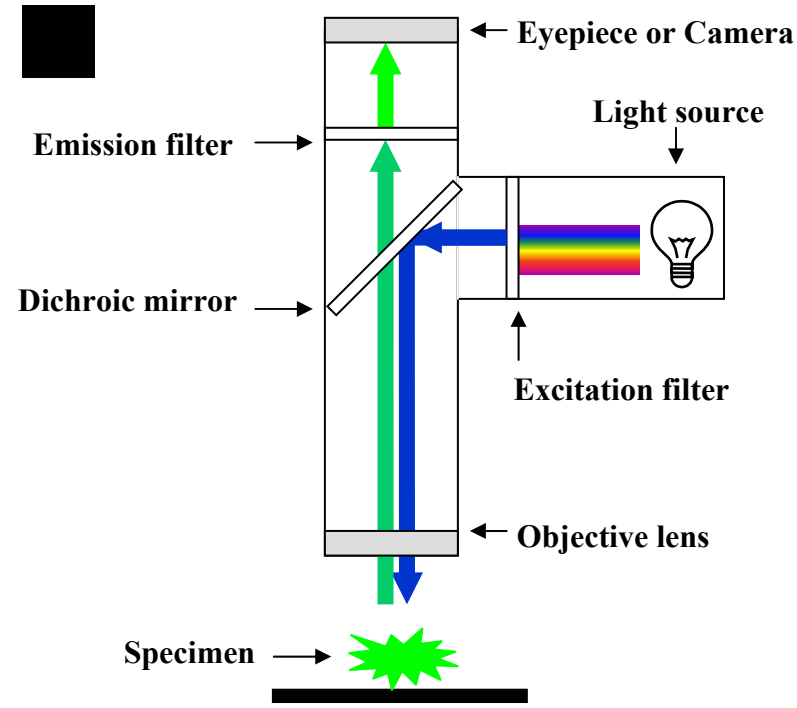
2.7.3.2 Fusion protein technology

GFPs have been used as a genetic fusion partner to target proteins in order to monitor their localization. GFP gene is fused with the gene encoding the target protein, resulting chimeric protein expressed in cell or organism of interest. The ideal is to link the target protein with a GFP while the target protein still maintains its normal functions and localizations. The fused target protein can be easily detected and monitored by green fluorescence of the fused GFP. GFP has been successfully introduced into every major cell organelle, including plasma membrane, nucleus, endoplasmic reticulum, Golgi apparatus, secretory vesicles, mitochondria, peroxisomes, vacuoles, and phagosomes. (Tsein, 1998).

2.7.4 Fluorescence microscope and GFP visualization

In stead of a normal light microscope that is used to study samples or specimens by using reflection and absorption, a fluorescence microscope has a set of filters and specific light source to detect fluorescence from specimens of interest. Typical components of a fluorescence microscope are the light source, which is Xenon or Mercury arc-discharge lamp, excitation filters, dichroic mirror, and emission filter (Figure 2.7). The specimen is specifically labeled with or has fluorophores as its component. Firstly, high energy (or short wavelength) light is separated from wide spectrum of light by an excitation filter and reflects to dichroic mirror. The dichroic mirror then brings the high energy light passes to and is absorbed by fluorophores in the specimen, causing them to emit much weaker energy (or longer wavelength) light. The phenomenon is known as fluorescence. The light of specific wavelength is separated from the weaker emitted fluorescence by an emission filter and reaches to

microscope eyepieces or detector. The filters and the dichroic are chosen to match the spectral excitation and emission characteristics of the fluorophore of the specimen. Most fluorescence microscopes now used are epi-fluorescence microscopes which excitation and observation of the fluorescence are located above the specimen.



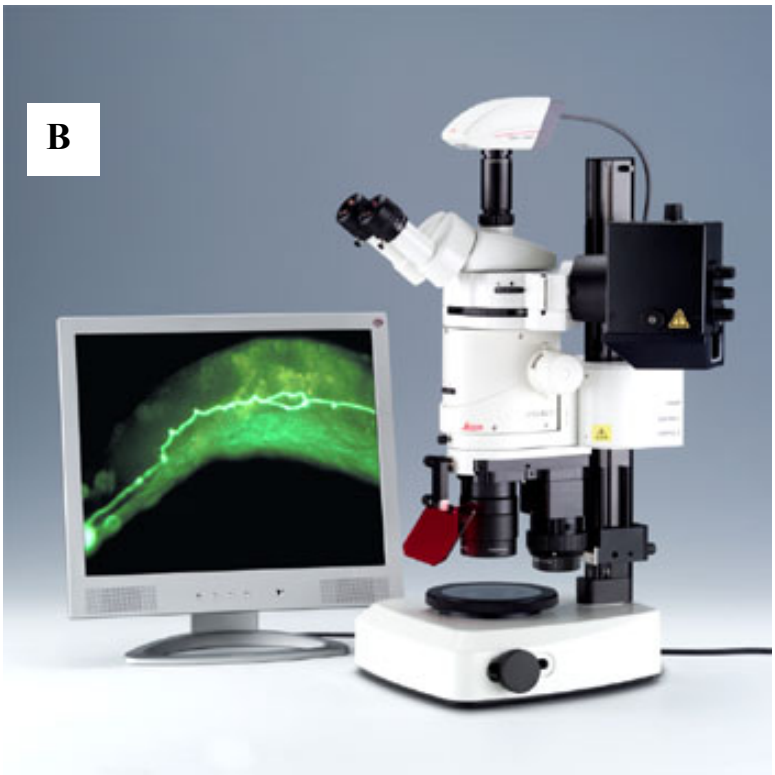


Figure 2.7 Simple model of fluorescence microscope (A) and fluorescence microscope model Leica FluoCombi III (B) of BioRad Company.

2.8 Conventional grape breeding versus grape genetic engineering

2.8.1 Improvement of existing grape cultivars

Numbers of grape cultivars have been produced commercially through conventional breeding. 'Cabernet Sauvignon', 'Chardonnay', and 'Thompson Seedless' are excellent examples of high quality commercial grapes that have been cultivated worldwide. Those cultivars however are susceptible to diseases so that disease resistance needs to be established. Theoretically, backcross is the only breeding technique potentially used to add resistance trait to the cultivars without significantly modifying their genetic material. Unfortunately, it requires more than a lifetime of a breeder to complete the breeding program, making the improvement impossible in reality. Besides, for wine grapes, new cultivar resulting from conventional breeding are always assigned new name, making slow acceptance in the market (Kikkert et al., 2001). Genetic engineering, on the other hand, is able of transfering gene encoding desirable trait into target grape cultivar without modifying its desired commercial attributes (Colova-Tsolova et al., 2001). By transformation approach, transgenic 'Chardonnay', which was the same 'Chardonnay' that has recognization of wine industry but with disease resistibility, was produced (Mauro et al., 1995; Kikkert et al., 2000; Vidal et al., 2003). However, to date, none of transgenic grape has been released for commercial cultivar.

2.8.2 Availability of genetic resource

As a sexual reproductive-based method, conventional breeding is merely limited to use genetic resource from different organisms, even different plant species is almost

impossible. It is not an obstacle for genetic transformation that allows movement of genetic material from any organism to any other organisms. Fungal (Kikkert et al., 2000), insect (Torregrosa, et al., 2002), and plant (Yamamoto et al., 2000; Harst et al., 2000) genes have already been transferred to grape cultivars mainly for improvement of disease resistance. The ability of using unlimited genetic resource is the most advantage of genetic engineering to establish genetic traits that have never been available for conventional breeding.

2.8.3 Regulation of gene encoding desirable traits

Conventional plant breeding employs natural gene transfer as genes encoding desirable traits are purely unmodified. Expression of those genes is therefore regulated by nature biological mechanism like native genetic material in plant cell. Genetic engineering is different. The gene, termed as transgene, is constructed in plasmid vector under controlling of promoter that designs condition and intensity of transgene expression. The 35S gene of cauliflower mosaic virus (CaMV 35S) is used very frequently for strong constitutive promoter. Its transgene is highly expressed throughout the whole plant, resulting in tremedrous degree of desired trait performance. To date, a number of promoters are available in order to express a transgene in certain tissue target, quantity, and time.

2.8.4 Time requiement

This has to be emphasized that conventional grape breeding is laborious and time consuming. As described, it takes up to 20 years to develop one cultivars through interspecific hybridization and decades if backcross. Unlike conventional grape

breeding, genetic engineering of grape needs less than 5 years to produce transgenic grape and additional 5 years for evaluation.

2.8.5 Financial investment

Conventional grape breeding typically generates hundreds of hybrid plants and all of them need to be grown and maintained in vineyard for many years of evaluation processes. This requires huge financial investment for land, field equipments, pesticide, fertilizer, and labour. *Agrobacterium*-mediated transformation, the most commonly used for grape genetic engineering, costs much cheaper because the experiment is literally conducted in laboratory and generally needs basic lab materials and chemicals.

2.9 *In vitro* selection for salt tolerance

2.9.1 Soil salinity

Salt-affected soil can be divided into two categories, including sodic and saline. The sodic soil is dominated by excess sodium on exchange sites and a high concentration of carbonate/bicarbonate anions. It has a high pH, normally greater than 8.5 and may up to 10.8, with a high sodium absorption ratio (SAR) and poor soil structure. The saline soil is also generally dominated by sodium ions, but with the dominant chloride and sulphate anions. Its pH values and SAR are much lower and electrical conductivities are higher than in sodic soils. Salt-affected soils contain sufficient concentrations of soluble salts to reduce plant growth (Flowers and Flowers, 2005).

In Thailand, there are a total of 17.8 million rai or 16.73% of saline soil spreading over 17 provinces of Northeastern. The saline soil can be classified as 12.6 million rai of low saline soil, 3.7 million rai of moderate saline soil, 1.5 million rai of extreme saline soil, and another 19.4 million rai of potential spreading saline soil (Land and Development, www, n.d.).

2.9.2 Effects of salinity to plants

Salinity of soil, which is caused by excessive amounts of salts, directly affects plants as decreasing of productivity or death. Suppression of growth is generally observed in all plants, however, their tolerance levels and growth reducing rate under salt stress vary due to defense mechanism of different plant species. In plants, salt stress affects major processes such as growth, photosynthesis, protein synthesis, and energy metabolism. The effects were discussed in a review of Parida and Das (2005) and some of those that are important presented below.

2.9.2.1 Effect on growth

When plants are subjected to salt stress, rate of leaf surface expansion is immediately reduced as salt concentration increases (Wang and Nil, 2000). Salt stress generally results in a considerable decrease in the fresh and dry weights of leaves, stems, and roots (Hernandez et al., 1995; AliDinar et al., 1999; Chartzoulakis and Klapaki, 2000). Many experiments have been conducted in plants under salt stress in order to understand and manage the effects. Mohammad et al. (1998) found that increasing salinity is accompanied by significant reductions in shoot weight, plant height, number of leaves per plant, root length, and root surface area per plant in

tomato. Increased NaCl levels results in a significant decrease in root, shoot, and leaf growth biomass and increase in root/shoot ratio in cotton (Meloni et al., 2001). Salt stress results in a clear stunting of plants (Hernandez et al., 1995; Cherian et al., 1999; Takemura et al., 2000).

2.9.2.2 Effect on nitrogen metabolism

Nitrogen is the most importance among many nutrients for plant growth and devopment, especially vegetative stage. Nitrate reductase is a key enzyme for nitrogen metabolism. In leaves, nitrate reductase activity (NRA) decreases in many plants under salt stress (AbdElBaki et al., 2000; Flores et al., 2000). The NRA reduction in leaves is caused by a specific effect associated with Cl^- salts in external medium. Under salinity, nitrate content of leaves and their NRA decreases in corn (*Zea mays*) (AbdElBaki et al., 2000). Salinity is also found to be a considerable problem in nitrogen fixing plants. In chickpea (*C. arietinum* L.), salinity inhibits nitrogen fixation by reducing nodulation and nitrogenase activity (Soussi et al., 1999). Serraz et al., (1998) found that when nodulated roots of legume plants such as soybean, common bean, and alfalfa were exposed to NaCl, their growth is observed as rapidly decreasing. This also associated with a short-term inhibition of both nodule growth and nitrogenase activity.

2.9.2.3 Effect on water content

Many experiments (Morales et al., 1998; Hernandez et al., 1999; Khan et al., 1999; Khan, 2001; Meloni et al., 2001; Romeroaranda et al., 2001) have found that water potential and osmotic potential of plants become more negative but turgor

pressure increases with an increasing of salinity. Water potential and osmotic potential of leaves decline due to the osmotic potential of rooting medium and stress imposition. A greater decline in osmotic potential compared with the total water potential led to turgor maintenance in plants under progressive or prolonged NaCl stress (Rajasekaran et al., 2001).

2.9.2.4 Effect on photosynthetic pigments

Under salt stress, chlorophyll and total carotenoid contents in leaves are observed as decreasing in general. The oldest leaves start to develop chlorosis and fall with prolonged period of salt stress (Gadallah, 1999; Hernandez et al., 1995, 1999; Agastian et al., 2000). Kennedy and De Fillippis (1999) reported a significantly reducing of protochlorophyll, chlorophylls, and carotenoids under NaCl stress, however in this case, the rate of decline of protochlorophyll and chlorophyll is greater than that of Chl-*a* and carotenoids in *Grevilea*. In leaves of tomato, the contents of total chlorophyll (Chl-*a*+*b*), Chl-*a*, and *β* carotene decrease by NaCl stress (Khavarinejad and Mostofi, 1998).

2.9.2.5 Effect on photosynthesis

Plant growth as biomass production is a measure of net photosynthesis. Therefore, salt stress affecting growth also affects photosynthesis. High salt concentration in soil and water create high osmotic potential, resulting in reduction of water availability to plants use. Decreasing of water potential reversibly inactivates photosynthetic electron transport via shrinkage of intercellular space which is due to efflux of water through water channels in plasma membrane (Allakhverdiev et al.,

2000). Increasing of osmotic potential under high salt conditions causes leaking of Na^+ ions into cytosol (Papageorgiou et al., 1998) and also inactivates photosynthetic and respiratory electron transport (Allakhverdiev et al., 1999). Reduction in stomatal conductance from salt stress also causes reduction of photosynthetic rate, resulting in restricted CO_2 availability for carboxylation reactions (Brugnoli and Bjorkman, 1992). Stomatal closure minimizes loss of water by transpiration, however in the same time, this affects chloroplast light-harvesting and energy-conversion systems, leading to alteration in chloroplast activity (Iyengar and Reddy, 1996). Allakhverdiev et al. (2002) suggested that salt stress inhibits repairing of PSII via suppression of the activities of the transcriptional and translational machinery.

2.10 Approaches to enhance salt tolerance in plants

Salt tolerance, as well as other stress tolerances, is a combination of many characteristics (or technical term as a multigenic trait) of plants to survive under excess of NaCl. The multigenic trait is controlled by many genes or quantitative trait loci (QTLs) which limit crop improvement for salt tolerance through conventional plant breeding program. Besides, physiology of salt tolerance is also complex by a wide range of adaptations of halophytes and less tolerant plants. Flowers (2004) suggested that developing of salt tolerant crops is possible through seven approaches: (1) genetic engineering, (2) conventional plant breeding program, (3) use of *in vitro* selection, (4) pooling physiological traits, (5) interspecific hybridization, (6) develop halophytes as alternative crops, and (7) use of marker-aided selection. Although pooling physiological traits, interspecific hybridization, and marker-aided selection do not require long cycles of recurrent selection, they also deal with many of processes in

hybrid or progeny production and conventional screening in experimental field.

Between 1993 and early 2003, genetic engineering had been employed in order to produce transgenic plants tolerant to salinity. There were 68 experiments, conducting in many plant species. However, only four experiments contained quantitative data on the response of transformants comparing to wild-type, whereas half of all the experiments provided insights into components of tolerance but no claims of enhanced tolerance at whole plant level. Until this day, none of transgenic plants of any crop species has established or even tested in field (Flowers, 2004).

In vitro selection that uses the advantage of somaclonal variation has become an attractive alternative approach to produce salt tolerant plants. The technique does not require such deep knowledge of genetic basic yet much less expensive than conventional plant breeding and genetic engineering that needs to produce and carry lots of hybrids and is limited by high technology and financial investment, respectively. Somaclonal variation is the variation observed among plants regenerated through *in vitro* culture (Larkin and Scowcroft, 1981), and has been proven to be useful in crop improvement (Skirvin et al., 1993; Jain et al., 1998a; Jain et al., 1998b; Jain and De Klerk, 1998). The variation is unpredictable and can be both heritable (genetic) and non-heritable (epigenetic) in regenerated plants (Rout et al., 2006). Morphological variations generated by somaclonal variation have been intensively studied in several crop and fruit tree species (Tremblay et al., 1999). Somaclonal variation can broaden the genetic variation in number of crop plants, resulting in alteration of plant characteristics, including plant height, yield, number of flowers per plant, early flowering as well as resistance to diseases, insects, pests and salt (Rout et al., 2006).

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CHAPTER III

ESTABLISHING A PROTOCOL FOR SOMATIC EMBRYOGENESIS AND PLANT REGENERATION FROM SUSPENSION CULTURE IN A TABLE GRAPE 'AUTUMN ROYAL SEEDLESS' (*Vitis vinifera* L.)

3.1 Abstract

This experiment was aimed at establishing a system for grape somatic embryogenesis and plant regeneration which is the prerequisite of plant biotechnological breeding. Proembryonic mass (PEM) suspension culture of 'Autumn Royal Seedless' (*Vitis vinifera* L.) was established from primary somatic embryos at the second subculture in MSGGN liquid medium. The PEMs, yellowish in color, grew rapidly in the liquid medium. Browning was neither observed in the liquid medium nor the PEMs. Light had an extreme negative effect to somatic embryogenesis of PEMs. Activated charcoal facilitated somatic embryogenesis and development. When MS medium was used for somatic embryogenesis and plant regeneration, full-strength was superior to half-strength. More than 1,000 mature somatic embryos could be produced from approximately 50 mg of SPEMs within 14-16 weeks on FMSC solid medium. The somatic embryos tested were easily germinated (100%) on FMSC medium and up to 95% of them were converted into normal plantlets. Using the same

protocol, PEM suspension culture of 'Chardonnay' (*V. vinifera* L.) and 'Tara' (*V. rotundifolia*) were established. Characteristics of the PEM suspension culture had been consistently recorded. Among three cultivars, 'Autumn Royal Seedless' exhibited the highest performance in growth and regeneration. However, its growth tended to be expansion of proembryonic cell and enlargement of PEMs rather than new PEMs generation. Highly synchronous culture was obtained from 'Chardonnay'. The culture was homogeneous and provided preferable finely-PEMs. 'Tara' grew the poorest PEM suspension culture which browning was easily observed.

3.2 Introduction

Biotechnology has great potential for grape genetic improvement. However, successful implementations of grape biotechnologies such as transformation and *in vitro* selection are based on a high-yield productivity of synchronized somatic embryos as well as an efficient single cell regeneration system. Successful somatic embryogenesis has been reported on various *Vitis* species (Martinelli and Gribaudo, 2001; Gray et al., 2005). Numerous solid media have been formulated for somatic embryo induction (Mullins and Srinivasan, 1976; Mauro et al., 1986; Harst, 1995; Perl et al., 1995; Nakano et al., 1997; Torregrosa, 1998; Martinelli et al., 2001; Nakajima and Matsuta, 2003). Although somatic embryos could be multiplied by recurrently subculture on fresh solid medium, yield still remains low in general. In addition, somatic embryos are highly asynchronous in solid medium. Quality of somatic embryos is a main factor affecting regeneration frequency, which varies in each subculture on solid medium (Bornhoff and Harst, 2000; Wang et al., 2004).

Furthermore, somatic embryos on solid medium showed exhibitive dormancy, whereas in liquid medium they were not dormant and also showed higher plant regeneration efficiency (Jayasankar et al., 2003).

Plant suspension culture is a preferable approach in which produce large amounts of synchronous cells in short period of time. In recent years, suspension culture protocols have been successfully established for several grape cultivars, e.g. 'Cabernet Sauvignon' (Torregrosa et al., 2002), 'Chardonnay' (Jayasankar et al., 1999; Vidal et al., 2003), 'Dornfelder' (Bornhoff and Harst, 2000), 'Red Globe' (Wang et al., 2002), rootstock '41B' (Wang et al., 2004) and '110 Ritcher' (Wang et al., 2004), 'Sultana' (Franks et al., 1998), 'Thompson Seedless' (Jayasankar et al., 1999), *V. rupestris* Scheele (Martinelli et al., 2001).

A repeatable protocol for grapevine in suspension culture was reported by Jayasankar et al. (1999), where 'Chardonnay' somatic embryos were produced in liquid medium and 60% of them were regenerated. However, when the same protocol was applied for 'Thompson Seedless', somatic embryos did not develop beyond the heart stage, which might imply that liquid medium was not suitable for embryogenesis of the seedless grape. Suspension cultures have been used for producing embryogenic materials for grape transformation (Kikkert et al., 1996; Kikkert et al., 2000; Harst et al., 2000; Iocco et al., 2001; Vidal et al., 2003), cryopreservation (Wang et al., 2002; Wang et al., 2004), and protoplast fusion (Matt et al., 2000).

The objective of this study was to demonstrate a highly repeatable protocol for somatic embryogenesis and plant regeneration from proembryonic mass suspension culture of 'Autumn Royal Seedless' (*V. vinifera* L.), a new California seedless grape. The protocol consists of two major steps: 1) multiplication of proembryonic masses

(PEMs) in liquid medium and 2) regeneration of PEMs in solid medium. The procedure reported here is simple and highly efficient. At the end, the study is also focused on characteristic differences of PEMs suspension cultures of 'Autumn Royal Seedless' with others *Vitis* spp.

3.3 Materials and methods

3.3.1 Somatic embryogenesis

The initial somatic embryo culture of 'Autumn Royal Seedless' (*Vitis vinifera* L.) was induced from immature ovule in the same lab (Xu et al., 2005), at the Center for Viticulture and Small Fruit Research, Florida Agricultural and Mechanical University. The somatic embryo was cultured on half-strength MS medium (Murashige and Skoog, 1962) with 30 g l^{-1} sucrose, 1 mg l^{-1} NOA (β-naphthoxyacetic acid), 2 mg l^{-1} BA (6-benzylaminopurine), 3 g l^{-1} phytagel, adjusted pH to 5.7. The culture was maintained in darkness and 26±2 ^{o}C.

3.3.2 Development of suspension culture medium

To obtain the liquid medium for 'Autumn Royal Seedless', its primary somatic embryos was cultured in a variety of liquid media which are created by formulating medium components including sugar (sucrose and maltose), plant hormone (2,4-D 1 mg l^{-1}, 2,4-D 1 mg l^{-1} plus BA 0.5 mg l^{-1}, and NOA 1 mg l^{-1}), glutamine, glycerol, and pH (5.7 and 5.8). The liquid media were prepared in 1 litter screw bottle. The media, Erlenmeyer flasks sealed with double-folded aluminum foil, and other equipments were sterilized by autoclaving at 121^{o}C and 1.1 kg pressure cm^{-2} for 20 min. Fifty and

100-ml media were then poured into 125-ml and 250-ml flasks, respectively. Approximately 500-mg of the primary somatic embryo of 'Autumn Royal Seedless' was initially incubated in 50 ml each medium. The contact area between the edge of aluminum foil and the neck of the flask was tightly sealed with 2-3 cm wide Parafilm. The flasks were then placed on a rotary shaker at 120 rpm, 26±2 ^{o}C and covered with a box for darkness. The experiment was repeated three times. After 3 weeks, the performance of liquid media was determined by visual scoring. The score was given upon ability of PEMs production which 0 was inability, 1 was poor, 2 was moderate, and 3 was high.

3.3.3 Establishment of proembryonic masses suspension culture

The newly developed MSGGN liquid medium consisted of MS medium, plus 20 g l^{-1} maltose, 500 mg l^{-1} glutamine, 5 ml l^{-1}glycerol, and 1 mg l^{-1} NOA, adjusted to pH 5.7. The MSGGN medium as well as liquid medium of Jayasankar et al. (1999) were used for the suspension culture. Medium preparation and culture protocol were described above.

3.3.4 Subculture

The cultures were subdivided at 3-week intervals to fresh MSGGN by using the procedure modified from Jayasankar et al. (1999). Briefly, flasks were transferred to lamina flow hood for 3-5 min in order to allow PEMs to precipitate down. Approximately 60% of the supernatant was gently decanted. The suspension culture remaining was swirled and filtered through a 1 mm stainless steel sieve. The filtered PEMs were briefly rinsed with fresh MSGGN medium in order to bring passable PEMs through sieve pore. The large cluster of proembryonic mass (LPEM, >1 mm

diameter) were retained on the sieve, whereas the flow-through liquid culture containing small cluster of proembryonic mass (SPEM, ≤1 mm diameter) was collected in a 150 ml beaker. The liquid culture was swirled and re-filtered through a single layer of Kimwipe EX-L that was relaxedly stretched in a 5 cm diameter plastic funnel. The mouth of the flasks was flamed before and after suspension culture or liquid medium transferring in order to prevent contamination. Approximately 200 and 400 mg of SPEMs was transferred by stainless steel spatula to 50 ml and 100 ml of fresh MSGGN flasks.

3.3.5 Factors affecting suspension cell in somatic embryogenesis

Three factors including, activated charcoal, darkness, and MS medium were tested. The media were consisted of full- or half-strength MS medium plus 30 g l^{-1} sucrose with or without 3 g l^{-1} activated charcoal (AC). After mixing of activated charcoal on a stirrer for 20 – 25 min, the media were adjusted to pH 5.8, solidified with 3 g l^{-1} phytagel, and then sterilized by autoclaving at 121^{o}C and 1.1 kg cm^{-2} for 20 min. The media were dispended into 20 ml in 100×15 mm Petri dish. SPEMs were spread out evenly on the media at approximately 200 mg per Petri dish. The cultures were kept in light at 16 h photoperiod or darkness. The somatic embryo appearance was examined under a stereomicroscope.

3.3.6 Somatic embryogenesis and plant regeneration

Approximately 50 mg of SPEMs was placed as 6 clumps in a 100×15 mm Petri dish filled with 20 ml FMSC solid medium, with a total of 10 Petri dishes. FMSC medium was consisted of full-strength MS plus 30 g l^{-1} sucrose and 3 g l^{-1} activated

charcoal. The medium was adjusted to pH 5.8 as described above and solidified with 3 g l^{-1} phytagel. The cultures were maintained at 26±2 ^{o}C in darkness for somatic embryogenesis. After 4 weeks, the cultures were subdivided by the following procedure; elongated somatic embryos (≥ 5 mm) were transferred to regeneration medium (see below) and 2 clumps were spreaded out evenly in a 100×25 mm Petri dishes containing 25 ml FMSC medium, and continued transferring to fresh medium every 4 weeks to promote somatic embryos development. The somatic embryos (≥ 5 mm) were harvested individually and transferred to Magenta GA7 boxes containing 80 ml FMSC medium for plant regeneration. The boxes were maintained in tissue culture shelves with 16 h photoperiod. There were 28 boxes and 9 somatic embryos per box. Somatic embryogenesis and plant regeneration were repeated twice.

3.3.7 Plantlet establishment

When the plantlets reached at least 7 cm high and developed at least 5 leaves, they were transferred to potting soil mixture in 3 inches plastic cup, each of which was covered with another plastic cup on top. The plants were watered daily with tap water. The top was gradually opened and completely removed after 4 weeks. The plants were maintained for additional 2 weeks before transferring to 5 inches plastic pots.

3.3.8 Comparative study with other *Vitis* spp.

3.3.8.1 Embryogenic materials

Primary somatic embryo of ‘Autumn Royal Seedless’ (*V. vinifera* L.) and ‘Crimson Seedless’ (*V. vinifera* L.), and embryogenic callus of ‘Tara’ (*V.*

rotundifolia) and 'Chardonnay' (*V. vinifera* L.) were tested. The primary somatic embryo and embryogenic calli were cultured on half strength MS macronutrient, (Murashige and Skoog, 1962), MS micronutrient, MS vitamins with 30 g l^{-1} sucrose, 1 mg l^{-1} NOA (*β*-naphthoxyacetic acid), 2 mg l^{-1} BA (6-benzylaminopurine), 3 g l^{-1} phytagel, adjusted pH to 5.7. The cultures were subdivided as 4-6 week intervals and maintained in darkness and 26±2 ^{o}C.

3.3.8.2 Establishment of suspension cultures and subculture

The primary somatic embryos or embryogenic callus of 'Autumn Royal Seedless', 'Crimson Seedless', 'Chardonnay', and 'Tara' were cultured in 125-ml Erlenmeyer flasks containing 50 ml of MSGGN medium. The newly developed formula containing half strength MS basal medium and B5 vitamin (Gamborg et al., 1968) were also applied for 'Crimson Seedless' and 'Tara'. The protocol for subculture was described as above.

3.3.8.3 Somatic embryogenesis, plant regeneration, and characteristic recording

The protocol of somatic embryogenesis and plant regeneration was described as above. During thirty-month-long consistently cultivation and regeneration, characteristics of the PEMs were recorded.

3.4 Results and discussion

3.4.1 Development of suspension culture medium

The result was shown in Table 3.1. Sucrose has been used as carbon source for grape suspension culture (Franks et al., 1998; Martinelli et al., 2001; Zlenko et al., 2002). However, it was found to be an unsuitable form of sugar for 'Autumn Royal Seedless' suspension culture. The somatic embryo inoculum rapidly turned brown and no PEM was produced. Glycerol demonstrated its importance of being a liquid medium component as most glycerol-containing media produced PEMs at moderate and high levels. This suggested that glycerol might facilitate cell growth as osmotic maintainer. Plant homone is another important component since it directs cell differentiation and development (Savidge, 1982). Among plant hormones tested, NOA gave the best production of PEM suspension culture, whereas the poorest was observed from BA. Besides 'Autumn Royal Seedless', 'Chardonnay' and 'Merlot' suspension culture have also been produced using glycerol and maltose liquid medium supplemented with NOA (GM-NOA) for transformation experiments (Kikkert et al., 2000; Vidal et al., 2003). These results indicated that glycerol, maltose, and NOA were essential components of liquid medium for high quality grape suspension culture.

Glutamine, an amino acid, significantly increased callus formation from anthers of 'Cabernet Sauvignon' (Mauro et al., 1986). In this study, when glutamine was incorporated into liquid media, high production of PEMs suspension cell was obtained. It was found that pH of liquid media affected cell color. Color of suspension cells became darker when they were cultured in liquid media at pH 5.8, whereas suspension cells cultured in liquid media at pH 5.7 showed blight-yellow color

Table 3.1 Composition of liquid medium tested.

Sugar (g l^{-1})		**glycerol**	**glutamine**	**Plant hormone** (mg l^{-1})			**pH**		**Medium**
maltose (20)	**sucrose** (30)	(5 ml l^{-1})	(500 mg l^{-1})	**2,4-D** (1)	**NOA** (1)	**BA** (0.5)	**5.7**	**5.8**	**determination**[a]
X	-	-	-	X	-	-	X	-	1
X	-	-	-	X	-	-	-	X	0
X	-	-	-	X	-	X	X	-	0
X	-	-	-	-	X	-	X	-	1
X	-	-	-	-	X	-	-	X	1
-	X	-	-	X	-	-	X	-	0
-	X	-	-	X	-	-	-	X	0
-	X	-	-	X	-	X	X	-	0
-	X	-	-	-	X	-	X	-	1
-	X	-	-	-	X	-	-	X	0

Table 3.1 (continued).

Sugar (g l^{-1})		**glycerol**	**glutamine**	**Plant hormone** (mg l^{-1})			**pH**		**Medium**
maltose (20)	**sucrose** (30)	(5 ml l^{-1})	(500 mg l^{-1})	**2,4-D** (1)	**NOA** (1)	**BA** (0.5)	**5.7**	**5.8**	**determination**[a]
X	-	X	-	X	-	-	X	-	2
X	-	X	-	X	-	X	X	-	1
X	-	X	-	-	X	-	X	-	2
X	-	X	-	-	X	-	-	X	2
-	X	X	-	-	X	-	X	-	2
-	X	X	-	-	X	-		X	1
X	-	X	X	-	X	-	X	-	3
X	-	X	X	-	X	-	-	X	2

X = medium component was chosen in each formula

[a] = sore was given upon ability of media in suspension cell production; 0: inability, 1: poor, 2: moderate, and 3: high.

to yellowish. The most suitable liquid medium composed of maltose, glycerol, glutamine, NOA (pH 5.7) was named MSGGN medium. For comparison purpose, the liquid medium of Jayasankar et al. (1999) was tested for 'Autumn Royal Seedless'. It was found that somatic embryo inoculum turned yellow-brown and suspension cell grew much slower than culturing in MSGGM medium.

3.4.2 Establishment of proembryonic mass

White PEMs appeared in the suspension 2 weeks after the somatic embryos were incubated in MSGGN medium. The PEMs grew rapidly and suspension culture was established in two subcultures. At this moment, the PEMs were yellowish and capable of rapid multiplication. The results demonstrated that the MSGGN medium with the culture techniques provided were highly efficient for producing proembryoic mass of 'Autumn Royal Seedless' (Figure 3.1). This result also confirmed the reports that not only embryogenic callus (Kikkert et al., 2000; Wang et al., 2002; Vidal et al., 2003; Wang et al., 2004) but also somatic embryos (Jayasankar et al., 1999; Bornhoff and Harst, 2000) were suitable materials for producing grape cell suspensions. Sieving was very helpful to synchronize suspension culture by separating of LPEMs (Figure 3.2A) and SPEMs (Figure 3.2B) during subculture.

To establish a suspension culture technology, cell browning in liquid media (Jayasankar et al., 1999) has been a major obstacle for grape (Bornhoff and Harst, 2000) and other crops producing a lot of polyphenolic compound such as oil palm (Teixeira et al., 1995) and mango (Litz et al., 1995). In this study, browning of neither PEMs nor liquid medium was observed during the culture initial and subsequent cultures in MSGGN medium. The browning reaction is a result of the enzymatic

action of polyphenol oxidase (PPO) by oxidizing phenolic compound to quinines which eventually become melanin pigments by polymerization (Macheix et al., 1990 quoted in Yoruk and Marshall, 2003). Therefore, adding antioxidants, chemicals inhibit oxidation activity of PPO, in liquid medium can be the solution to prevent or reduce cell browing. A combination of polyvinylpolypyrrolidone (PVPP) and dithiothreitol (DTT) were found to dramatically inhibit tissue browning and necrosis during cocultivation of *Agrobacterium*-mediated transformation in 'Superior Seedless' (Perl et al., 1996).

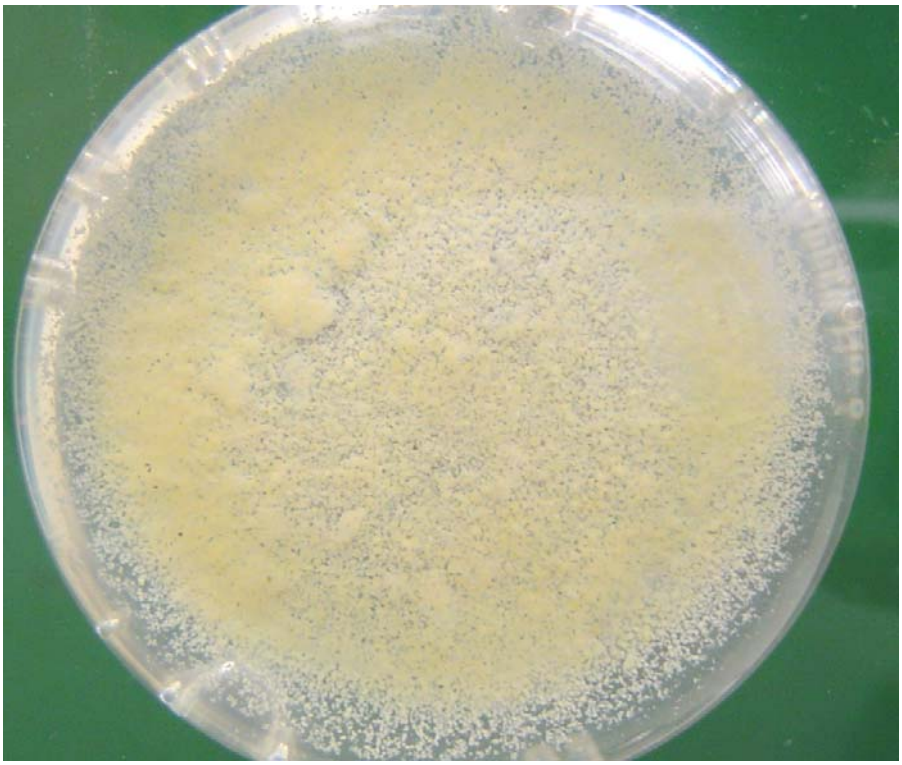

Figure 3.1 Proembryonic mass suspension culture in MSGGN medium at 3-week culture period in 125-ml flask.

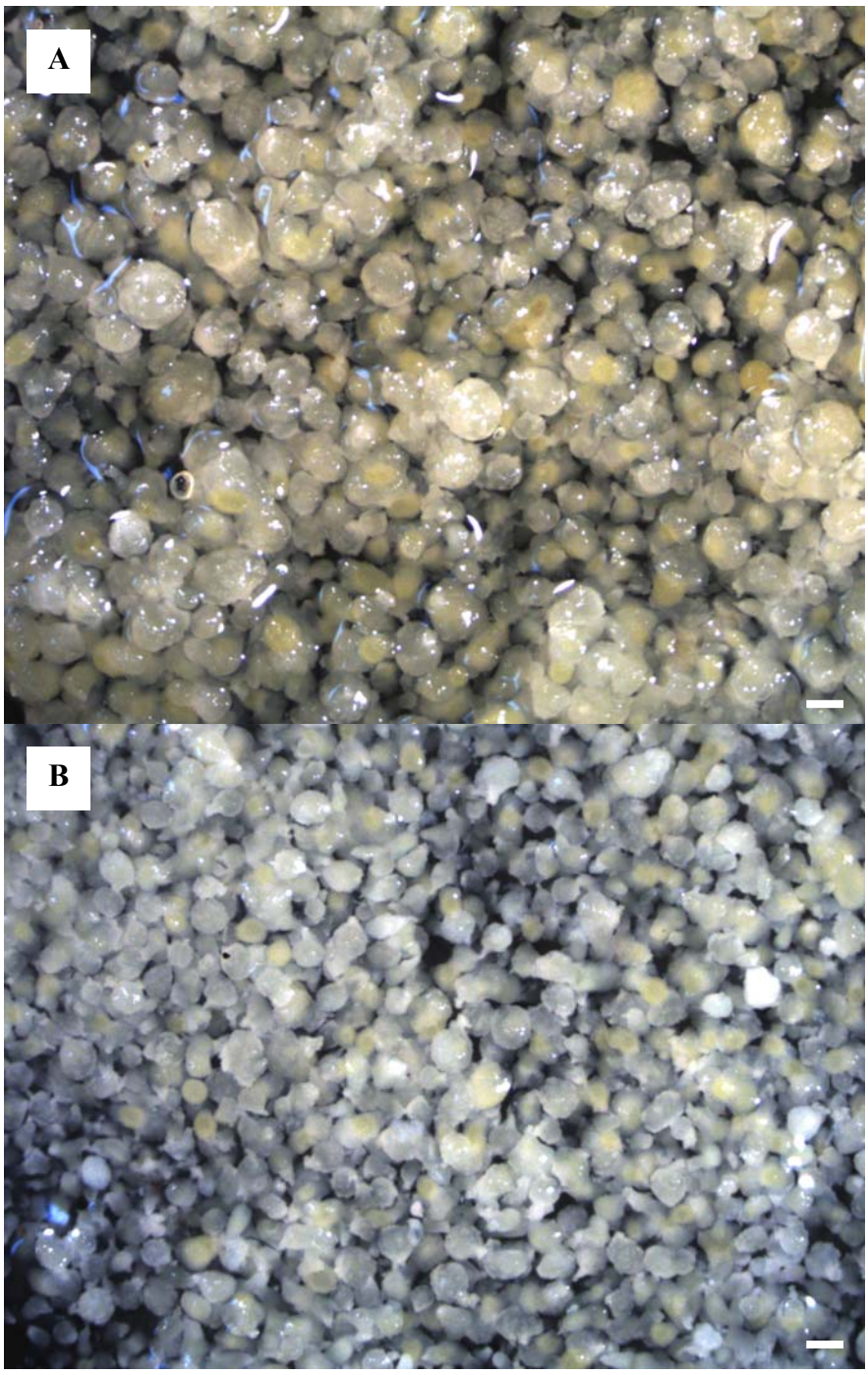

Figure 3.2 Large cluster of proembryonic masses (A) and small cluster of proembryonic masses (B) which were separated by sieving. Bars = 1 mm.

3.4.3 Somatic embryogenesis

Three factors, light, strength of MS medium and activated charcoal, were tested and all of them played important roles for grape somatic embryogenesis. Light was the most influenced factor among the three. After 4 weeks on FMSC, SPEMs mostly grew friable callus and somatic embryos were rarely observed under light condition, whereas numerous somatic embryos were fully developed under darkness (Figure 3.3A and B). This strongly indicated that light had a negative regulation to genes that involved in auxin production, which has been found to play a major role in embryogenesis (Souter and Lindsey, 2000). Strength of MS medium and AC also affected grape somatic embyogenesis.

In every combination tested, full-strength MS media were all superior to half-strength. The strength of MS medium to somatic embryogenesis could be observed under light (Figure 3.3A and C) and highly emphasized in darkness (Figure 3.3B and D). Activated charcoal had a positive effect for promoting somatic embryogenesis during embryo development (Figure 3.3E and F). This has been reported in different grape cultivars (Motoike et al., 2001). Extracellular proteins secreted by somatic embryos during embryogenesis, which inhibited embryo development, have been reported (Coutos-Thevenot et al., 1992). It was possible that the proteins could be inactivated by being absorbed to activated charcoal.

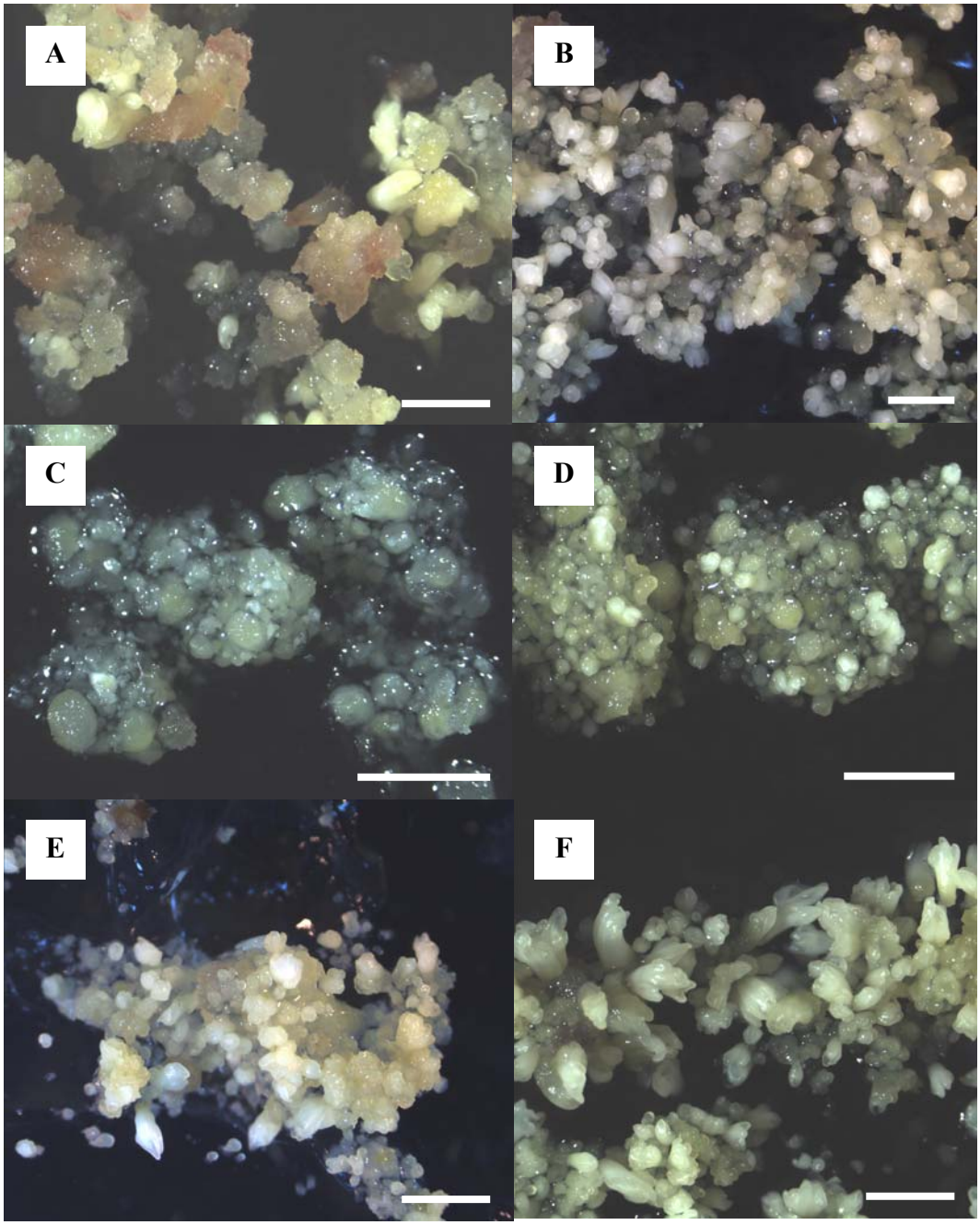

Figure 3.3 Development of somatic embryos 4 weeks on FMSC medium with light (A) and without light (B) and on HMSC medium with light (C) and without light (D). Somatic embryo development in dark after 3 weeks in FMS medium without AC (E) and with AC (F). Bars = 5 mm.

Two weeks after transferring SPEMs to the FMSC medium, somatic embryos appeared at various stages including globular, torpedo, and early cotyledonous (Figure 3.4A). The somatic embryos developed asynchronously, which have been reported in other grape cultivars (Matsuta and Hirabayashi, 1989; Jayasankar et al., 1999; 2003) as well as other perennial plant species (Litz and Gray, 1992). For example, some somatic embryos precociously elongated into 1-2 cm within 4 weeks before first subculture while the majority was about 0.5-2 mm at length (Figure 3.4B and C). More than 1,000 somatic embryos were obtained from approximately 50 mg of SPEMs within 14-16 weeks. LPEMs generated far less and highly asynchronous somatic embryos within the same period of time.

3.4.4 Plant regeneration

Plant regeneration from somatic embryos composed of two continuous processes: (1) somatic embryo germination and (2) conversion. Somatic embryo germination has been characterized by cotyledon expansion, chlorophyll formation and following by radicle development (Merkle and Wiecko, 1990). Shoot meristem development and subsequent vegetative leaf initiation has been termed as conversion (Nickle and Yeung, 1994). Based on these definitions, all of the somatic embryos of 'Autumn Royal Seedless' germinated in about 2 weeks after transferring to FMSC medium in Magenta boxes (Figure 3.5A).

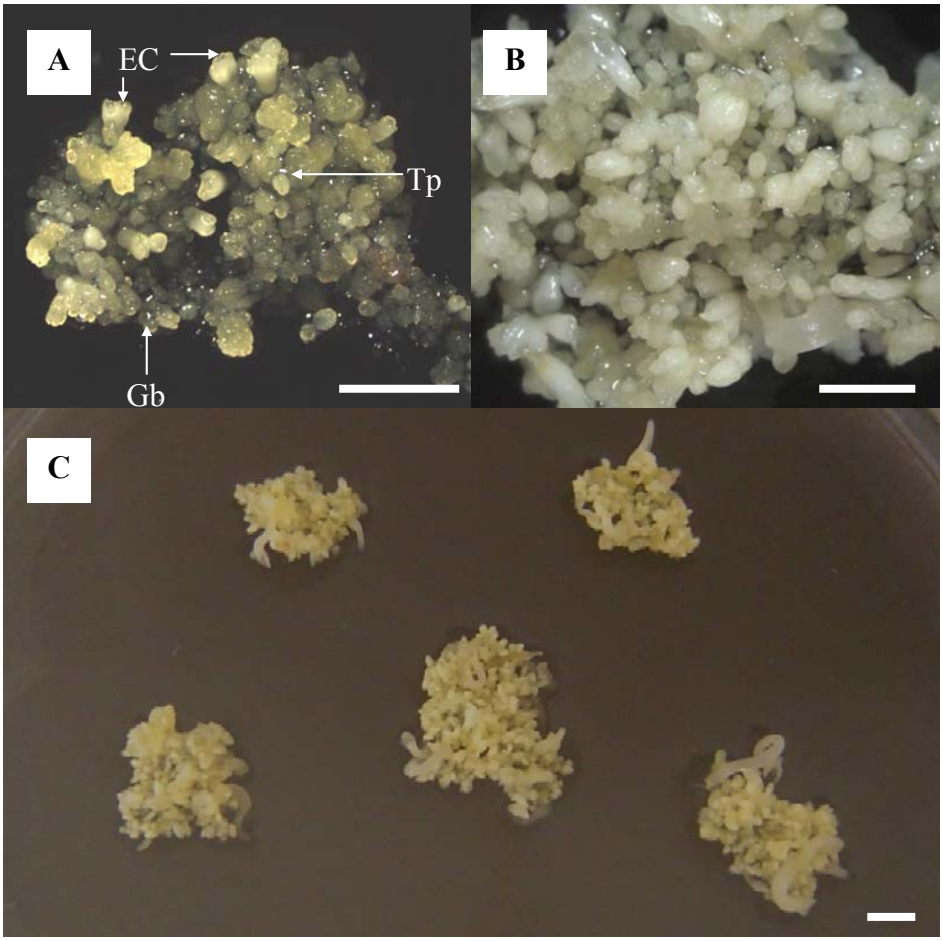

Figure 3.4 Somatic embryos appeared at various stages including globular (Gb), torpedo (Tb), and early cotyledonous (EC) on FMSC medium after 2 (A) and 4 weeks (B and C). Bars = 5 mm.

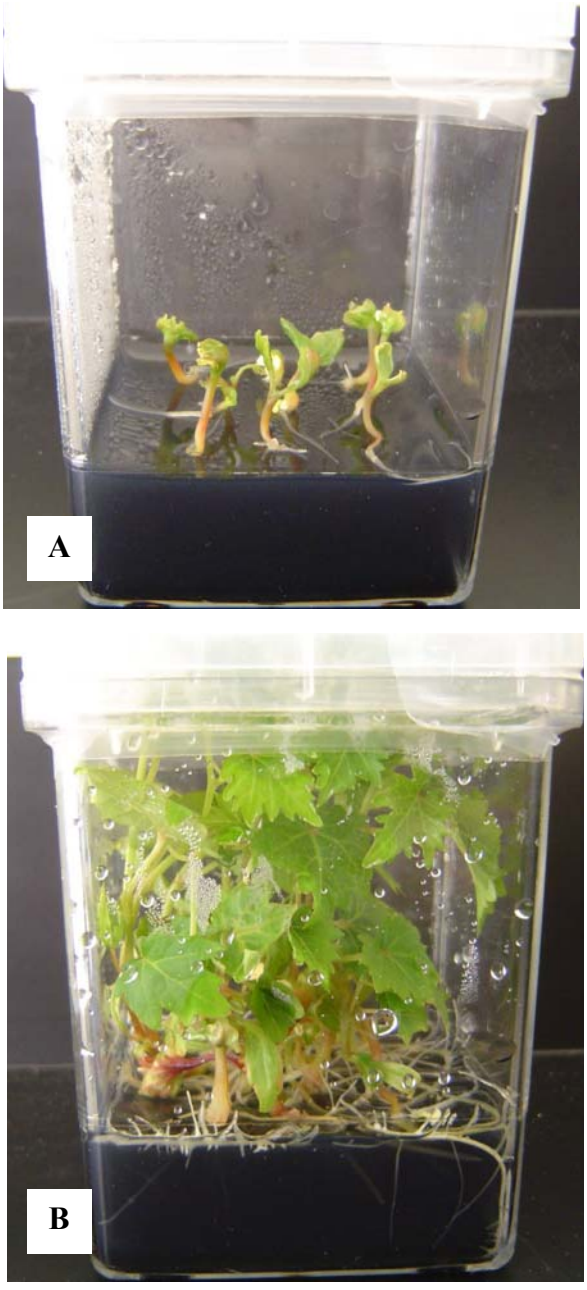

Figure 3.5 Germinated somatic embryos on FMSC medium in Magenta box 2 weeks after being transferred (A) and converted to normal plantlets (B).

The germinated somatic embryos were developed into regenerated plants with roots, shoots, and leaves in another week. Somatic embryo germination and conversion rate varied depending upon its quality (Motoike et al., 2001). In this study, cotyledon was the most important factor for somatic embryo conversion. Somatic embryos with perfect cotyledon (unfolded, expanded and green) were converted much faster into plantlets than those with poor cotyledon (folded, unexpanded, and yellowish with green at the tip). Some somatic embryos with poor cotyledon did not start vegetative growth until 8 weeks after culture.

Somatic embryogenesis was traditionally conducted on semisolid medium. Although, Jayasankar et al. (1999) successfully produced somatic embryos in liquid medium, the percentage of somatic embryos developed into regenerated plants were lower than somatic embryos producing from semisolid medium. The germination rate of somatic embryos derived from semisolid medium typically ranged from 5-30% (Matsuta and Hirabayashi, 1989; Perl et al., 1995; Salunkhe et al., 1999; Motoike et al., 2001). On the other hand, more than 60% of suspension cell-derived somatic embryos grew into plant (Jayasankar et al., 1999). Salunkhe et al. (1999) reported that each gram of embryogenic callus yielded more than 400 somatic embryos and 86.3% of which were not converted to plantlets.

In this study, approximately 50 mg of SPEMs generated more than 1,000 somatic embryos and more than 95% of the germinated somatic embryos converted into normal plants (Figure 3.5B), which are highly efficient. Dormancy of somatic embryos was not observed. The regenerated plants were successfully grown in pots within 6 weeks.

Results of this study strongly indicated that 'Autumn Royal Seedless' proembryonic mass suspension culture could provide high quality and quantity of somatic embryos for plant regeneration. In our laboratory, the suspension cultures have been maintained and subcultured for over two years but they still retained highly multiplicable and regeneratiable capability. This protocol offers a simple yet highly efficient suspension culture technique for producing synchronized somatic embryo cell materials for grape biotechnological researches such as genetic transformation, protoplast fusion, and *in vitro* selection.

3.4.5 Comparative study of PEM

3.4.5.1 Genotype specificity

Genotype specificity to MSGGN and newly developed liquid medium was demonstrated among 'Autumn Royal Seedless', 'Crimson Seedless', 'Chardonnay', and 'Tara'. This similar result was previously reported (Jayasankar et al., 1999; Bornhoff and Harst, 2000). 'Autumn Royal Seedless' was always the first cultivar established in MSGGN medium, whereas 'Crimson Seedless' had never been succeeded in both media. Although 'Tara' PEM suspension culture could be established in new medium, it had the lowest growth among grape cultivars in our suspension cell record. Comparing media composition between MSGGN and new medium (shown in Appendix), thiamine hydrochloride (thiamine·HCl) in the new medium was 100 times higher than it appeared in MSGGN medium and glycine was not a component of the new medium. This suggested that thiamine·HCl and glycine were necessary for 'Tara' (Table 3.2). As a *Mascadinia* grape (*V. rotundifolia*), 'Tara' might need different nutrients and hormomes from *Euvitis* grape to generate its

Table 3.2 Characteristics of proembryonic mass suspension culture.

Characteristic of PEM	Genotype		
	Autumn Royal (*vinifera*)	**Chardonnay** (*vinifera*)	**Tara** (*rotundifolia*)
Establishment of suspension culture	4 weeks	8 weeks	8 weeks
Size	0.2-3 mm	0.2 mm	0.2 mm
Color	Yellow	White-Creamy	Opaque-Gray
Uniformity of suspension culture	Low	High	Moderate
Growth	Autumn Royal Seedless > Chardonnay >> Tara		
Culture without subdivision[1]	6 weeks	10 weeks	5 weeks
Browning of suspension culture[2]	No	No	Yes
Somatic embryogenesis[3]	3 weeks	8-10 weeks	10-12 weeks
Plant regeneration[4]	2 weeks	2 weeks	4 weeks
Medium compositions	**MSGGN**	**MSGGN**	**New**
- MS basal medium	Full strength	Full strength	Half strength
- Vitamin	MS	MS	B5

[1] Maximum time before browning was observed; [2] Observation in regular subculture;

[3] Somatic embryos appeared; [4] Chlorophyll and shoot development were observed

suspension cells

3.4.5.2 Color and size

Color and size of 'Autumn Royal Seedless', 'Chardonnay', and 'Tara' PEM were relatively different. The color might not be directly involved with potential usage in biotechnological studies, but it showed cell vitality and was a significant indicator of cell browning. Size of PEM was a parameter affecting the efficiency of nutrient or chemical absorption in which finely divided PEM was preferable. Since small cluster of PEM contained fewer number of internal proembryonic cells than the large one, it offered more control of exposing between the cells and chemical treatment or environmental parameters. This was a key for *in vitro* selection (Gu et al., 2004; Jayasankar et al., 2000), helped reduce chimera in transformation experiment (Vidal et al., 2003), and also provided high yields for protoplast isolation.

3.4.5.3 Uniformity and growth

Uniformity of suspension cell is important (Gu et al., 2004) since it was employed as an experimental unit. PEM suspension culture of 'Chardonnay' was highly synchronous. The culture could be subdivided without sieving for over 3 months. On the other hand, it was necessary in every subculture for 'Autumn Royal Seedless' to removed expanding PEMs from suspension culture in order to keep the suspension culture synchronously, and stimulate multiplication.

3.4.5.4 Browning

Spontaneous differentiation of PEMs and cell browning were substantial obstacles (Bornhoff and Harst, 2000; Jayasankar et al., 1999; Litz et al., 1995; Teixeira et al., 1995). It has been reported that high cell density and phenolic compound generated the problem. The browning was easily observed in 'Tara' suspension culture if without subculture. Muscadine grape has been known as high phenolic producing species, therefore, browning of 'Tara' was expected. 'Chardonnay' and 'Autumn Royal Seedless' suspension culture could be maintained up to 10 and 6 weeks, respectively, without subculture.

3.4.5.5 Somatic embryogenesis and plant regeneration

Somatic embryogenesis and plant regeneratability are highly important since they directly determine achievement of plant biotechnological breeding (Martinelli and Gribaudo, 2001). It took only three weeks for perfect cotyledon stage of somatic embryo of 'Autumn Royal Seedless' to be observed. In the following two weeks under light condition, the somatic embryo simply developed into a regenerated plant. PEMs of 'Chardonnay' and 'Tara' could also be regenerated but at lower frequency. Differentiation of PEMs of 'Autumn Royal Seedless' spontaneously occurred in liquid and solid culture, which provided high efficiency somatic embryogenesis and plant regeneration.

'Autumn Royal Seedless' was the highest performance cultivars for proembryonic mass suspension culture, which provided the highest yield and plant regeneration frequency. However, 'Chardonnay' suspension culture was more preferable because it was highly synchronous. The culture has been used for in *vitro*

selections and transformation. More suitable condition was needed for ‘Tara’ to extablished its suspension cell cuture.

3.5 Conclusion

Proembryonic mass (PEM) suspension culture of ‘Autumn Royal Seedless’ was successfully established from primary somatic embryos in MSGGN medium. The results strongly indicated that activated charcoal had a positive effect in facilitating somatic embryogenesis and development, whereas light had an extreme negative effect on somatic embryogenesis of PEMs. Full strength was superior to half strength MS medium in both somatic embryogenesis and plant regeneration. Three grape cultivars performed differently in MSGGN liquid medium. ‘Autumn Royal Seedless’ showed the highest performance among three cultivars in growth and regeneration. An approximately 50 mg of its SPEM generated more than 1,000 somatic embryos and more than 95% of the germinated somatic embryos converted into normal plants. Uniformity of cell suspension was observed from ‘Chardonnay’ and ‘Tara’ showed no preferable character for being a suspension culture material at this moment.

3.6 References

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CHAPTER IV

GENETIC TRANSFORMATION OF A TABLE GRAPE 'AUTUMN ROYAL SEEDLESS' (*Vitis vinifera* L.) USING ENHANCED GREEN FLUORESCENT PROTEIN (EGFP) GENE

4.1 Abstract

Agrobaterium-mediated transformation was developed for 'Autumn Royal Seedless' (*Vitis vinifera* L.) with SPEM and primary somatic embryo as the target tissue materials. The transformation system was obtimized using EGFP gene encoding enhanced green fluorescent protein as a reporter element. No transformed cell was obtained when SPEM was used as target tissue. Two months after transformation, some embryogenic calli and globular somatic embryos showed green fluorescent light on selective medium. The somatic embryos did not grow beyond cotyledonous stage within a year of plant regeneration attempt. Partially transformed globular somatic embryos were also observed. EGFP was proven to be very helpful to monitor transformed cells. Using this protocol with 'Autumn Royal Seedless', putative transgenic somatic embryos were produced at low transformation frequency. Although the experiment failed to produce transgenic plant, several factors affecting transformation efficiency have been studied.

4.2 Introduction

Grapes (*Vitis vinifera* L.) are one of the major commercially cultivated fruit crops worldwide. Gene delivery has been employed for genetic improvement of several characteristics such as disease resistance and stress tolerance (Colova-Tsolova et al., 2001). *Agrobacterium*-mediated transformation is the most common method for grape species (Kikkert et al., 2001). Although a number of grape transformation attempts have been successfully reported, numerous genotypes have not been tested especially newly released cultivars. Furthermore, transformation efficiency generally appears to be genotype specific (Iocco et al., 2001; Torregrosa et al., 2002).

Green fluorescent protein (GFP) has been widely used as a reporter protein to develop and optimize transformation protocol in plant species. Unlike β-glucuronidase (GUS) that requires exogenous substrates and destructive assay (Jefferson, 1987), the GFP is self-catalyzed forming a fluorescent chromophore and requires only excitation under ultraviolet or blue light to emit a bright green fluorescence (Chalfie et al., 1994). The most advantages of GFP are non-toxic and real-time assay. We here report *Agrobacterium*-mediated transformation of enhanced *Aequorea victoria* green fluorescent protein (EGFP) gene to 'Autumn Royal Seedless', a new California grape cultivar.

4.3 Materials and methods

4.3.1 Plant materials

Primary somatic embryo culture of 'Autumn Royal Seedless' (*Vitis vinifera* L.) (Figure 4.1) was provided by Prof. Dr. Jiang Lu at the Center for Viticulture and Small Fruit Research, Florida Agricultural and Mechanical University. The somatic embryo was cultured on half-strength MS (Murashige and Skoog, 1962) macronutrient, full-strength MS micronutrient and vitamin with 30 g l^{-1} sucrose, 1 mg l^{-1} NOA (β-naphthoxyacetic acid), 2 mg l^{-1} BA (6-benzylaminopurine), 3 g l^{-1} phytagel, adjusted pH to 5.7. The culture was maintained in darkness at 26±2 ^{o}C.

Proembryonic mass suspension culture (Figure 4.1) was produced and subcultured in MSGGM medium as described by Jittayasothorn et al. (2007) (shown in Appendix). Small cluster of proembryonic mass (SPEM, <1 mm diameter) were separated from the medium by filtration through a 1 mm stainless steel sieve, collected on a Kimwipe paper, and cultured on somatic embryo solid medium. Primary somatic embryos, SPEM, and SPEM cultured on solid medium for 6 months (SPEM6) were used as tissue targets.

4.3.2 Bacterial strain and vector construction

The EGFP gene was amplified from plasmid pEGFP (shown in Appendix) and subsequently cloned into a binary vector pBI-d35S-D4E1-nos (shown in Appendix) by replacing D4E1 gene. The vector carried neomycin phosphotransferase (*npt*II) gene driven by nos promoter and a transgene driven by double CaMV 35S promoter in T-DNA region (Figure 4.2). The Newly engineered binary vector was multiplied in

Esherichia coli strain DH5α (Gibco-BRL, Bethesda, MD, USA) and verified by DNA sequencing. The binary vector was introduced into *Agrobacterium tumefaciens* strain EHA105 (Gibco-BRL) by freez-thaw method (Burrow et al., 1990).

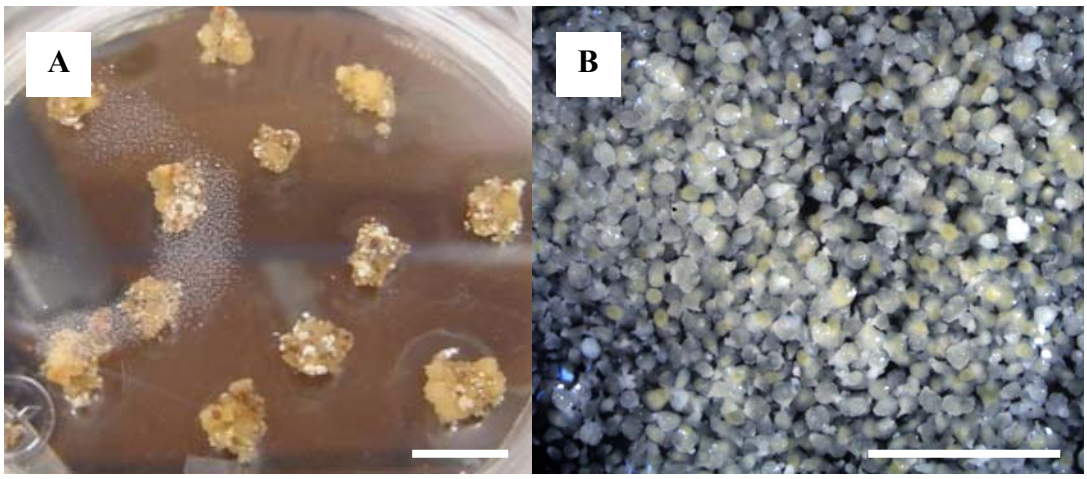

Figure 4.1 Primary somatic embryos (A) and small cluster of proembryonic masses (B) of 'Autumn Royal Seedless'. Bars = 1 cm.

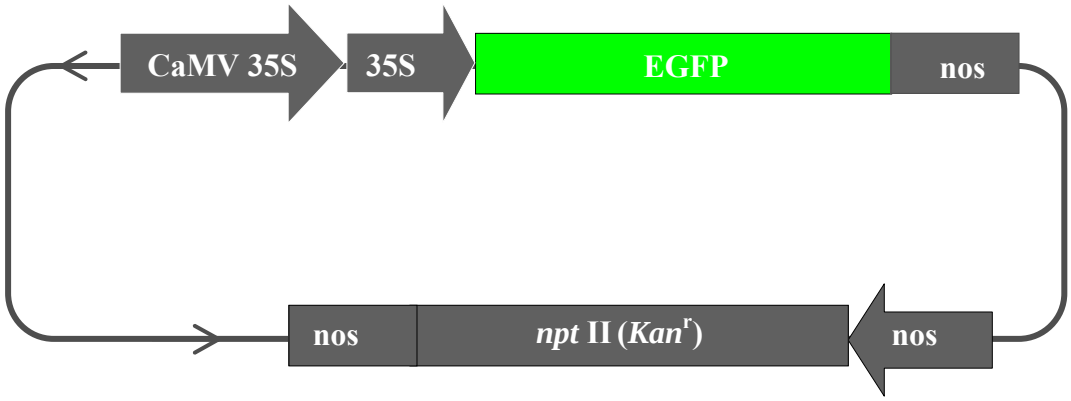

Figure 4.2 Schematic map of the pBI-d35S-EGFP-nos binary vector.

4.3.3 Transformation

4.3.3.1 Optimization of transformation factors

Genotype specificity has been known as one of many obstacles for grape transformation. To obtain *Agrobacterium*-mediated transformation protocol for 'Autumn Royal Seedless', each transformation factor including type of target tissue, composition of *vir* gene induction medium, time of induction, acetosyringone, and cocultivation temperature was combined to create a set of transformation conditons (Table 4.1).

4.3.3.2 Standard protocol of transformation

Agrobacterium was cultured overnight at 28°C and 200 rpm in 30 ml of LB medium (Sambrook et al., 1989) containing 50 μg ml^{-1} ampicillin and 100 μg ml^{-1} kanamycin for selecting disarmed and binary vector, respectively. Concentrated bacterial culture was diluted to 0.6 at OD_{600} in 40 ml and centrifuged at 4,000 rpm for 10 min at 4°C to recover bacterial cells. Cell pellet was resuspended in 30 ml in *vir* genes induction medium (adjusted to pH 5.6) supplemented with acetosyringone at 0 or 100 or 1,000 μM. The induction was performed for 2 or 6 hr at 125 rpm in darkness at 25°C and used as inoculum.

Primary somatic embryos and SPEMs were dried out on sterile Whatman filter papers for 15 and 30 min, respectively, and then submerged in bacterial inoculum in sterile 80 ml beaker for 20 min at 25°C with periodically forceps-stirring to break off somatic embryo clumps. The infected plant materials were collected by Kimwipe filtration and blotted dry on sterile filter papers to remove excess bacterial inoculum and cultured in darkness at 25 or 28°C on somatic embryo culture medium but without

plant hormones. After 2 days, cocultivated plant materials were washed twice with sterile water containing 100 μg ml^{-1} kanamycin and 500 mg l^{-1} cefotaxime. The plant materials were blotted dry on sterile filter papers, and cultured on selection media (below).

4.3.4 Antibiotic selection

Cocultivated tissues were cultured on selective medium supplemented with 500 mg l^{-1} cefotaxime. The tissues were entirely transferred to fresh medium every 2 weeks or longer depending on bacterial growth. After 4 weeks, 100 μg ml^{-1} kanamycin was added to the medium and cefotaxime was reduced to 250 mg l^{-1}. The medium was refreshed every 4 weeks. After 12 weeks, plant tissues were transferred to FMSC regeneration medium (Jittayasothorn et al., 2007).

4.3.5 Microscopic GFP detection

EGFP expression in putative transformed somatic embryos was visualized using a LEICA *MZFLIII* stereo fluorescent microscope and Leica IM50 software. The stereomicroscope is equipped with a Leica GFP Plus filter (480/40 nm excitation filter).

4.4 Results and discussion

4.4.1 Optimization of transformation factors

All the transformation conditions were attempted on every target tissue. Unfortunately, several conditions were impossible for primary somatic embryo and

SPEM6 because of their limited availability of tissue material (Table 4.1). SPEM collected freshly from liquid medium could be tested in every condition; however, no transformed cell was obtained. Franks et al. (1998) reported similar results in *Agrobacterium*-mediated transformation of seedless grape 'Sultana' (Thompson Seedless, Sultana). Three types of target tissues including embryogenic callus which its surface was covered with globular somatic embryos, proliferating somatic embryos at the development of heart to torpedo stage, and embryogenic suspension cells were cocultivated with *Agrobacterium* strains EHA101 and EHA105. Three and more than 50 transgenic plants were regenerated from proliferating somatic embryos and embryogenic callus respectively. No transgenic plant was obtained from suspension cells. Similarly, *Agrobacterium*-mediated transformation failed if PEM suspension cells of 'Chardonnay' were used as target tissue (S. Jayasankar, personal communication, Febuary, 2005). Hypothetically, cell wall of the suspension cells and SPEM cells in this study might become thicker after being cultured in liquid medium, which could possible make them unsusceptible to *Agrobacterium*.

To prove the cell wall hyphothesis, SPEM were removed from MSGGN medium and cultured on solid medium for 6 months (SPEM6). The SPEM was expected to generate new cells that exhibited thin or similar thickness of cell wall to general solid embryogenic cultures. Twenty transformation conditions were applied to SPEM6 but no transgenic cell was produced. Harst et al. (2000) successfully produced transgenic plants from suspension culture of grapevine 'Dornfelder' and 'Riesling' somatic embryos. The results suggested not only cell wall but also developmental stage of suspension cell that affected gene transfer mechanisim of *Agrobacterium*.

Primary somatic embryo was the only target tissue that gave successful

Table 4.1 *Agrobacterium*-mediated transformation conditions for 'Autumn Royal Seedless'.

***vir* gene induction medium** (g l^{-1} for sugar)					**Induction time (hr)**	**Aceto. (μM)**	**Cocultivation temp. (^{o}C)**	**GFP detection**		
Medium	**$NaPO_4$** (2 mM)	**glucose**	**galactose**	**xylose**				**PSE**	**SPEM**	**SPEM6**
1/10 LB	Yes	5	-	-	2	0	25	-	-	-
1/10 LB	Yes	5	-	-	2	0	28	-	-	-
1/10 LB	Yes	5	-	-	2	100	25	√	-	-
1/10 LB	Yes	5	-	-	2	100	28	-	-	-
1/10 LB	Yes	5	-	-	2	1,000	25	NA	-	NA
1/10 LB	Yes	5	-	-	2	1,000	28	NA	-	NA
1/10 LB	Yes	5	-	-	6	0	25	NA	-	NA
1/10 LB	Yes	5	-	-	6	100	25	NA	-	NA
1/10 LB	Yes	4	4	4	2	0	25	NA	-	NA
1/10 LB	Yes	4	4	4	2	0	28	NA	-	NA

Table 4.1 (continued).

***vir* gene induction medium** (g l^{-1} for sugar)					**Induction time (hr)**	**Aceto. (μM)**	**Cocultivation temp. (^{o}C)**	**GFP detection**		
Medium	**$NaPO_4$** (2 mM)	**glucose**	**galactose**	**xylose**				**PSE**	**SPEM**	**SPEM6**
1/10 LB	Yes	4	4	4	2	100	25	-	-	-
1/10 LB	Yes	4	4	4	2	100	28	-	-	-
1/10 LB	Yes	4	4	4	6	100	25	NA	-	-
1/10 LB	Yes	4	4	4	6	100	28	NA	-	-
1 LB	No	5	-	-	-	0	25	NA	-	-
1 LB	No	5	-	-	-	100	25	NA	-	-
1 LB	No	5	-	-	-	100	28	-	-	-
1 LB	No	5	-	-	-	1,000	25	-	-	-
1 LB	Yes	5	-	-	2	0	25	-	-	-
1 LB	Yes	5	-	-	2	0	28	-	-	-

Table 4.1 (continued).

***vir* gene induction medium** (g l^{-1} for sugar)					**Induction time (hr)**	**Aceto. (µM)**	**Cocultivation temp. (^{o}C)**	**GFP detection**		
Medium	**$NaPO_4$** (2 mM)	**glucose**	**galactose**	**xylose**				**PSE**	**SPEM**	**SPEM6**
1 LB	Yes	5	-	-	2	100	25	-	-	-
1 LB	Yes	5	-	-	2	100	28	-	-	-
1 LB	Yes	5	-	-	2	1,000	25	-	-	-
1 LB	Yes	5	-	-	2	1,000	28	-	-	-
1 LB	Yes	4	4	4	2	100	25	NA	-	NA
1 LB	Yes	4	4	4	2	100	28	NA	-	NA
Iocco	Yes	5	-	-	2	100	25	-	-	-
Iocco	Yes	5	-	-	2	100	28	-	-	-

√ = GEF sinal was detected: NA = the treatment was not performed: PSE = primary somatic embryo:

Iocco = *vir* genes induction medium from Iocco et al. (2001)

transformation. However, the success was merely obtained by 1 transformation conditions from the total of 16 conditions tested (Table 4.1). The transformation protocol started with activation of *vir* genes for 2 hr in induction medium containing 1/10 LB, 2 mM sodium phosphate ($NaPO_4$), 5 g l^{-1} glucose, and 100 µM acetosyringone. The somatic embryos were then submerged in bacterial inoculum and cocultivated at 25°C for 2 days. Mark Thomas and his group in 1998 (CSIRO Plant Industry Horticulture, Australia) simply used LB supplemented with 10 g l^{-1} glucose to produce transgenic *vinifera* cv. Sultana (Franks et al., 1998). Later in their next publications (Table 2.2), transgenic plants were obtained from 7 (Iocco et al., 2001) and 4 (Torregrosa et al., 2002) important grapevines using new transformation protocol. In the new protocol, AB salt (Chilton et al., 1974) supplemented with 2 mM sodium phosphate, 40 mM MES, and 100 µM acetosyringone was used as *vir* gene induction medium. *Agrobacterium* strain EHA101 and EHA105 were cultured in the induction medium prior cocultivation with embryogenic callus. However, no transformed cell was produced when their induction medium was applied to 'Autumn Royal Seedless' target tissues. Genotype specificity might be responsible for a part of transformation success.

Composition of induction media was a major difference between transformation protocol of Thomas's group and the transformation protocol developed for 'Autumn Royal Seedless'. Instead of AB salt, the developed protocol composed of 1/10 LB medium. This suggested that diluted LB medium provided suitable form and concentration of nutrients for *Agrobacterium* to transfer its recombinant T-DNA to 'Autumn Royal Seedless' cell. When the successfully developed protocol was compared to other 5 failed-1/10 LB-based conditions for primary somatic embryo, it

suggested that sodium phosphate, acetosyringone and cocultivation at 25°C were the necessary components to obtain transformed cells (Table 4.1).

4.4.2 Production of transgenic somatic embryos

Agrobacterium-mediated transformation method had been developed for 'Autumn Royal Seedless'. Necrotic cells were detected in all target tissues within 2-3 days after cocultivation, but transformed tissue was only observed from primary somatic embryos materials. Perl et al. (1996) suggested that the necrogenesis was due to a hypersensitive response, which was a result of oxidation caused by elevated level of peroxidase activity, of grape cells to *Agrobacterium*. The hypersensitive response causing grape necrotic tissue in grape was generally observed in *Agrobacterium*-mediated transformation (Kikkert et al., 2001).

GFP was highly helpful to monitor transformed cells (Figure 4.3). Two months after transformation, some embryogenic calli showed fluorescent signal on selective medium (Figure 4.4). Green fluorescent globular somatic embryos could also be observed at this moment (Figure 4.5). Some of the somatic embryos were partially transformed (Figure 4.6). Partially transformed somatic embryos of grapevines (Nakano et al., 1994; Iocco et al., 2001; Torregrosa et al., 2002) and chimeric transformed shoots of *V. vinifera* L. (Baribault et al., 1990; Berres et al., 1992) were also observed from *Agrobacterium*-mediated transformation. The fluorescent somatic embryos as well as untransformed coculitvated somatic embryos did not grow beyond early cotyledonous stage within a year of plant regeneration attempt (Figure 4.7). Interestingly, in typical routine of plant regneration, somatic embryos of 'Autumn Royal Seedless' rapidly developed to plantlet within 2 month after transferring to

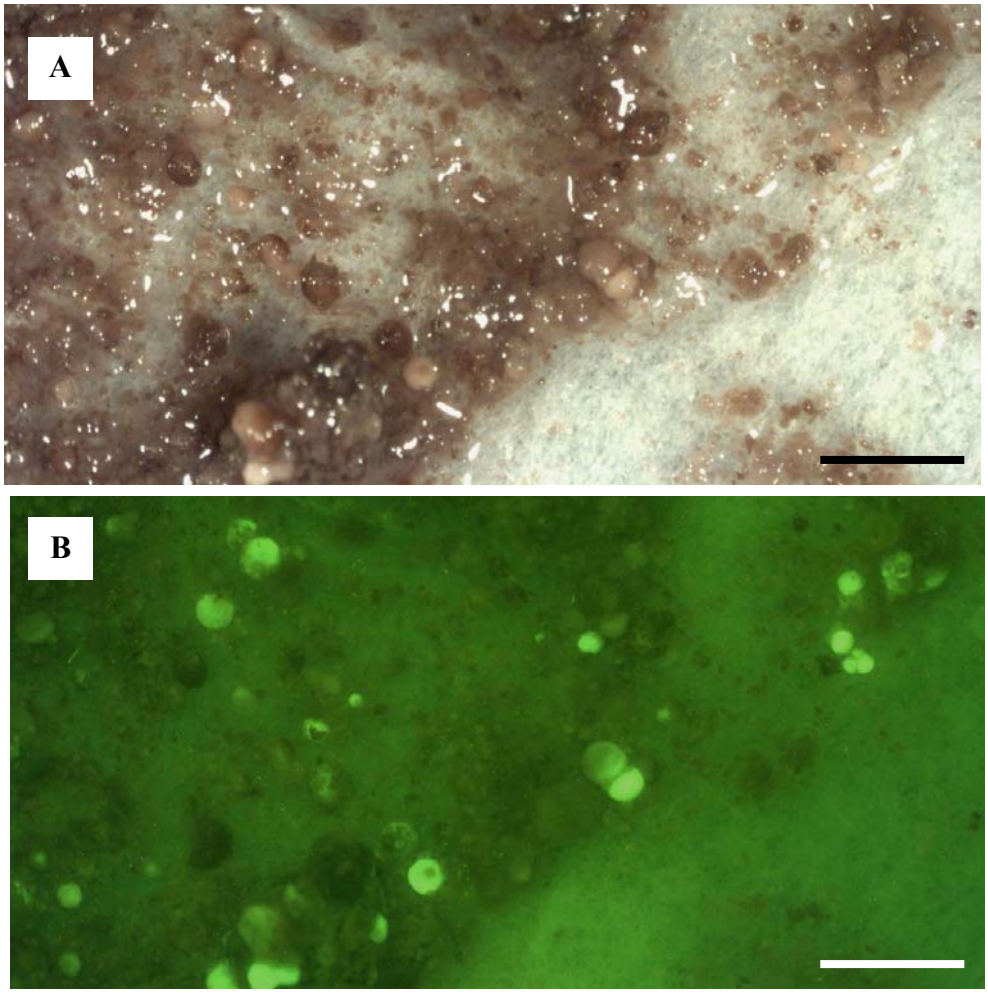

Figure 4.3 Transformed embryogenic callus and somatic embryo expressing EGFP gene: white light (A) and blue light (B). Bars = 5 mm.

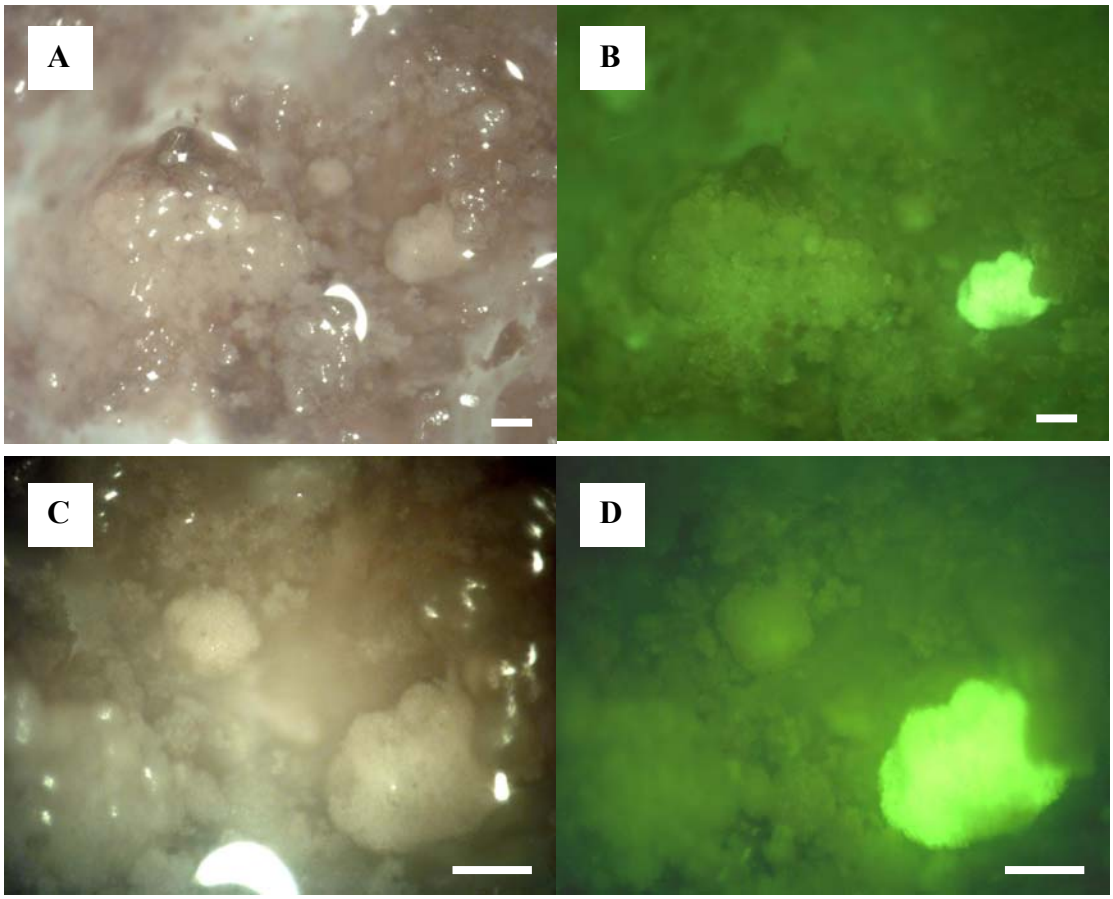

Figure 4.4 Transformed embryogenic callus on selective medium 2 months after transformation: white light (A and C) and blue light (B and D). Bars = 2 mm.

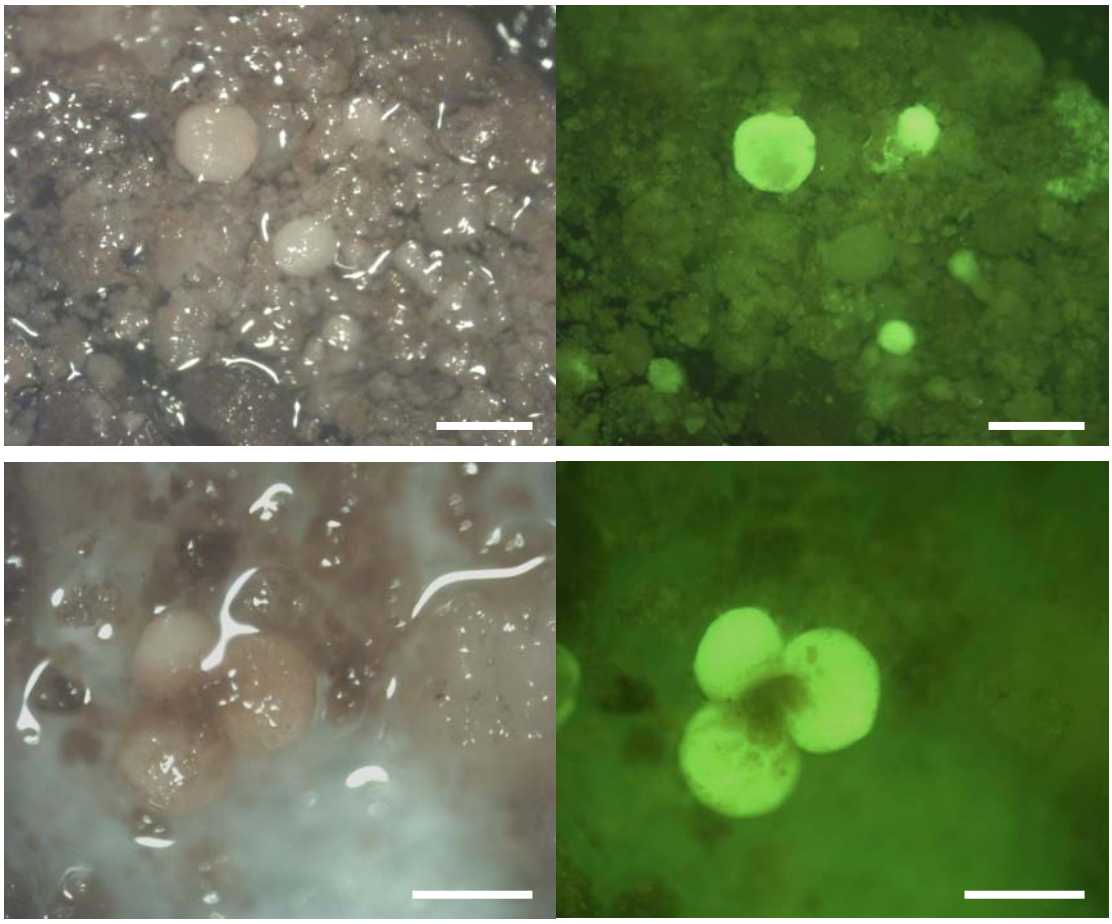

Figure 4.5 Transformed globular somatic embryos on kanamycin-free medium 2 months after transformation: white light (A and C) and blue light (B and D). Bars = 1 mm.

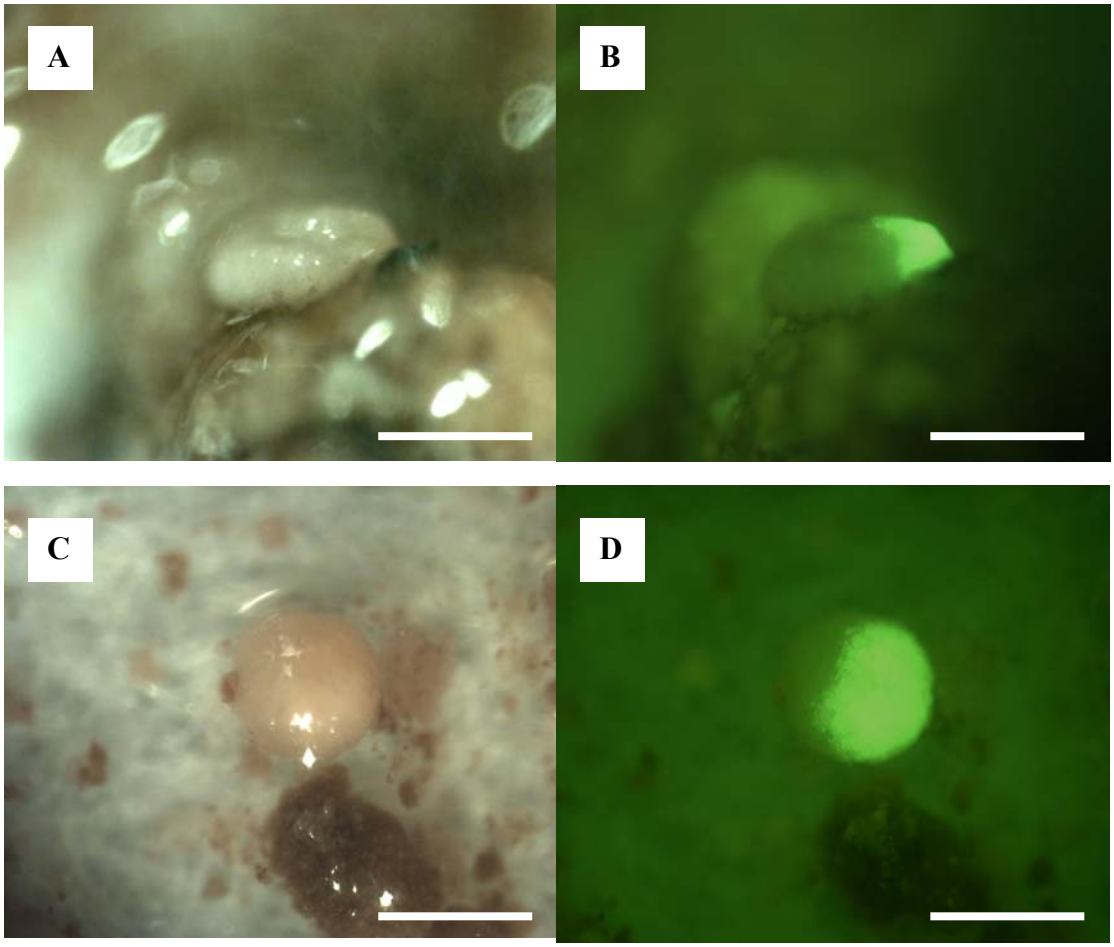

Figure 4.6 Partially transformed globular somatic embryos: white light (A and C) and fluorescent light (B and D). Bars = 1 mm.

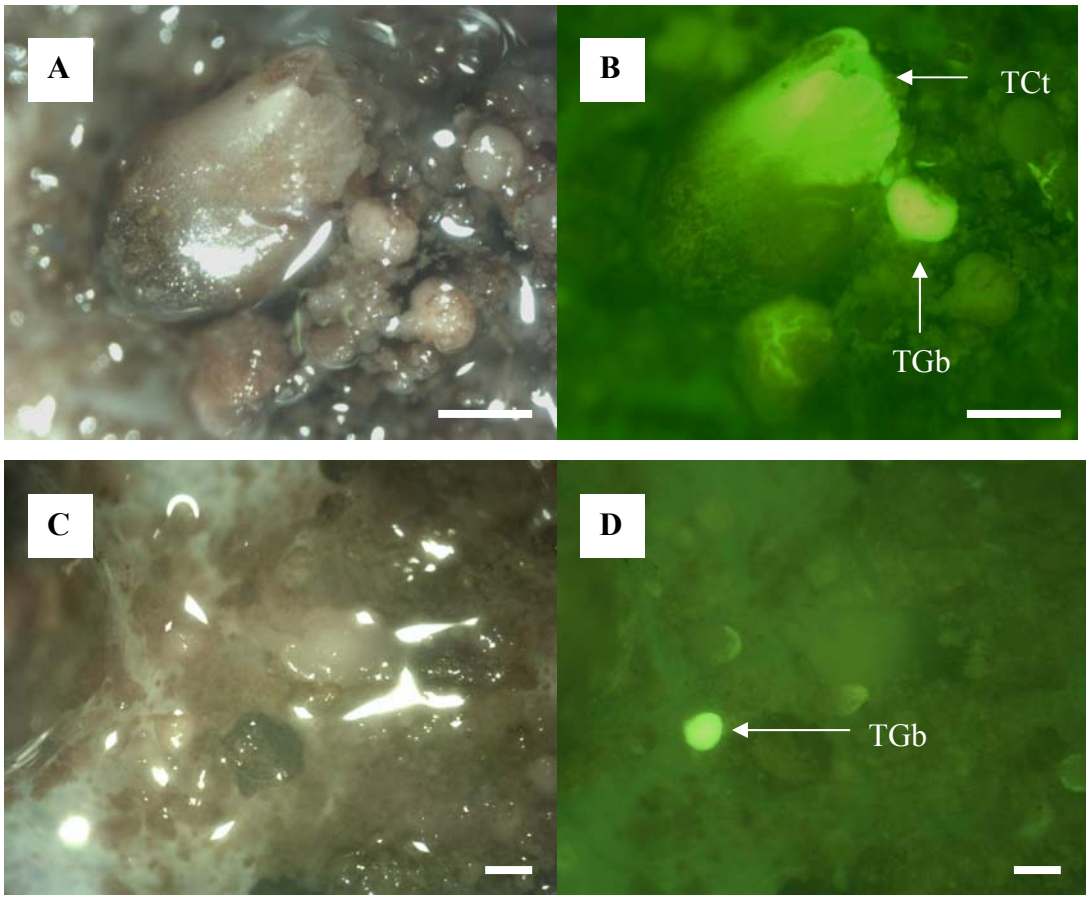

Figure 4.7 Transformed globular (TGb) and cotyledonous (TCt) somatic embryos on kanamycin-free medium 3 months after transformation: white light (A and C) and blue light (B and D). Bars = 1 mm.

regeneration medium. The result suggested that *Agrobacterium* inoculum might affect the somatic embryo development. Iocco et al. (2001) reported the similar result that transformed somatic embryos of grapevine 'Semillon' and 'Pinor Noir' did not developed to germination stage while transgenic plants were obtained from other 7 cultivars (Table 2.3).

Using this protocol with 'Autumn Royal Seedless', more than 50 putative transgenic somatic embryos were produced in low transformation frequency. Since regenerated plant has not been obtained, DNA-based method could not be performed to verify gene integration. Further improvement needs to be done for successful *Agrobacterium*-mediated transformation in 'Autumn Royal Seedless'. Special treatments prior to cocultivation of somatic embryos such as culturing them on preconditioning medium (Zhijian et al., 2001) were suggested to make the somatic embryos susceptible to *Agrobacterium*. Wounding of somatic embryos though did not give an increasing number of transformed somatic embryos in *V. rupestris* with *Agrobacterium* strain LBA4404 (Martinelli and Mandolino, 1994). It is however interesting to apply the technique to *V. vinifera* grape 'Autumn Royal Seedless' with supervirulent EHA105 *Agrobacterium*.

4.5 Conclusion

The protocol for *Agrobacterium*-mediated transformation was developed for 'Autumn Royal Seedless'. Transformation conditions were applied to a number of SPEM suspension cells, but only primary somatic embryo was the tartget tissue that gave successful transformation. Transformed embryogenic calli and globular somatic

embryos showed fluorescent signal on selection medium after two months of selection. The somatic embryos did not grow beyond cotyledonous stage. Using this protocol, more than 50 putative transgenic somatic embryos were produced at low transformation frequency.

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CHAPTER V

IN VITRO SELECTIONS FOR SALT TOLERANCE OF TABLE GRAPES 'TARA' (*Vitis rotundifolia*) AND 'CHARDONNAY' (*Vitis vinifera* L.)

5.1 Abstract

Soil salinity has been known as an abiotic stress affecting major processes of plant species. In order to produce salt tolerant grapes, 'Tara' embryogenic calli and 'Chardonnay' suspension cells were *in vitro* selected on solid media supplemented with NaCl. Five (0.5, 1.0, 1.5, 2.0, and 2.5%) and 2 (0.5 and 1.0%) concentrations of NaCl were used for recurrent and progressive selections, respectively. The best result was obtained from recurrent selection. More than 95% of calli turned brown when they were cultured on 1.0% NaCl-containing medium for 10 weeks, whereas 100% browning was observed among the calli cultured on 1.5 and 2.0% NaCl-containing media within 5 weeks. Ten salt tolerant (ST) lines of 'Tara' embryogenic callus were initially obtained from 6-month selection on 1.0% NaCl-containing medium as they generated new white embryogenic cells. However, only ST1 showed further growth while other 9 lines turned brown after 2 subcultures. Somatic embryos from ST1 could be observed as early as 3 weeks after being transferred to FMSC medium. The somatic embryos reached 0.5 cm long within 8 weeks. Approximately 10% of somatic

embryos developed from ST1 could survive and germinate on FMSC medium containing 1.0% NaCl for double selection. Distinct change in polypeptide patterns was observed: (a) increasing level of 51- and 24-kDa polypeptides and (b) reducing levels of 48- and 27-kDa polypeptides. High level of 26-kDa polypeptide was detected in early stage but became lower at late stage of salt tolerance. For 'Chardonnay', 0.5% of NaCl had the least effect on growth of the PEM suspension cells. Color of the cells was slightly darker and their multiplication was noticeable within 4 weeks. No suspension cell growth was observed from higher NaCl concentrations. Although somatic embryos were obtained from 0.5% NaCl-containing medium, their root turned brown, shoots stunted, and they died after culturing on FMSC medium containing NaCl. No ST line was obtained from progressive selection from 1.0% NaCl-containing medium.

5.2 Introduction

Grape is one of the major fruit crops. Numerous grape cultivars have been grown in various viticulture areas throughout the world. However, a given variety may only adapt to certain areas due to the biotic and abiotic stresses. Soil salinity is a major abiotic stress in plant agriculture worldwide (Zhu, 2001), which limits the productivity of agricultural crops. Salinity reduces the ability of plants to absorb water, causing rapid reductions in growth rate, along with a suit of metabolic changes identical to those caused by water stress (Borsani et al., 2003).

Over three decades that biochemical response of plant cell to salt stress has been investigated. It has been found that salt tolerance could be established in plant as

there was evidence that new proteins were produced and accumulated in plant cell under salt stress. Scientists have attempted to produce salt tolerant plant species using tissue culture systems including suspension culture, callus and shoot culture (Woodward and Bennett, 2005).

As a fruit crop, grape conventional breeding is hampered by long juvenile period which requires carrying seedlings for several years before evaluation. Furthermore, salt tolerance has been known as a quantitative trait, which is hard to improve by traditional methods (Foolad and Lin, 1997) or genetic transformation for true tolerance. *In vitro* selection is therefore becoming an attractive alternative approach for producing salt tolerant grape cultivars. We report here the first *in vitro* selection for salt tolerance in muscadine grape from embryogenic cell culture.

5.3 Materials and methods

5.3.1 Plant embryogenic materials

Embryogenic calli of 'Tara' (*Vitis rotundifolia*) were induced from immature ovule by Xu et al. (2005) and cultured on solid medium containing half-strength MS macronutrient, full-strength MS micronutrient and vitamin plus 30 g l^{-1} sucrose, 2 mg l^{-1} BA (6-benzylaminopurine), and 1 mg l^{-1} NOA (β-naphthoxyacetic acid). The medium was adjusted to pH 5.7 prior to autoclaving and solidified with 3 g l^{-1} phytagel. The embryogenic calli were subdivided 6-8 weeks interval. Suspension culture of 'Chardonnay' (*V. vinifera* L.) was maintained in MSGGN medium as described by Jittayasothorn et al. (2007).

5.3.2 Selections for NaCl-tolerant embryogenic cell lines of 'Tara'

5.3.2.1 Recurrent selection

The embryogenic calli were recurrently selected on solid media supplemented with 5 concentrations of sodium chroride (NaCl) including 0.5, 1.0, 1.5, 2.0, and 2.5% (w/v). Ten clumps of embryogenic calli, approximately 2 g each, were cultured on the media. The similar set of typical embryogenic calli was cultured on the medium without and with NaCl for 1 month as controls. The embryogenic calli were subcultured at 6 weeks interval by the following procedure: entire embryogenic calli were transferred to fresh media for the first two subcultures. Enlarging clumps of embryogenic callus were half divided. Start from the 3rd subculture, only white or light brown embryogenic calli were subdivided to fresh media for further selection. After 4 such subcultures, surviving embryogenic calli were named as salt tolerant lines and maintained in their original NaCl-containing medium.

5.3.2.2 Progressive selection

Embryogenic calli obtained from culturing on 0.5% (w/v) NaCl-containing medium were transferred to medium plus 1.0% (w/v) NaCl. They were subcultured and selected as described above.

5.3.3 Selections for NaCl-tolerant suspension cell lines of 'Chardonnay'

Each 500 mg of small cluster of proembryonic masses (SPEMs, ≤ 1 mm diameter), which had been maintained over a year from previous experiment, was subjected to salt-containing liquid medium. The liquid medium was consisted of MSGGN medium (Jittayasothorn et al., 2007) supplemented with NaCl at 4 concentrations including 0.5, 1.0, 1.5, and 2.0%. Suspension medium was renewed every 10 days for the first 2

months to prevent cell browing and later every 21 days. Each treatment was comprised of 12 flasks.

5.3.4 Somatic embryogenesis and plant regeneration of salt tolerant plants

5.3.4.1 Tara

Salt tolerant embryogenic calli were transferred to FMSC medium (Jittayasotorn et al., 2007) with and without NaCl in a 100x25 mm Petri dishes for embryogenesis. Somatic embryos were harvested and cultured on the same medium in Magenta box. Somatic embryogenesis and plant regeneration were conducted under darkness and at 16 h photoperiod, respectively.

5.3.4.2 Chardonnay

After 6 months, SPEMs were removed from liquid media and rinsed with MSGGN liquid medium. Approximately 100 mg of washed SPEMs in 1.0 – 1.3 ml of MSGGN medium were pipette-transferred onto Whatman filter paper on FMSC medium with or without the same NaCl concentration as their original liquid medium in a 100x15 mm Petri dish. The cultures were maintained in darkness at 26^{o}C. Approximately 1 cm long somatic embryos were harvested and transferred into FMSC medium containing 1.0% NaCl. The culture was maintained at 16 h photoperiod.

5.3.5 Protein extraction

Total protein was extracted from salt tolerant embryogenic calli using Plant Total Protein Extraction Kit (Sigma). Two hundred mg of tissue sample was ground in liquid nitrogen to a fine powder. Frozen-still tissues were transferred to a 2 ml v-

bottom freezing vial and held at –20°C. A 1.5 ml of methanol solution was added to the sample. The mixture was vortexed for 15-30 seconds and incubated for 5 minutes at –20°C with periodic vortexing. The suspension was centrifuged at 16,000 x *g* for 5 minutes at 4°C and the supernatant was removed with a pipette. Addition of methanol solution was repeated for 2 additional times.

The tube was inverted over a clean paper towel to remove any visible methanol solution. A 1.5 ml of prechilled –20°C acetone was added to the pellet. The mixture was vortexed for 15-30 seconds and incubated for 5 minutes at –20°C. The suspension was centrifuged at 16,000 x *g* for 5 minutes at 4 °C. The supernatant was removed with a pipette and the pellet was allowed to dry for 5-10 minutes at room temperature. After drying the sample, freezer vial was weighed and the predetermined tare mass was subtracted from the vial weight to determine the tissue mass. A 4 µl of reagent type 2 working solution was added to each mg of tissue pellet. The pellet was completely resolved by vortexing and the suspension was gently mixed at ambient temperature. The suspension was centrifuged at 16,000 x *g* for 30 minutes to remove tissue debris. The supernatant containing total protein was transferred to new tube with a pipette.

5.3.6 SDS-PAGE

SDS-PAGE was carried out following the procedure of Laemmli (1970). Slab gels (15 cm x 10 cm x 1.5 mm) with a 4% polyacrylamide stacking gel and a 12% polyacrylamide separating gel were used and equal amount of protein was applied in each lane. Electrophoresis was performed with maximum of 150 V at a constant current of 30 mamp in stacking gel and was increased to 50 mamp before the

bromophenol blue reached the separating gel about 1-2 mm. After electrophoresis, gels were stained with either silver staining or Coomssie brilliant blue R-250. Molecular weight of polypeptides was determined according to their Rf.o

5.4 Results and discussion

5.4.1 Selection for salt tolerant embryogenic calli of 'Tara'

Among the NaCl concentrations used, 0.5% NaCl apparently had no effect on embryogenic cells as it was evident that they grew as well as control. Therefore, they were not designated as tolerant lines. The embryogenic calli generally turned brown earlier when higher concentrations of NaCl were applied. More than 95% of calli turned brown when they were cultured 1.0% NaCl-containing medium for 10 weeks, whereas browning was fully observed among the calli cultured on 1.5 and 2.0% NaCl-containing media within 5 weeks (Figure 5.1). Browning was initially observed at embryogenic cells located on surface of the clump while those cells attached to the media were still normal. This could be caused by evaporation and lose of water potential of the cells. Browning later appeared inside and was fully detected in the whole clump if none of embryogenic cells was tolerant to NaCl.

Interestingly, approximately 40% of embryogenic calli on 2.5% NaCl-containing medium remained white-yellow color but without growth. These embryogenic calli were slightly wilted. Hypothetically, NaCl concentration at 2.5% might help preserve color of the cells by inactivating enzymatic activity of polyphenol oxidase (PPO), which catalyes browning reaction in plants (Mayer, 1987). The browning reaction is a result of the catalytic action of PPO by oxidizing phenolic compounds to quinones

which eventually become melanin pigments by polymerization (Macheix et al., 1990 quoted in Yoruk and Marshall, 2003).

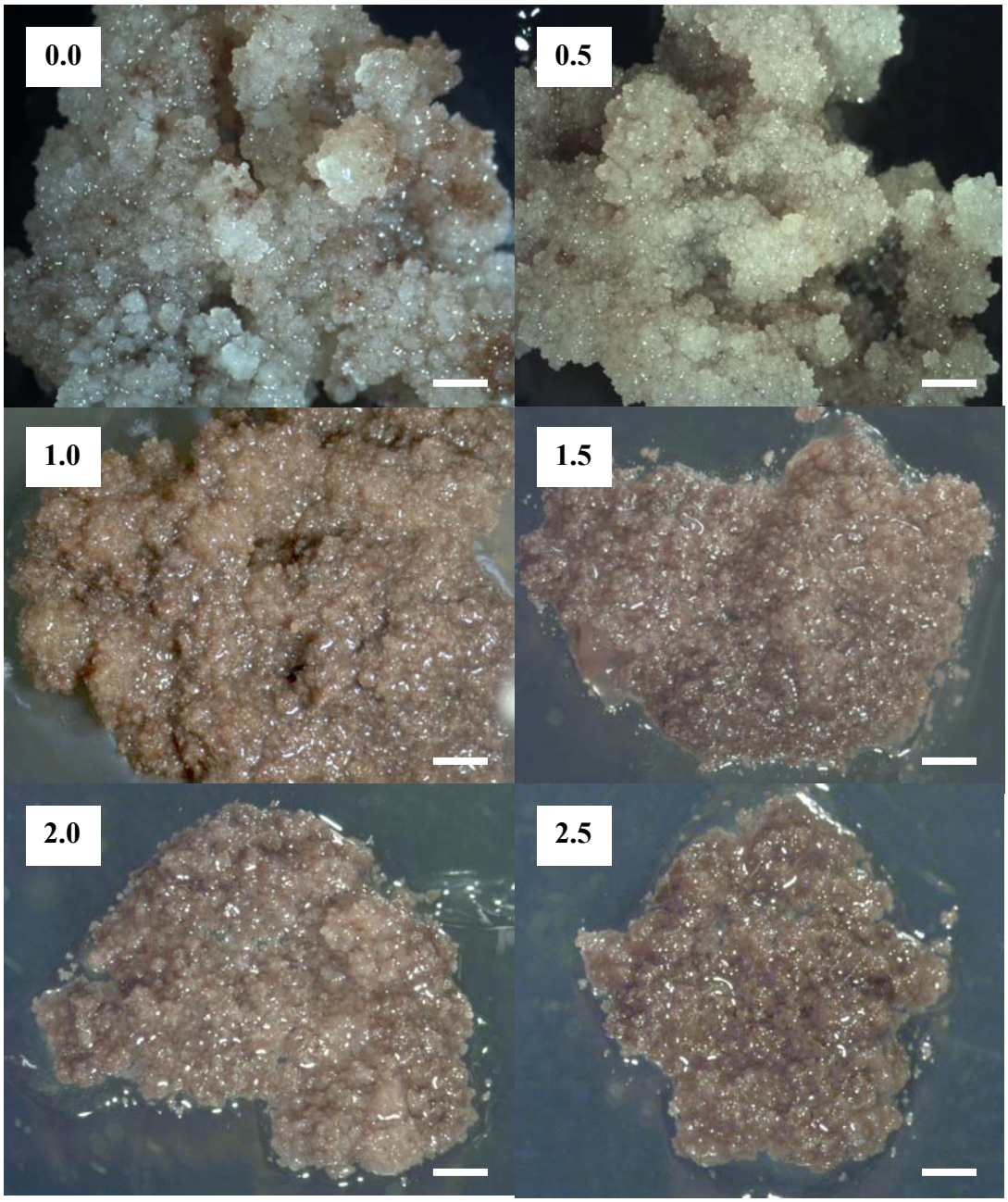

Figure 5.1 'Tara' embryogenic calli cultured on 0, 0.5, 1.0, 1.5, 2.0, and 2.5% NaCl-containing media for 5 weeks. Bars = 1 mm.

At the 4th subculture, some embryogenic calli on 1.0% NaCl generated new white embryogenic cells (Figure 5.2), which contained 10 putative salt tolerant lines. Two lines were obtained from the same clump of embryogenic callus but different location, while the rest was individually obtained from different clumps. The salt tolerant lines were separated from their original clumps and transferred into fresh medium in order to stimulate embryogenic cell division. Some embryogenic calli of each line was transferred to FMSC medium for somatic embryogenesis. After 2 subcultures, only ST1 showed further growth while other 9 lines turned brown. The results suggested that selection on NaCl-containing medium for 6 months might be too early to obtain embryogenic calli that were truly tolerant to NaCl. No ST line on 1.0% NaCl-containing medium was obtained from progressively selection. The results indicated that induction of adaptabililty of embryogenic calli on 0.5% NaCl-containing medium was unnecessary for them to survive in medium containing higher NaCl concentration.

5.4.2 Somatic embryogenesis and plant regeneration of 'Tara'

Three weeks after being transferred to FMSC medium, some of the ST1 developed somatic embryos from globular to early cotyledonous stage (Figure 5.3), while others from different clumps turned brown. Two weeks later, somatic embryos were observed from other ST lines. The somatic embryos reached 0.5 cm long as early as 8 weeks on FMSC medium. Surprisingly, no somatic embryogenesis was observed from embryogenic calli cultured on 0% NaCl-containing medium within 12 weeks. It has been known that compatible molecules were increasingly synthesized and accumulated in plant cells while they were cultured in medium containing NaCl. Some of the molecules might have positive effect on somatic embryogenesis.

Figure 5.2 ‘Tara’ embryogenic calli on 1.0% NaCl-containing medium generated new white embryogenic cells at the 4th subculture. Bars = 1 mm.

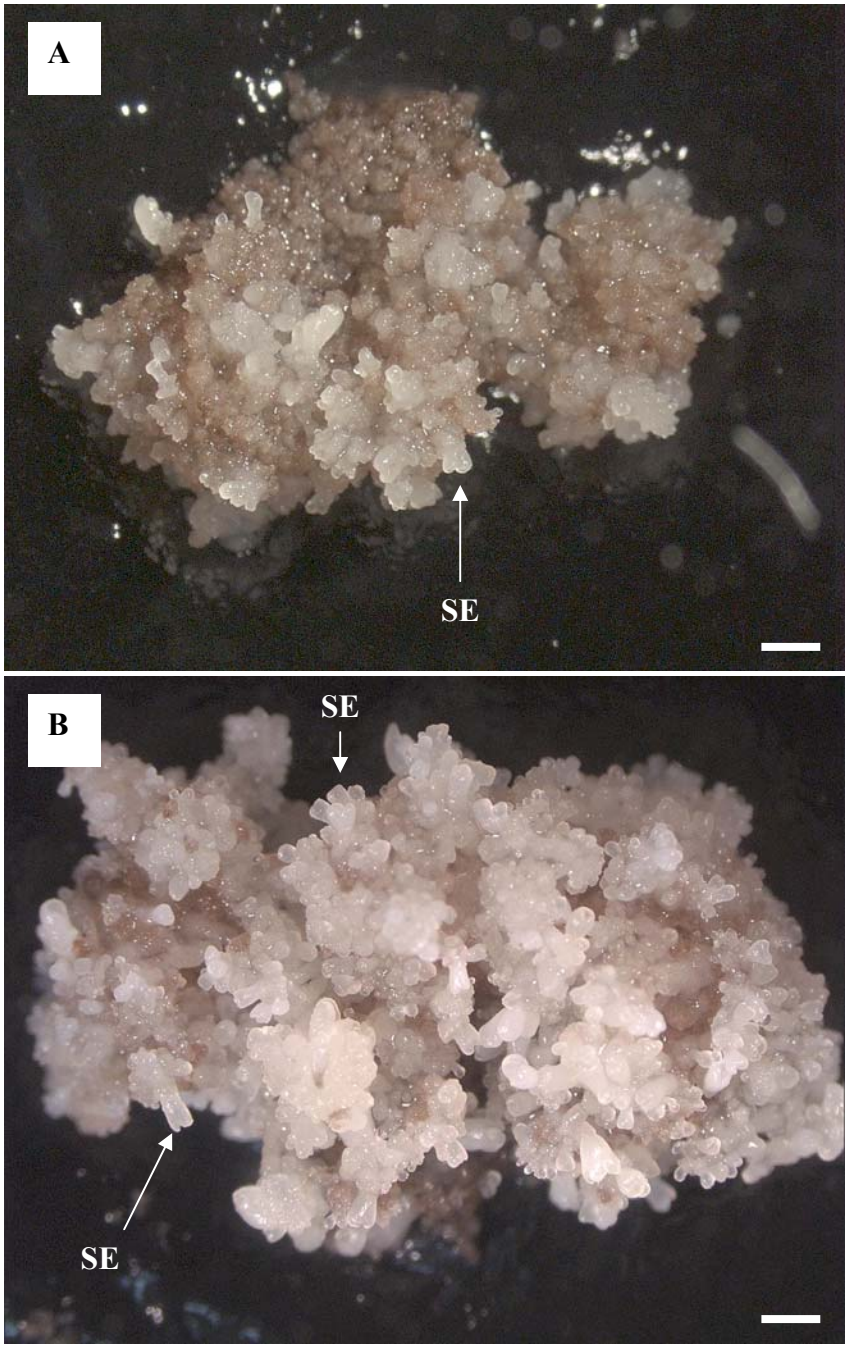

Figure 5.3 Somatic embryos (SE) developed from ST1 line after being transferred to FMSC medium for 3 (A) and 5 (B) weeks. Bars = 1 mm.

Proline has been known as an amino acid that was highly accumulated in tissue or cell of several plant species cultured under salt stress such as *Populus euphratica* callus (Zhang et al., 2004), *Medicago media* cv. Rambler suspension cell (Chaudhary et al., 1997), *Eucalyptus camaldulensis in vitro* shoot (Watanabe et al., 2000; Woodward and Bennett, 2005), and also grape *in vitro* leaf (Singh et al., 2002). Many experiments demonstrated that frequencies of somatic embryogenesis and plant regeneration were significantly increased when proline was added into regeneration media (Ronchi et al., 1984; Skokut et al., 1985; Rao et al., 1995; El-Itriby et al., 2003). According to those discoveries, in this experiment, it was possible that proline was accumulated in embryogenic cells while they were selected in NaCl-containing medium. When transferred to regenerating medium where there was no influence from NaCl, the accumulated proline then stimulated somatic embryogenesis and embryo development.

Within a week, all somatic embryos showed chlorophyll development. However after a week later, only approximately 10% of somatic embryos developed from ST1 could survive and germinate in FMSC medium containing 1.0% NaCl for double selection, whereas somatic embryos from other ST lines died (Figure 5.4). The results indicated that most somatic embryo developed from salt tolerant embryogenic calli was not truly tolerant to NaCl.

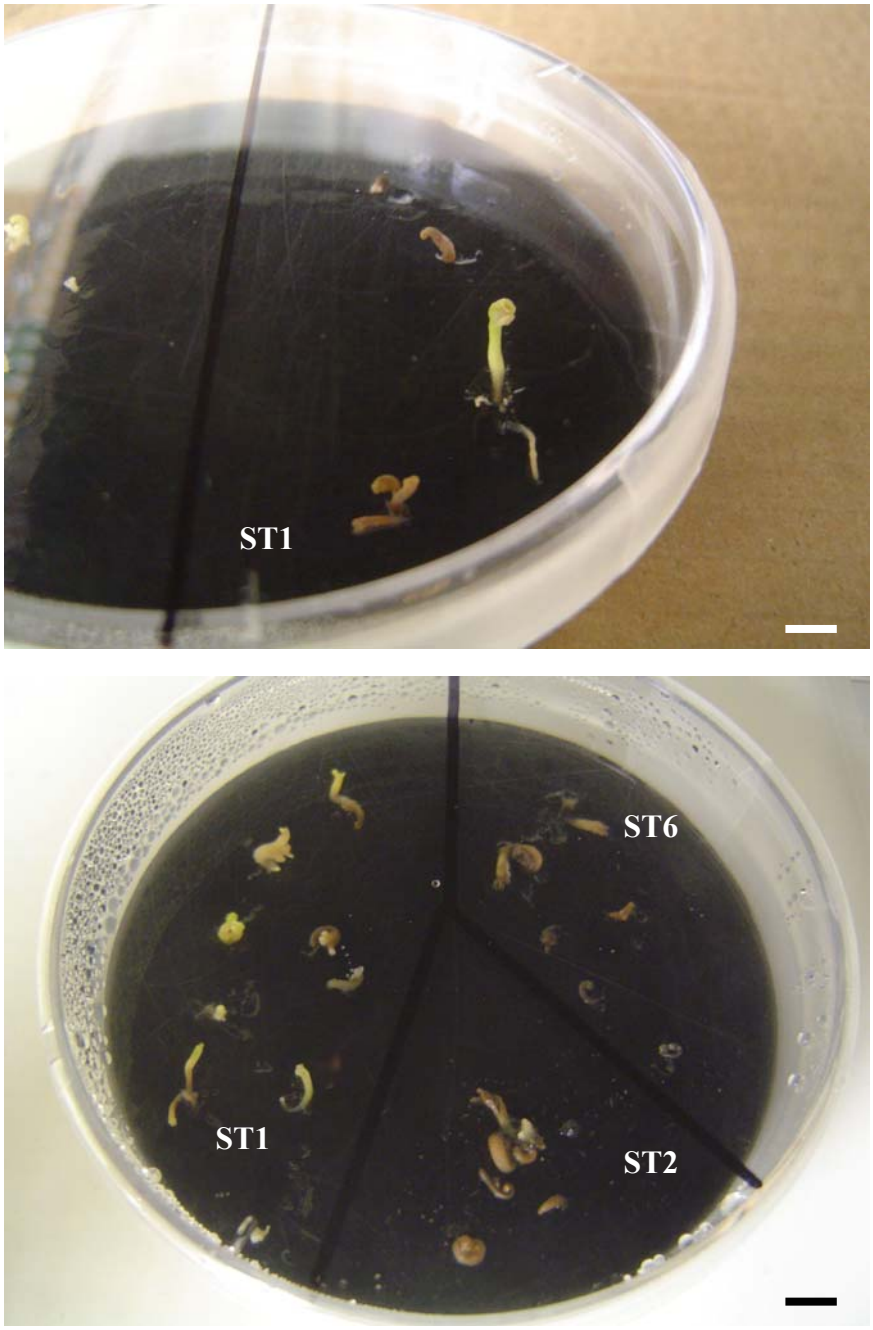

Figure 5.4 Some 'Tara' somatic embryos developed from ST1 survived and germinated on FMSC medium containing 1.0% NaCl for double selection while all somatic embryos of ST2 and ST6 turned brown and died. Bars = 5 mm.

5.4.3 SDS-PAGE

Polypeptide bands were nicely revealed on SDS-PAGE gel stained with Coomassie blue. There was no difference in type of salt stress induced polypeptides between tolerant callus cultured in 1.0% NaCl (SC1%) and tolerant callus cultured in 0% NaCl (SC0%) (Figure 5.5). However, distinct changes in patterns of the polypeptides were observed in response to salt stress: (a) increasing levels of 51- and 24-kDa polypeptides and (b) reducing levels of 48- and 27-kDa polypeptides. Similar finding was also found in tobacco suspension cells cultured on NaCl-containing medium (Singh et al., 1985). The patterns suggested that cells enhanced synthesis of some polypeptides (51 and 24 kDa) in order to survive under salt condition, whereas synthesis of some other polypeptides (48 and 27 kDa) was inhibited by the effect of NaCl accumulation. The increasing polypeptides have been known to be involved in cell morphological changes such as large vacuolar volume (Mimura, 2003), dense cytoplasm, and large nucleus (Gu et al., 2004).

High level of 26-kDa polypeptide was detected in early stage (TC1%) and became lower at late stage (SC1%) (Figure 5.5). This indicated that the polypeptide was responsible for salt tolerance in short-term period right after embryogenic cell was subjected to salt stress condition. Singh et al. (1985) reported that the 26-kDa polypeptide was involved in adaptation of unadapted tobacco cells cultured in NaCl-containing medium but it turned over rapidly. It was found that salt tolerant and typical calli cultured on 0% NaCl-containing medium, (SC0%) and (TC0%) repectively, gave the identical polypeptide pattern. The results could be interpreted that salt tolerance might not appear in genetic level, but it was inducingly regulated by native genes when plant cell was subjected to salt stress condition.

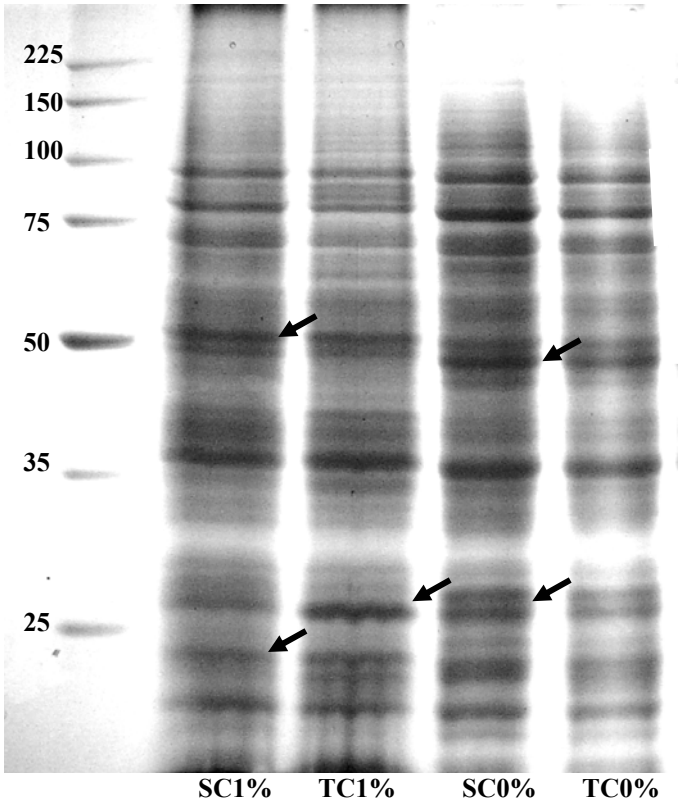

Figure 5.5 SDS-PAGE separation of polypeptides from typical and salt tolerant embryogenic calli cultured on NaCl-containing medium at 0 and 1.0%. Arrows indicated major differences in polypeptide bands of 51, 24, 26, 48, and 27 kDa from top to bottom and left to right. SC0% = salt tolerant callus culture in 0.0% NaCl for 1 month, SC1% = salt tolerant callus culture in 1.0% NaCl, TC0% = typical callus cultured in 0.0% NaCl, and TC1 = typical callus cultured in 1.0% NaCl for 1 month.

5.4.4 Selection for NaCl-tolerant suspension cell lines of 'Chardonnay'

As expected, 0.5% of NaCl had the least effect to growth of the SPEM suspension cells. Color of the cells was slightly darker and their multiplication was noticeable within 4 weeks (Figure 5.6 and 5.7). No suspension cell growth was observed from higher NaCl concentrations. Suspension cells in MSGGN media containing 1.0% and 1.5% NaCl turned opaque after 30 and 20 days, respectively. As the NaCl concentration increased to 2.0%, it took only 4-5 days for the color to change, and the suspension cells became completely dark within 2 weeks. Slower growth is an adaptive feature of plant survival under stress because it allows plants to rely on multiple resources (e.g. building blocks and energy) to combat stress (Zhu, 2001).

5.4.5 Somatic embryogenesis and plant regeneration of 'Chardonnay'

Somatic embryogenesis was observed as early as 6 weeks from embryogenic calli cultured in MSGGN medium containing 0.5% NaCl (Figure 5.8A). Within the following 2 weeks, mature somatic embryos were obtained. No somatic embryo was induced from SPEM selected at other concentrations and control at this time.

Somatic embryos showed chlorophyll development and some also grew the first shoot within few days after being transferred to 16 h photoperiod, however, their root started turning brown and their shoots stunted about 2 weeks later (Figure 5.8B).

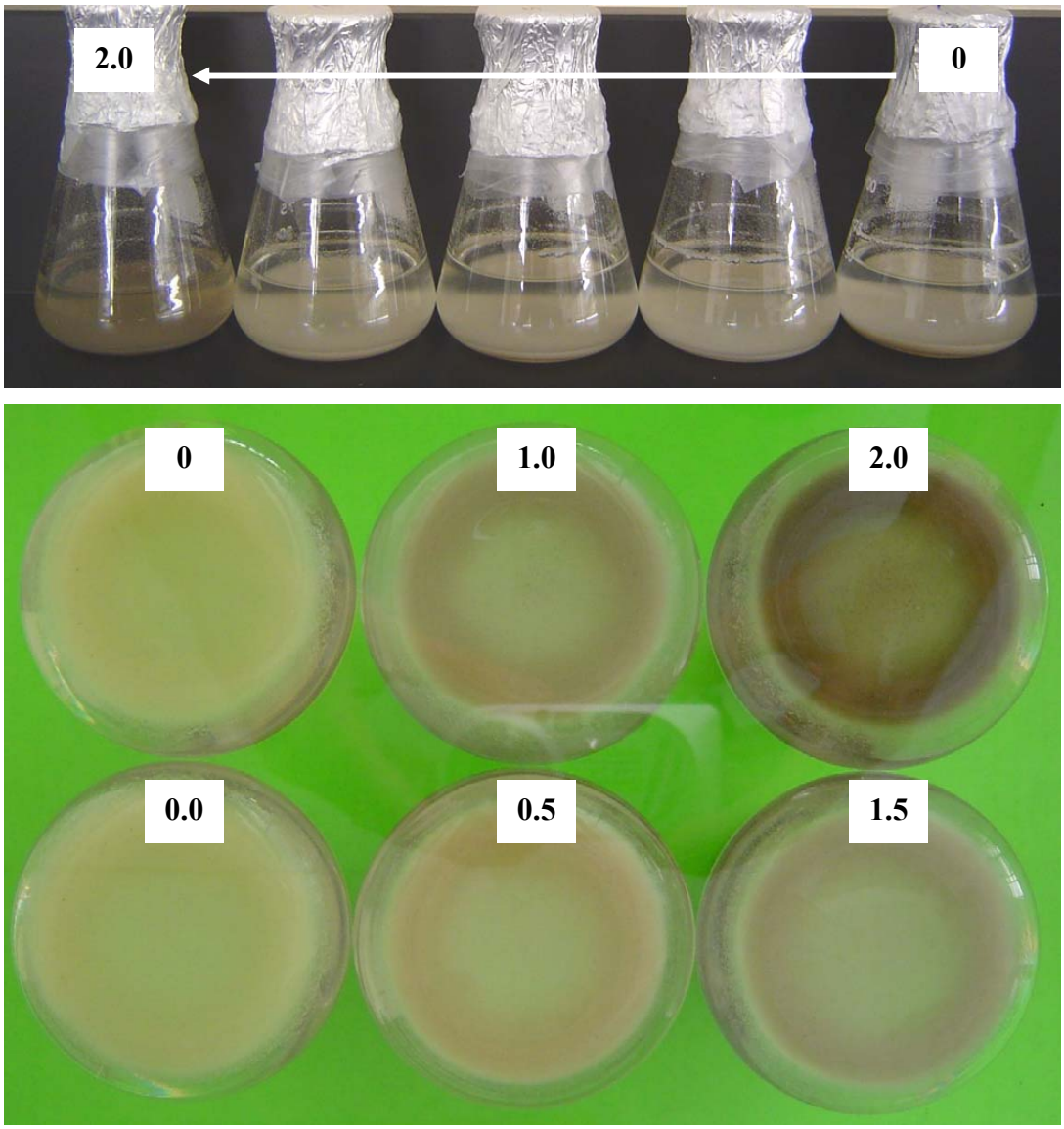

Figure 5.6 'Chardonnay' SPEM cultured in MSGGN media containing 0, 0.5, 1.0, 1.5, and 2.0% NaCl for 6 weeks.

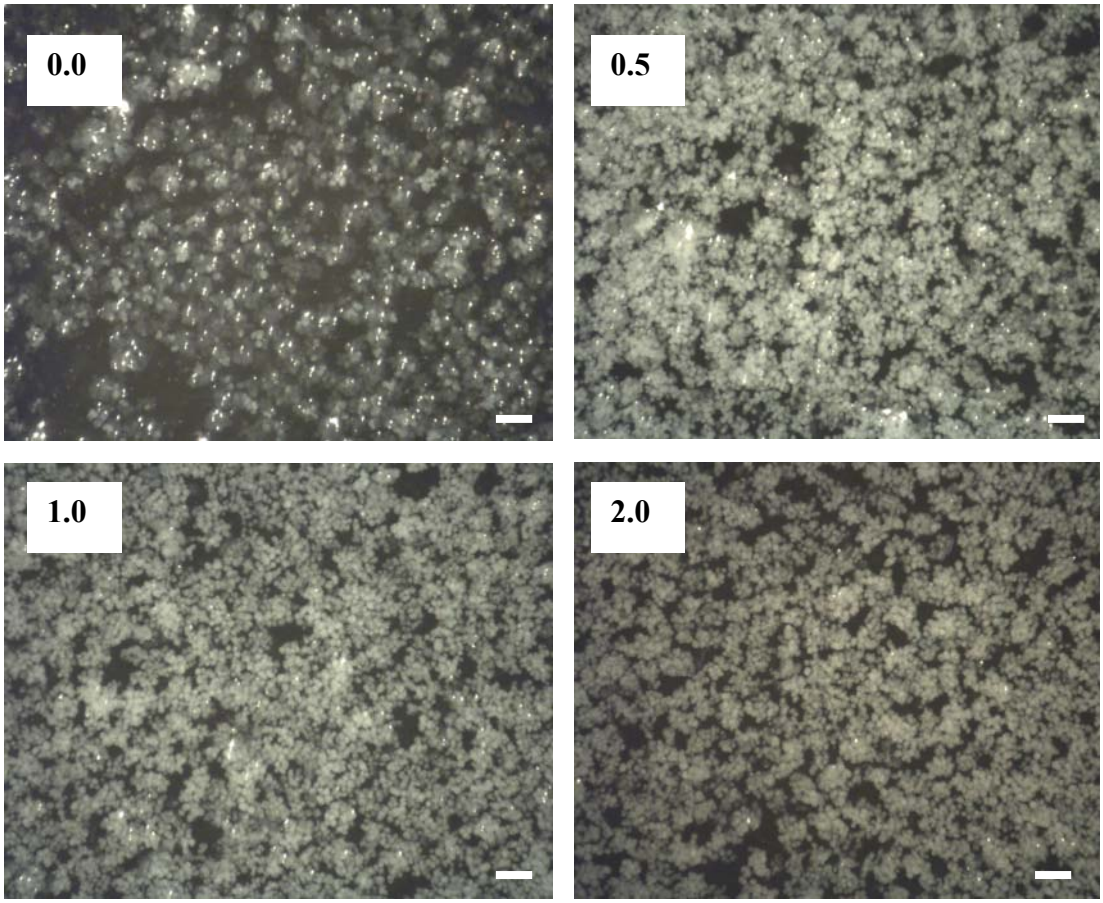

Figure 5.7 'Chardonnay' SPEM cells cultured in MSGGN media containing 0, 0.5, 1.0, and 2.0% NaCl for 6 weeks.

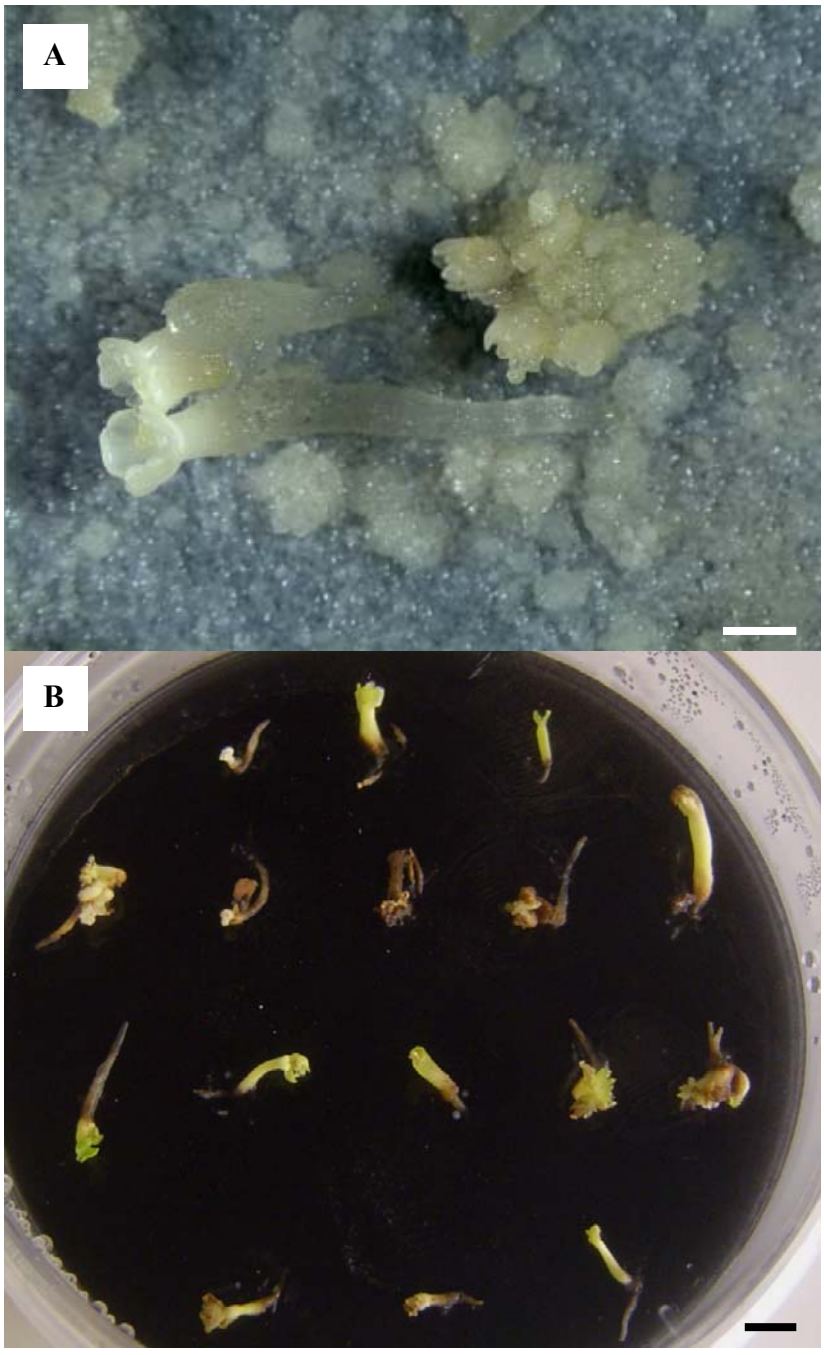

Figure 5.8 'Chardonnay' somatic embryos developed on FMSC medium containing 0.5% NaCl after 2 months (A) and the somatic embryo were transferred to FMSC medium containing 1.0% NaCl for double selection (B). Upper bar = 1 mm and lower bar = 5 mm.

5.5 Conclusion

Using *in vitro* selection strategy, salt tolerant somatic embryos of a muscadine grape 'Tara' were obtained from solid medium containing 1.0% NaCl in recurrent selection. Increasing and decreasing levels of polypeptides extracted from salt tolerant somatic embryos were revealed on SDS-PAGE. Salt tolerance might not appear in genetic level but it was an inducible regulation of cells to survive under salt stress condition. No suspension cell growth of 'Chardonnay' was observed from liquid media containing 1.0% NaCl and higher concentrations.

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CHAPTER VI

OVERALL CONCLUSIONS

Grape is one of the most important fruit crops in the world. It is one of the most widely adapted species and is capable of being cultivated commercially in over 70 countries with a total area of 10 million hectares. Although grapes have been registered as a commercial fruit in Thailand since 1960's, somatic embryogenesis, which is the prerequisite for biotechnological breeding, and its applications have never been reported in the country. This has unfortunately limited grape improvement through genetic transformation and *in vitro* selection which those two rely on somatic embryogenesis that allows whole plant is regenerated from single cells. To accelerate grape breeding in Thailand, this research was aimed at establishing a highly efficient protocol for somatic embryogenesis and plant regeneration, developing a transformation system, and producing salt tolerant plant using *in vitro* selection.

In the first experiment, the complete system of somatic embryogenesis and plant regeneration via proembryogenic mass suspension culture was accomplished. Proembryonic mass (PEM) suspension culture of 'Autumn Royal Seedless' was successfully established from primary somatic embryos cultured on solid medium. The results of factors affecting somatic embryogenesis of PEM cells strongly indicated that activated charcoal had a positive effect in facilitating somatic embryogenesis and development, whereas light had tremendously negative effect to somatic embryogenesis. Full strength was superior to half strength MS medium for both

somatic embryogenesis and plant regeneration. Although suspension cultures of ‘Chardonnay’ and ‘Tara’ were also established in liquid media, ‘Autumn Royal Seedless’ showed the most desirable performance in growth and regeneratability. Uniformity of cell suspension was observed from ‘Chardonnay’ while ‘Tara’ had yet no appreciable character for being a suspension culture material.

The PEMs and primary somatic embryos were subsequentialy used as plant tissue materials in transformation experiment. *Agrobactium*-mediated transformation was employed to transfer EGFP gene to ‘Autumn Royal Seedless’. After 2 months of transformation, some embryogenic calli and globular somatic embryos fluoresced green light on selection medium when primary somatic embryos were used as target tissue. None of the transformed cell was obtained from SPEMs. The fluoresced somatic embryos, however, did not grow beyond cotyledonous stage making DNA analysis of gene integration in regenerated plant impossible. This transformation protocol yielded putative transgenic ‘Autumn Royal Seedless’ somatic embryos at low transformation frequency. The last experiment was *in vitro* selection for salt tolerance. ‘Tara’ embryogenic calli were cultured on solid medium containing 5 concentrations of NaCl including 0.5, 1.0, 1.5, 2.0, and 2.5%. Salt tolerant (ST) lines of embryogenic callus was obtained from 1.0% NaCl liquid medium after 6 months selection, however, only ST1 maintained its growth after subculturing for 2 more months. Increasing and decreasing level of polypeptides extracted from salt tolerant somatic embryos were revealed on SDS-PAGE. Using *in vitro* selection strategy, salt tolerant somatic embryos of a muscadine grape ‘Tara’ was obtained. Although ‘Chardonnay’ had decent character for suspension culture and seemed to be a good source of cell variation, no suspension cell was tolerant to 1.0% NaCl or higher concentration.

Besides difficulty in nature of the experiments, time was another important limited factor. The whole experiment was purely conducted in USA within 3 years and 6 months, according to the maximum time of stay of J1-Visa Research Scholar. However, more than 90% overall of the experiment were achieved. This research provided new information and knowledge of biotechnological grape breeding at using new *vinifrea* grape 'Autumn Royal Seedless' in transformation experiment and especially this was the first report of *in vitro* selection of muscadine grape.

APPENDIX

Table 1A Compositions of MS and B5 basal medium in mg/l.

Medium components	**Nutrient compositions**	**Chemical formula**	**MS medium**	**B5 medium**
Macronutrient	Ammonium nitrate	NH_4NO_3	1,650	-
	Ammonium sulfate	$(NH_4)_2SO_4$	-	134
	Calcium chloride anhydrous	$CaC1_2$	332.2	113.24
	Magnesium sulfate anhydrous	$MgSO_4$	180.7	122.09
	Potassium nitrate	KNO_3	1,900	2,500
	Potassium phosphate monobasic	KH_2PO_4	170	-
	Sodium phosphate monobasic	$NaH_2PO_4.H2O$	-	150
Micronutrient	Boric acid	H_3BO_3	6.2	3
	Cobalt chloride-$6H_20$	$CoC1_2.6H_2O$	0.025	0.025
	Cupric sulfate-5 H_20	$CuSO_4.5H_2O$	0.025	0.025
	Na_2EDTA-2 H_20	Na_2EDTA	37.26	37.26
	Ferrous sulfate-$7H_20$	$FeSO_4.7H_2$	27.8	27.8
	Manganese sulfate-H_20	$MnSO_4.4H_2O$	16.9	10
	Molybdic acid-$2H_20$	$Na_2M_0O_4.2H_2O$	0.25	0.25
	Potassium iodide	KI	0.83	0.75
	Zinc sulfate-$7H_20$	$ZnSO_4.7H_2O$	8.6	2

Table 1A (continued).

Medium components	Nutrient compositions	Chemical formula	MS medium	B5 medium
Vitamin	Glycine (Free base)	-	2	-
	Myo-Inositol	-	100	100
	Nicotinic acid (Free base)	-	0.5	1
	Pyridoxine-HCl	-	0.5	1
	Thiamine-HCl	-	0.1	10

The publication in Vitis 46(1): 45-46

Vitis **46** (1), 45–46 (2007)

Research Note

A simple and highly efficient protocol for somatic embryogenesis and plant regeneration from proembryonic mass suspension culture in 'Autumn Royal Seedless'

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Introduction: Although somatic embryos could be multiplied by recurrent subculture on fresh solid medium, in general, yield still remains low. In addition, somatic embryos are highly asynchronous in solid medium. Quality of somatic embryos predominantly affects regeneration frequency, which varies in each subculture on solid medium (BORNHOFF and HARST 2000). Furthermore, somatic embryos on solid medium showed exhibitive dormancy, whereas in liquid medium they were not dormant and also showed higher plant regeneration efficiency (JAYASANKAR *et al*. 2003).

In this report we demonstrated a highly repeatable protocol for somatic embryogenesis and plant regeneration from proembryonic mass suspension culture of 'Autumn Royal Seedless' (*V. vinifera* L.). The protocol consists of two major steps: (1) multiplication of PEMs in liquid medium and (2) regeneration of PEMs in solid medium.

Material and Methods: Establishment of PEMs suspension culture: MSGGN liquid medium consisted of MS (MURASHIGE and SKOOG 1962) medium plus 20 g l^{-1} maltose, 500 mg l^{-1} glutamine, 5 ml l^{-1}glycerol, and 1 mg l^{-1} NOA, adjusted to pH 5.7. The liquid medium, Erlenmeyer flasks sealed with double-folded aluminum foil, and other equipments were sterilized by autoclaving at 121 °C and 1.1 kg cm^{-2} pressure for 20 min. Fifty and 100 ml medium was then poured into 125 ml and 250 ml flasks, respectively. Approximately 500 mg of 'Autumn Royal Seedless' primary somatic embryos, induced in the same lab (XU *et al*. 2005), was initially incubated in 50 ml MSGGN medium. The contact area between the edge of aluminum foil and the neck of the flask was sealed with Parafilm. The flasks were placed on a rotary shaker at 120 rpm in 26 ± 2 °C and maintained in darkness.

Subculture: The cultures were subdivided at 3-week-intervals to fresh MSGGN by using the modified procedure of JAYASANKAR *et al*. (1999). Briefly, flasks were transferred to aluminum flow hood for 5 min in order to allow PEMs to precipitate. Approximately 60 % of the supernatant was gently decanted. The suspension culture remaining was swirled and filtered through a 1 mm stainless steel sieve. The filtered PEMs were briefly rinsed with fresh MSGGN medium in order to bring passable PEMs through sieve pores. The large cluster of proembryonic masses (LPEMs, >1 mm diameter) were retained on the sieve, whereas the flow-through liquid culture containing small cluster of proembryonic masses (SPEMs, <1 mm diameter) was collected in a beaker. The liquid culture was swirled and re-filtered through a single layer of Kimwipe in a funnel. Approximately 200 and 400 mg of SPEMs were transferred by stainless steel spatula to 50 ml and 100 ml of fresh MSGGN flasks.

Somatic embryogenesis and plant regeneration: Approximately 50 mg of SPEMs in 6 clumps were placed in a 100 x 15 mm Petri dish filled with 20 ml FMSC medium (MS medium plus 30 g l^{-1} sucrose with 3 g l^{-1} activated charcoal, pH 5.8). The cultures were maintained at 26 ± 2 °C in darkness for somatic embryogenesis. After 4 weeks, the cultures were subdivided by the following procedure: somatic embryos (≥5 mm) were transferred to regeneration medium (see below) and 2 clumps were spread out evenly in a 100 x 25 mm Petri dishes containing 25 ml FMSC medium; this was transferred to fresh medium every 4 weeks to promote somatic embryo development. The somatic embryos (≥5 mm) were harvested individually and transferred to Magenta GA7 boxes containing 80 ml FMSC medium for plant regeneration. The boxes were maintained in a 16 h photoperiod. There were 9 somatic embryos per box.

Plant establishment: When plantlet length reached at least 7 cm and they had developed at least 5 leaves, they were transferred to potting soil mixture in 3 inches plastic cups, each of which was covered with another plastic cup on top.

Results and Discussion: Establishment of PEMs suspension culture: Whitely PEMs appeared in the suspension 2 weeks after the somatic embryos were incubated in MSGGN medium. The PEMs grew rapidly and suspension culture was established in two subcultures. At this time, the PEMs were yellowish and capable of rapid multiplication. The results demonstrated that the MSGGN medium with the culture techniques were highly efficient for producing proembryoic mass of 'Autumn Royal Seedless' (Figure, A). Sieving was very helpful to synchronize suspension culture by separating of SPEMs (Figure, B) and LPEMs during subculture.

Cell browning has been a major obstacle in establishing a suspension culture for grape (JAYASANKAR *et al*. 1999, BORNHOFF and HARST 2000). In this study, browning of neither PEMs nor liquid medium was observed during the initial and subsequent cultures.

Somatic embryogenesis: Two weeks after being transferred to the FMSC medium, the SPEMs developed into somatic embryo stages including globular, heart-shape, torpedo, and early cotyledon. The somatic embryos

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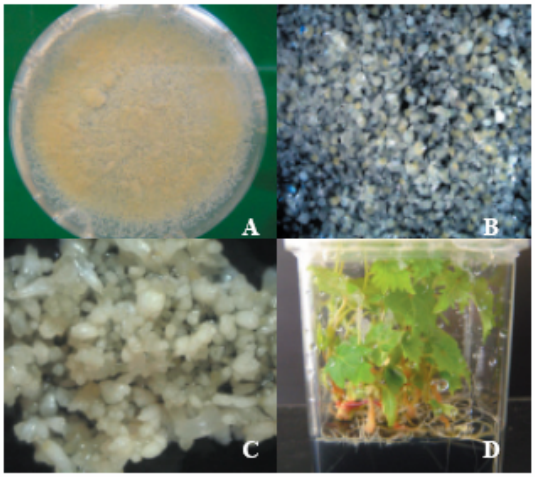

Figure: PEMs suspension culture in MSGGN medium after a 3-week-culture period in 125 ml flask (**A**). SPEMs separated by sieving (**B**). Somatic embryos developed asynchronously on FMSC medium in for 4 week (**C**). Regenerated plants in a Magenta box (**D**).

appeared asynchronously (JAYASANKAR *et al.* 1999), some somatic embryos precociously elongating to 1-2 cm within 4 weeks before the first subculture while the length of the majority was about 0.5-2 mm (Figure, C). More than 1,000 somatic embryos were obtained from approximately 50 mg of SPEMs within 14-16 weeks. LPEMs generated far less and highly asynchronous somatic embryos within the same time.

Plant regeneration: The somatic embryos of 'Autumn Royal Seedless' germinated in about 2 weeks after transferring to Magenta boxes in FMSC medium. The germinated somatic embryos were converted into plantlets with roots, shoots and leaves in another week. Somatic embryo germination and conversion rate varied depending upon quality (MOTOIKE *et al.* 2001). In this study, cotyledons were found to be the most important factor for somatic embryo conversion. Somatic embryos germinated into perfect cotyledons (unfolded, expanded and green) were converted into plantlets much faster than those with poor cotyledons (folded, unexpanded, and yellowish with some green at the tip).

In this study, approximately 50 mg of SPEM generated more than 1,000 somatic embryos and more than 95 % of the germinated somatic embryos converted into normal plants (Figure, D), which is highly efficient. Dormancy of somatic embryos was not observed. The regenerated plants were successfully established in pots within 6 weeks.

Acknowledgements: This work was supported by Florida Viticulture Advisory Council, USDA/CSREES, and the Royal Golden Jubilee Ph.D Program (RGJ) of Thailand. The authors are grateful to Dr. S. JAYASANKAR and Dr. L. MARTINELLI for valuable critiques of the manuscript.

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Received January 18, 2006

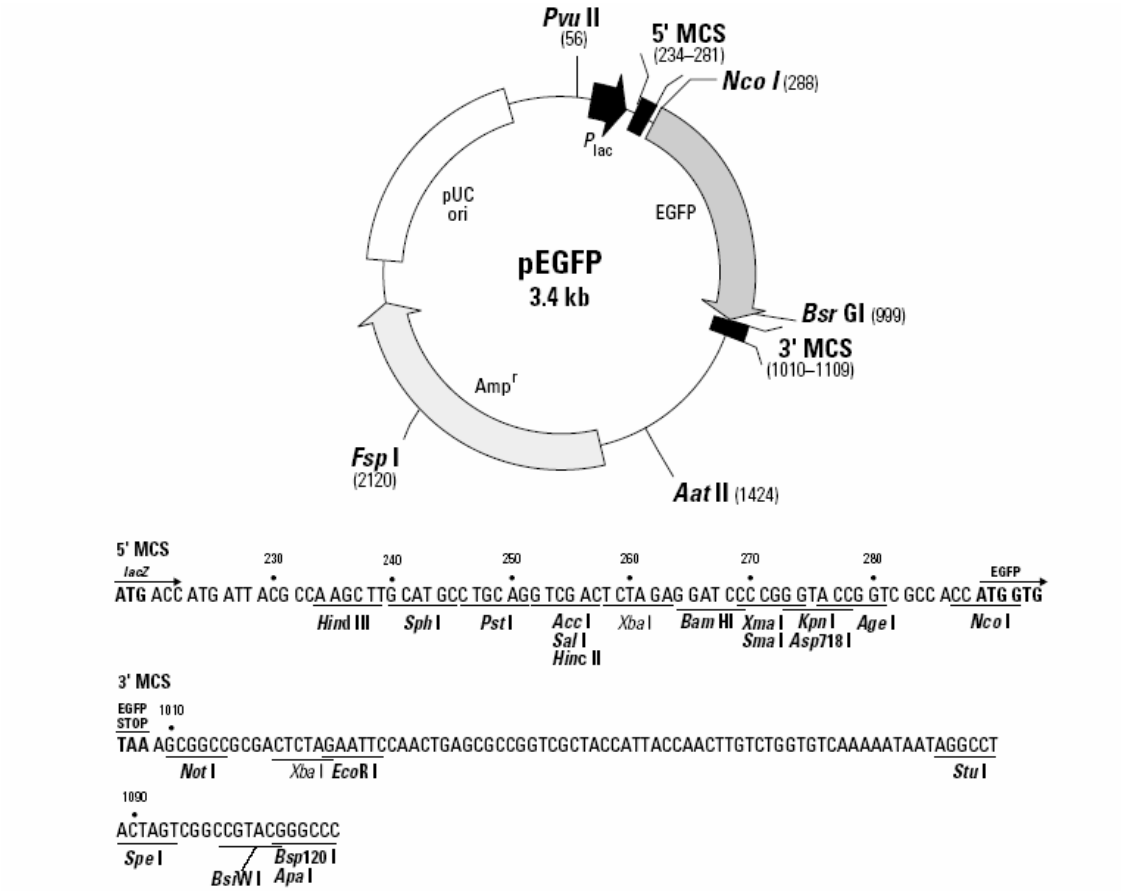

Figure 1A pEGFP vector information and its description from Clontech Lab. Inc., Palo Alto, CA, USA (www.clontech.com/images/pt/dis_vector/PT3078-5.pdf)

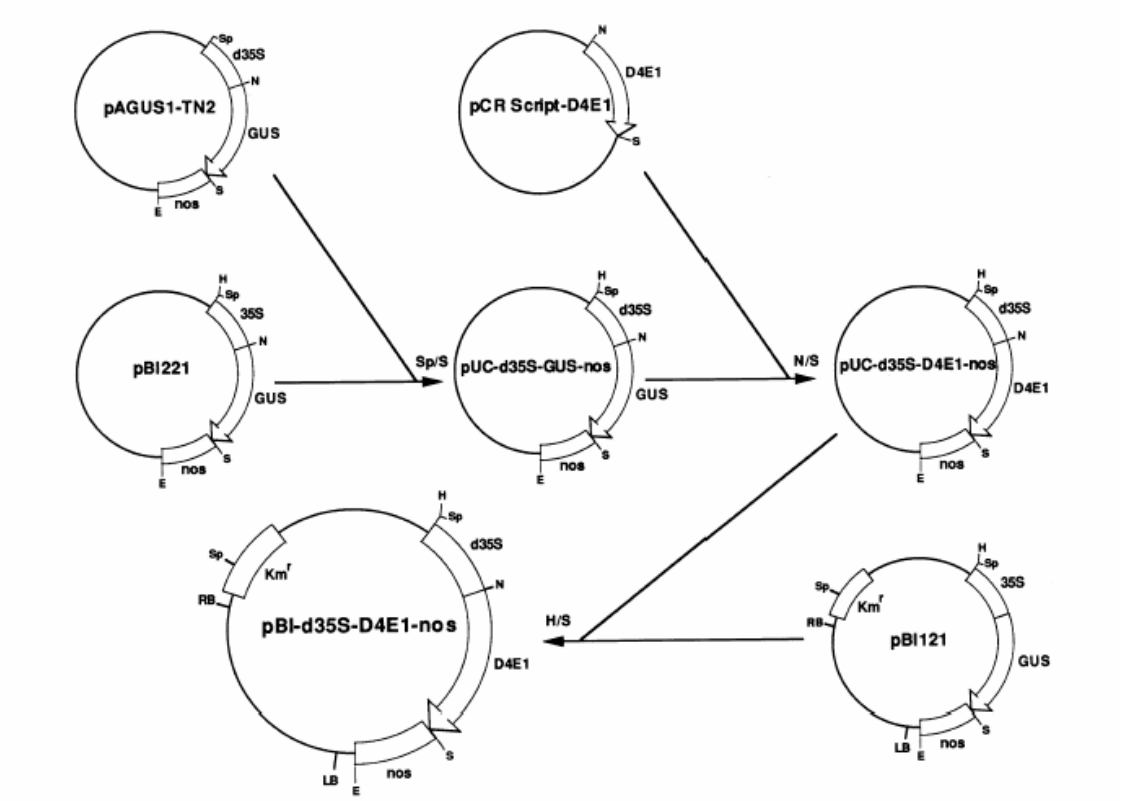

Figure 2A Diagram depicting the plasmids and enzymes used in the contruction of the binary vector pBI-d35-D4E1-nos (Cary et al., 2000).

BIOGRAPHY

Mr. Yingyos Jittayasothorn was born on September 17, 1977 in Roi-Et, Thailand. In 1995, he finished high school from Yasothorn Pittayakom, Yasothorn. He graduated the bachelor's degree of science with second class honor in Crop Production Technology from Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima in 1999. He have pursued his Doctoral degree in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology and received the Royal Golden Jubilee (RGJ) scholarship from the Thailand Research Fund in 2001. In 2002, he got outstanding of very good oral presentation in the 3rd National Symposium on Graduate Research, Thailand in the topic of "Using SSR Analysis for Grape Cultivar Identification". Then he went to Center for Viticulture and Small Fruit Research, Florida A&M University, USA to conduct his thesis research for 3 years and 4 months. While being in the US, he had poster presentations of his research projects in several international conferences including, (1) In Vitro Biology Meeting 2005, Maryland, USA, (2) International Symposium on Biotechnology of Temperate Fruit Crops and Tropical Species 2005 (co-author), Florida, USA, (3) American Society for Horticultural Science Annual Conference 2006, Louisiana, USA, (4) 9th International Conference on Grape Genetics and Breeding 2006, Udine, Italy, (5) World Congress of International Association for Plant tissue Culture and Biotechnology 2006, Beijing, China, and (6) International Horticultural Congress and Exhibition 2006, Seoul, Korea.

Moreover, he got the first place for outstanding achievement in emerging technology for graduate student competitive posters in the 1890 Land-Grant Universities Association of Research Directors 14th Biennial Research Symposium 2006, Georgia, USA under the topic of "A simple and efficient protocol for in vitro micropropagation of grape cultivars via shoot tuft production." One of his works has been published in Vitis (Journal of Grapevine Research) under the topic of "A simple and highly efficient protocol for somatic embryogenesis and plant regeneration from proembryonic mass suspension culture in 'Autumn Royal Seedless' and another 4 have been accepted for publication in Acta Horticulturae, a scientific series of International Society of Horticultural Science, in the topics of "Establishment of an efficient somatic embryogenesis and regeneration system in muscadine grape (*Vitis rotundifolia*)" (co-author), "Isolation and culture of grape protoplasts from somatic embryogenic suspension cultures and leaves of *Vitis vinifera* and *Vitis rotundifolia*" (co-author), "Somatic embryogenesis and plant regeneration from seed integument of seedless grape cultivars (*Vitis*)" (co-author), and "Genetic transformation of a seedless grape cultivar 'Autumn Royal' (*Vitis vinifera* L.)".