A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Plant Regeneration *via* Somatic Embryogenesis and *Agrobacterium*-mediated Transformation in 'Miyagawa Wase' Satsuma Mandarin

Seong-Beom Jin

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A thesis submitted in partial fulfillment of the requirement of the degree of Doctor of Philosophy

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This thesis has been examined and approved by

_____

Chairperson of the supervising committee

Professor Kwan Jeong Song, Ph.D., College of Applied Life Sciences, Cheju National University

_____

Professor Key Zung Riu, Ph.D., College of Applied Life Sciences, Cheju National University

_____

Professor Hyo Yeon Lee, Ph.D., College of Applied Life Sciences, Cheju National University

_____

Director Sang Jo Kang, Ph.D., National Institute of Subtropical Agriculture

_____

Professor Jong Tae Song, Ph.D., College of Applied Life Sciences, Kyungpook National University

Department of Agricultural Chemistry

GRADUATE SCHOOL

CHEJU NATIONAL UNIVERSITY

CONTENTS

SUMMERY IN KOREAN

온주밀감 (*Citrus unshiu* Marc.)은 배우자 불임, 다배성 및 긴 유년성 등의 식물학적 특성으로 인해 교잡 육종이 매우 어려운 작물이다. 그러므로 기존의 우량 형질을 유지하고 특정형질만을 도입하는 형질전환기술이 대안으로 제시되어 왔다. 형질전환을 위해서는 재분화와 유전자전이 방법이 확립되어야 한다. 온주밀감의 경우 다른 감귤에 비하여 식물체 재분화와 유전자 전이가 특히 어려운 것으로 알려져 있다. 따라서 본 연구에서는 온주밀감 주요 품종의 하나인 궁천조생을 이용하여 체세포배분화를 통한 식물체 재분화계와 *Agrobacterium*을 이용한 형질전환기술 체계를 확립코자 수행하였다.

온주밀감의 재분화 식물체는 캘러스 유기, 체세포배 분화, 자엽 유도, 신초 유도, 접목을 거쳐 얻을 수 있다. 본 실험에서는 성숙과실의 미발육 종자로부터 캘러스를 유기하였으며, 캘러스 유기 및 증식에 적합한 배지를 선발하고 체세포배 분화능에 미치는 캘러스 세포타입, agar농도, 탄수화물의 영향과 자엽형성에 미치는 adenine의 영향 그리고 식물체분화에 미치는 생장조절제의 영향을 조사하여 궁천조생의 식물체 재분화계를 확립하였다.

캘러스 유기 배지로서 MT, MS, 변형 MT Ⅰ, Ⅱ 및 Ⅲ 등 5종류를 검토하였는데 캘러스 형성율 (전체 종자 중 캘러스를 형성하는 종자의 비율)이 MT배지에서 가장 높았다. 유기된 캘러스의 증식에 적합한 sucrose

농도 (1, 2, 3, 5, 10, 15 및 20%)를 조사하였는데 배지 종류와 관계없이 sucrose 5%에서 캘러스 생육이 가장 양호하였다.

캘러스세포의 체세포배 분화 정도는 배지의 agar 농도 (0.8, 1.0, 1.2, 1.4 및 1.6%)에 따라서도 달라졌는데 agar 1.4%일 때 가장 높게 나타났다. 배지의 탄수화물 종류와 농도 역시 캘러스세포의 체세포배 분화에 영향을 주었는데 lactose (3, 5, 7, 9 및 11%) 와 sucrose (3, 5, 10, 15 및 20%)을 비교했을 때 sucrose 보다는 lactose의 영향이 컸고 lactose의 경우 7%일 때 체세포배 분화율이 가장 높았다. 체세포배 분화능이 우수한 세포를 선발하고자 캘러스를 percoll 농도구배 (20, 30, 40, 50 및 60%)를 이용하여 세포의 비중에 따라 5가지 타입으로 분리하고, 타입별 체세포배 분화능을 조사하였다. 체세포배 분화능은 세포의 타입에 따라 상당한 차이를 보였는데 percoll 40% 층에서 분리한 세포들이 가장 높았다.

온주밀감의 경우 캘러스 체세포배로부터 정상 식물체를 직접 얻기는 어렵고 일단 자엽을 형성시킨 다음 자엽조직 절편을 다시 배양하여 식물체 지상부를 얻게 된다. 자엽 유기 조건을 규명하기 위하여 체세포배의 자엽형성에 미치는 영향을 조사하였는데, adenine 농도 (0.3, 0.5, 0.7, 0.9 mg/L)가 0.7 mg/L인 배지에서 자엽형성이 가장 양호하였다. 자엽절편으로부터 식물체를 유기하는데 영향을 주는 요인으로 두 가지 생장조절제 naphthalene acetic acid (NAA: 0.00, 0.01 mg/L)와 benzyl adenine (BA: 0.00, 0.10, 0.50 및 1.00 mg/L)에 대하여 조사하였는데 BA 단용했을 때 보다 BA와 NAA의 혼용했을 때 식물체 재분화율이 높았으며, BA 0.10 mg/L와 NAA 0.01 mg/L의 혼용했을 때 가장 높았다. 자엽절편으로부터 유기된

지상부를 탱자 *(Poncirus trifoliana*)대목에 기내접목하여 순화과정을 거쳐 완전한 식물체를 얻을 수 있었다.

*Agrobacterium*을 이용한 온주밀감의 형질전환계를 확립하기 위하여 캘러스세포의 형질전환율에 미치는 요인들과 형질전환체 선발 조건을 조사하고, 적정화된 조건으로 형질전환식물체를 얻은 다음 도입유전자의 발현과 도입유전자의 DNA를 확인하였다. 캘러스는 궁천조생 성숙과실의 미수정 미발육 종자로부터 유기시켰고 kanamycin 저항성 유전자 (*nptII*), hygromycin 저항성 유전자 (*hpt*)및 *β-glucuronidase* 유전자 (*gus*)가 들어있는 plasmid vector (pIG121)를 가진 *Agrobacterium tumefaciens* strain LBA4404를 이용하여 형질전환 시켰다. 형질전환효율은 캘러스에 발현되는 GUS 반점의 수로 평가하였다.

형질전환체 선발물질로 항생제인 kanamycin과 hygromycin 그리고 비항생제인 sorbitol을 검토하였다. 형질전환 되지 않은 캘러스 세포의 생육이 저해되는 최저농도는 kanamycin (0, 25, 50, 100, 150 및 200 mg/L)의 경우 100 mg/L, hygromycin (0, 5, 10, 15, 20 및 25 mg/L)의 경우 10 mg/L, sorbitol (0, 2.5, 5.0 및 7.5%)의 경우 5%였다. 그러나 kanamycin의 경우 100 mg/L이상에서도 상당한 기간 캘러스의 생육이 지속되었고, sorbitol의 경우에 5% 농도 이상에서 캘러스 세포의 증식은 억제되지만 체세포배가 형성되는 경향이 있었다. 반면 hygromycin은 10 mg/L부터 세포생육이 억제되기 시작했고 25 mg/L에서는 생육이 정지되거나 갈변되어 죽기 때문에 형질전환체 선발에는 25 mg/L hygromycin이 적합하였다.

형질전환율에 미치는 요인으로 균의 접종밀도와 접종시간,

acetosyringone (AS)의 농도, 공동배양기간을 조사하였다. 균 접종밀도는 조사된 OD_{600}=0.2, 0.4, 0.6, 0.8 및 1.0 중 OD_{600}=0.6일 때 형질전환율이 가장 높았다. 균 접종시간은 0, 10, 20, 30, 40, 50 및 60 min 중 20 min에서 가장 높았다 균 접종시간은 0, 10, 20, 30, 40, 50 및 60 min 중 20 min에서 가장 높았다. AS의 농도는 조사된 0, 50, 100, 150 및 200 μM 중 100 μM 일 때 가장 높은 형질전환율을 나타내었다. 공동배양기간은 조사된 범위(0, 2, 3, 4, 5 및 6일)내에서 기간이 길수록 형질전환율이 높았으나 6일 이상 공동배양 하는 경우에 *Agrobacterium*의 오염이 관찰되어 공동배양기간은 5일이 적당하였다.

*Agrobacterium*을 접종하여 공동배양이 끝난 세포를 선발배지 (MT 기본배지에 500 mg/L malt extract, 7% lactose, 25 mg/L hygromycin, and 250 mg/L cefotaxime을 첨가한 배지)에 옮겨 6주간 배양하여 지속적인 성장을 보이는 hygromycin저항성 체세포배를 선발하였다. 선발된 체세포배는 다시 자엽유도배지 (MT 기본배지에 0.1 M sorbitol, 0.1 M galactose, 0.7 mg/L adenine, 25 mg/L hygromycin, 250 mg/L cefotaxime, 0.2% gelrite을 첨가한 배지)에 옮겨 8주 배양하여 자엽을 분화시켰다. 이 자엽은 짙은 녹색으로 계속 배양하더라도 정상적인 식물체로 분화되지는 않았으며, 항생제를 넣지 않고 0.10 mg/L BA및 0.01 mg/L NAA을 첨가한 MT배지에 자엽절편을 다시 배양했을 때 약 8 ~ 12주 후 정상적인 식물체 지상부로 분화되었다. 이들 식물체 지상부가 2 cm정도로 성장했을 때 탱자 대목에 기내 접목하여 잠정 형질전환 식물체를 얻었다.

Hygromycin 배지에서 선발한 식물체에 대해 도입된 유전자의 DNA와

발현을 분석하였다. GUS에 대한 조직화학적 분석결과 형질전환식물의 잎뿐만 아니라 유식물 전체에서 도입유전자가 발현되는 것이 확인되었다. GUS 양성인 식물체의 genomic DNA를 분리하여 polymerase chain reaction (PCR)으로 hygromycin저항성 유전자(*hpt*)와 β-glucuronidase 유전자(*gus*)에 대해 분석한 결과 조사된 8개 식물체 모두 양성이었다. 그리고 식물체 genomic DNA의 Southern분석을 통해 도입유전자를 재확인 하였는데 그 결과 조사된 6개 식물체 중 적어도 4개의 식물체에서 상기 2 유전자가 있는 것으로 나타났다.

LIST OF ABBREVIATIONS

AS	:	acetosyringone
BA	:	benzyl adenine
CaMV	:	cauliflower mosaic virus
EDTA	:	ethylene diamine tetra acetate
GA_3	:	gibberellin
GFP	:	green fluorescent protein
GUS	:	β-glucuronidase
HCl	:	hydrochloric acid
HPT	:	hygromycin phosphotransferase
NAA	:	naphthalene acetic acid
NaCl	:	sodium chloride
NH_4OAc	:	ammonuim acetate
NOS	:	nopaline synthase
NPTII	:	neomycin phosphotransferase II
PCR	:	polymerase chain reaction
PEG	:	poly ethyelene glycol
SDS	:	sodium dodecyl sulfate
TE	:	Tris-EDTA
Tris	:	Tris(hydroxymethyl)aminomethane

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plants using the ^{32}P-labeled GUS gene probe.

Figure 32. Southern blot analyses of genomic DNAs of putative transgenic plants using the ^{32}P-labeled HPT gene probe.

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SUMMARY

Satsuma mandarin has unique characteristics of gamete sterility, nucellar embryony, polyembryony, and long juvenility. Due to these characteristics of satsuma mandarin, there has been a great difficulty in the application of conventional sexual hybridization methods to breeding of these species. In these plants, genetic transformation has been proposed as a substitute technique to maintain their useful traits and replace defective ones. For the successful transformation in a crop, the establishment of plant regeneration and gene introduction system is essential. In this study, a genetic transformation system was established in 'Miyagawa Wase', a cultivar of satsuma mandarin (*Citrus unshiu* Marc.). The factors related to plant regeneration from callus and to *Agrobacterium*-mediated transformation were examined in the first and the second part, respectively.

The important steps in obtaining regenerated plants of satsuma mandarin includes callus induction, somatic embryogenesis of callus, development of cotyledon from embryogenic callus, shoot regeneration from cotyledon section and micro-grafting of shoot onto rootstock. Therefore the proper medium was screened for callus induction from unfertilized and undeveloped seeds in mature fruit. Then the effects of cell type, agar concentration and carbohydrates on embryogenic capacity of callus cell were examined. The

effects of adenine on cotyledon formation and growth regulators on shooting were also studied.

To screen an optimum medium for callus induction, five basal media [MS, MT, modified MTⅠ (EME designated by Grosser and Gmitter, 1990a), MTⅡ (1/2EME designated by Grosser and Gmitter, 1990a), and MTⅢ (ECM designated by Ling et al., 1990)] were evaluated. Of the tested media MT medium was the most effective in callus formation of seeds. The effect of sucrose concentration on callus proliferation was tested over the range of 1, 2, 3, 5, 10, 15, or 20%, and the highest callus growth was observed at 5% of sucrose in media.

For screening embryogenic cells the cultured callus cells were fractionated into five types according to cell density using percoll gradient. The applied concentrations of percoll were 20, 30, 40, 50, or 60%. When the embryogenic capacity was assessed by the number of callus cells forming green globular embryo, the cells collected from the layer of percoll 40% showed higher embryogenic capacity than those from the others.

The concentration of agar in the culture medium affected somatic embryogenesis of callus cells. Over the tested range of agar concentration (0.8, 1.0, 1.2, 1.4, or 1.6%), the highest embryogenesis was obtained at 1.4% of agar. Somatic embryogenesis of callus cells was also affected by carbohydrates in the culture medium. The effect of lactose was higher than that of sucrose. The highest ratio of somatic embryo formation was observed

at 7% of lactose.

The embryogenic callus formed cotyledon when cultured on a medium containing adenine. The tested concentrations of adenine were 0.3, 0.5, 0.7, or 0.9 mg/L, and the highest cotyledon formation was observed at 0.7 mg/L. The developed cotyledons at this stage showed abnormal morphology and did not grow further into normal plants. However, normal shoots could be regenerated when the cotyledon sections were cultured on a medium containing growth regulators.

To optimize the condition for shoot induction, the effects of naphthalene acetic acid (NAA) and benzyl adenine (BA) were examined on shoot formation of cotyledon sections. Two level of NAA, 0.00 and 0.01 mg/L, and four levels of BA, 0.00, 0.10, 0.50, or 1.00 mg/L, were tested. For shoot induction, the combined use of NAA with BA appeared to be better than single use of each. The optimum concentrations of NAA and BA were 0.01 mg/L and 0.10 mg/L, respectively. Shoots were regenerated in 8 weeks from cotyledon sections at this condition. Shoots were micro-grafted onto rootstocks of *Poncirus trifoliana* and normal plants were obtained.

In transformant screening, three kinds of selective agents, two of antibiotics and one of non-antibiotics, were examined. Kanamycin and hygromycin as antibiotics and sorbitol as a non-antibiotics were used. The tested concentrations of kanamycin were 0, 25, 50, 100, 150, or 200 mg/L. The minimal concentration was 100 mg/L for inhibition of growth in

non-transformed callus, however some of callus grew for a significant period even at this minimal or higher level. In case of hygromycin, the test range of concentration was 0, 5, 10, 15, 20, or 25 mg/L. The minimal concentration was 10 mg/L, callus growth was completely inhibited at 25 mg/L or higher level, and hygromycin showed sharp selectivity. Sorbitol was tested for the concentration of 0, 2.5, 5.0, or 7.5%. The minimal concentration was 5%, but embryogenesis was observed at this level although callus growth was suppressed. Therefore hygromycin of 25 mg/L was used for transformant selection.

To establish an *Agrobacterium*-mediated transformation system in 'Miyagawa Wase' satsuma mandarin (*Citrus unshiu* Marc.), the factors such as bacterial density, inoculation time, co-cultivation period and acetosyringone (AS) concentration which known to affect transformation efficiency were investigated. Embryogenic callus obtained from undeveloped seeds of mature fruits was used for genetic transformation. Before bacterial inoculation, embryogenic callus was cultured in liquid MS medium with kinetin 10 mg/L and sucrose 6% for 4 weeks, and then passed by 1 mm mesh and fraction by percoll gradient of 30%, 40%, or 50%. Embryogenic callus cells in the layer of percoll 40% were collected and used for inoculation. *A. tumefaciens* strain LBA4404 carrying pIG121 vector harboring *hpt* and *hpt* genes was used in this study. The number of GUS spot in callus was counted after co-cultivation and used as criteria of transformation efficiency.

The effect of bacterial density on transformation efficiency was studied over the range of OD_{600}=0.2, 0.4, 0.6, 0.8, or 1.0. The transformation efficiency was the highest where the bacterial density of OD_{600}=0.6 was used for innoculation. Innoculation time was examined for 0, 10, 20, 30, 40, 50, or 60 min. The optimum time was 20 min. The effect of AS in the medium for innoculation and co-cultivation was tested for the concentration of 0, 50, 100, 150, or 200 μM, and 100 μM of AS showed the highest transformation efficiency. Co-cultivation period also influenced on transformation efficiency. Over the tested range of 2, 3, 4, 5, or 6 days, the efficiency was increased with time. However, bacterial over-growth was observed at 6 days of co-cultivation. The 5 days of co-cultivation gave the highest transformation efficiency without bacterial over-growth.

The co-cultivated callus was transferred onto a selective medium (MT medium supplemented with 500 mg/L malt extract, 7% lactose, 25 mg/L hygromycin, and 250 mg/L cefotaxime). On this medium hygromycin-resistant somatic embryos were obtained in 6 weeks. The selected somatic embryos were transferred again onto a MT medium containing 0.1 M sorbitol, 0.1 M galactose, 0.7 mg/L adenine, 25 mg/L hygromycin, 250 mg/L cefotaxime, and 0.2% gelrite. The cotyledons were formed in 8 weeks. Normal shoots were obtained in 8 ~ 12 weeks by the culture of cotyledon sections on a MT medium containing 3% sucrose, 0.01 mg/L NAA, 0.10 mg/L BA and 0.8% agar. The shoots of 2 cm were micro-grafted onto rootstocks of *P. trifoliana.*

The putative transgenic plants were subjected to histochemical GUS assay, PCR and Southern blot analysis. The histochemical GUS assay showed that the gene was expressed in leaf, petiole, and stem. PCR and Southern blot analyses for genomic DNAs from these plants using the *hpt* or *gus* gene-specific primers and probes indicated that *gus* and *hpt* genes were stably integrated into these plants.

RESEARCH BACKGROUND

Commercial citrus species and related genera are primarily evergreen species of subtropical and tropical origins belonging to the family Rutaceae. Rutaceae contains about 33 *Citrus* genera and 203 species (Pinhas Spegel-Roy 1996). C*itrus* species have been classified on five citrus groups of mandarin (*C. reticulata* Blanco and *C.* Marc.), sweet orange (*C. sinensis* [L.] Osb.), lemon (*C. limon* Burm. f.), lime (*C. aurantifolia* L.), and grapefruit (*C. paradisi* Macf.). Mandarin species had been divided into seven citrus species (Table 1) (Frederick and Gene, 1994). The mandarin group comprises numerous species as well as intergeneric and interspecific hybrids which possess several unique characteristics, including the requirement for cross-pollination for some cultivars to achieve commercially acceptable yields.

The term 'Mandarin' is used throughout most of the major citrus-producing regions including Japan (the major producer), China, Spain and Italy. The term 'tangerine' is used to refer to most mandarin-type citrus in most of the United states and refers to more deeply pigmented mandarins in Australia and China. Mandarins are referred to as soft citrus in South Africa. Mandarins are produced primarily for the fresh fruit market and as segments (Japan, Spain and China).

The common mandarine group (*C. reticulata* Blanco) represents an

assemblage of cultivars. Common mandarins differ morphologically from satsumas in having a more upright growth habit and in general small flowers and fruits. Laminae are also generally smaller than those of satsuman and its petiole size is also reduced. Fruits having typical mandarin characteristics of a hollow central axis, easy segmentation and green cotyledon, are more difficult to peel than satsumas or fruits in the Mediterranean group. However, the peel is more readily separated than that of sweet oranges. The firmer, more adherent peel also improves handling, storage and shipping characteristics compared with satsuma mandarins. The exceptions are 'Ponkan' and 'Dancy' which when grown under humid subtropical or tropical conditions may produce puffy, difficult-to-ship fruit.

Conventional breeding of Citrus species was mainly dependant on selection of naturally occurred mutation (Nishiura, 1965). However, it is very difficult to introduce good agronomic characteristics through traditional breeding method due to its long growing period and low mutation rates. Hybridization is useful for breeding of many plants, however there is an limitation in application of this method to some species of citrus including satsuma mandarin due to their male sterility, poly-embryony, and long juvenility (Grosser and Gmitter, 1990b; Swing and Reece, 1967). Therefore, genetic transformation seems to be much more convenient method for breeding of these citrus species. However, this technique is not frequently

used in some species of citrus including satsuma mandarin that tissue culture is difficult. Therefore, for applying this technique, efficient tissue culture system and genetic transformation system should be established.

Regeneration of *Citrus* was described almost 50 years ago (Since Maheswari and Ranganswamy, 1958) and is the basis for applying modern genetics to this crop. Since many citrus types are polyembryronic and adventive embryos are produced in vivo from nucellar tissue, most studies on *in vitro* somatic embryogenes have involved the culture of adventitious embryos, isolated nucellar embryos. Embryogeneic citrus cultures have been obtained from entire fertilized ovules or nucellus of fertilized ovules (Sabharwai, 1963) Kochba *et al*, (1972) obtained embryogenic callus from unfertilized ovules of 'Shamouti' orange in which there was no evidence of nucellar or zygotic embryos (Spiegel-Roy and Kochba, 1973).

But, satsuma mandarin species which are important at citrus industry in Jeju-do and Japan had been known to be especially difficult not only to obtain callus from nucellar tissue but also to obtain hybrid because of poly-embryony and male sterility. Fortunately, some studies succeeded in inducing embryogenie callus by culture of undeveloped ovules excised from mature fruit of satsuma mandarin and in obtaining regenerated plants from the embryogenic callus (Ling *et al*., 1990; Kunitake *et al*., 1991; Han *et al*., 2002). Also, plants could be obtained by culture of embryogenic callus derived from juice vesicle of satsuma mandarin (Nito and Iwamasa, 1990).

Development of molecular biology and transformation method have been offer possibility for citrus species improvement. Several methods have been studied for citrus transformation including direct uptake of naked DNA by protoplasts, physical delivery of foreign DNA by microprojectile bombardment and biological introduction of foreign gene by *Agrobacterium*-mediated procedure.

Transformation using protoplast were investigated by several investigators (Kobayashi and Uchimiya, 1989; Vardi *et al*., 1990). Uptake of DNA into protoplast could be achieved by treatments polyethylene glycol (PEG) or electroporation (Hidaka and Omura, 1993). These methods dose not have the host range limitations. However, there were several disadvantages due to high toxicity of PEG to protoplast of several species (Constable *et al*., 1975), difficulty in separation and cultivation of protoplasts and low regeneration efficiency (Genga *et al*., 1991; Van *et al*., 1995) which resulted in low transformation efficiency. The transformation efficiency using this technique was estimated to be approximately 1.0 x 10-6 (Kobayashi and Uchimiya, 1989). Micro-projectile bombardment (Sanford *et al*., 1987) dose not has host range and can be applied to any type of plant cells. But transgenic plants obtained by using this method have usually multiple copy numbers.

Although ***Ag****robacterium*-mediated method has disadvantages of limited host range and low plant regeneration efficiency due to using carbenicillin or cefotaxime for removal of bacteria after infection (Chee and Slighton, 1992),

it has an important advantage which produce transgenic plants with single copy number of introduced gene. Recently, some investigators tried to establish *Arobacterium*-mediated transformation in several species of citrus (Pena *et al*., 2001, Kobayashi *et al*., 1996; Cervera *et al*., 2000; Yang *et al*., 2000, Cervera *et al*., 1998a; Welition *et al*., 2003). Some of them applied *Arobacterium*-mediated method to orange species and obtained significant results (Kochba and Spiegel-Roy, 1973; Kobayashi *et al*., 1983, 1985, 1988, Vardi and Spiegel- Roy, 1982).

In contrast to orange species satsuma mandarin has known to have lower stability of callus cells in embryogenic capacity and be much more difficult in plant regeneration (Han *et al*., 2005). Until present, no successful transformation system was established in satsuma mandarin. In this study, factors related to plant regeneration from callus and to *Agrobacterium*-mediated transformation were examined, and a genetic transformation system was established in 'Miyagawa Wase', a cultivar of satsuma mandarin (*Citrus unshiu* Marc.).

Table 1. A comparison of taxonomic systems for commercially important citrus scion species.

Common name	Scientific name				
	Biochemical/ genetic (Scora)	Swingle	Hodgson	Tanaka	Balley and Balley
Sweet orange	*C. reticulata*	*C. sinensis*	*C. sinensis*	*C. sinensis*	*C. sinensis*
Grapefruit	*C. reticulata*	*C. paradisi*	*C. paradisi*	*C. paradisi*	*C. × paradisi*
Lemon	*C. medica*	*C. limon*	*C. limon*	*C. limon*	*C. limon*
Lime	*C. medica*	*C. aurantifolia*	*C. aurantifolia*	*C. latifolia*	*C. aurantifolia*
Pummelo	*C. maxima*	*C. grandis*	*C. grandis*	*C. grandis*	*C. maxima*
Mandarins					
Satsuma	*C. reticulata*	*C. reticulata*	*C. unshiu*	*C. unshiu*	*C. reticulata*
Ponkan	*C. reticulata*	*C. reticulata*	*C. reticulata*	*C. reticulata*	*C. reticulata*
Dancy	*C. reticulata*	*C. reticulata*	*C. reticulata*	*C. tangerina*	*C. reticulata*
Clementine	*C. reticulata*	*C. reticulata*	*C. reticulata*	*C. clementina*	*C. reticulata*
Willowleaf	*C. reticulata*	*C. reticulata*	*C. deliciosa*	*C. deliciosa*	*C. reticulata*
King	*C. reticulata*	*C. reticulata*	*C. nobilis*	*C. nobilis*	*C. × nobilis*
Temple	*C. reticulata*	*C. reticulata*	*C. temple*	*C. temple*	*C. × tangor*

(Frederick and Gene, 1994)

PART I. Plant Regeneration *via* Somatic Embryogenesis

Abstract

An effective plant regeneration system was established in 'Miyagawa Wase', a cultivar of satsuma mandarin (*Citrus unshiu* Marc.). The proper medium was screened for callus induction from unfertilized and undeveloped seeds in mature fruit, and then the effects of cell type, agar concentration and carbohydrates on embryogenic capacity of callus cell were examined. The effects of adenine on cotyledon formation and growth regulators on shooting were also studied. Among the tested five media (MT, MS, MTⅠ, MTⅡ, or MTⅢ), MT medium was most effective in callus formation of seeds. The effect of sucrose concentration on callus multiplication was tested over the range of 1, 2, 3, 5, 10, 15, or 20%, and the highest callus growth was observed at 5% of sucrose in media. The callus cells were fractionated into five types according to cell density by centrifuge in percoll gradient (20, 30, 40, 50, or 60%), the cells collected from the layer of percoll 40% showed higher embryogenic capacity than others. The concentration of agar in the culture medium affected somatic embryogenesis of callus cells. Over the tested range of agar concentration (0.8, 1.0, 1.2, 1.4, or 1.6%) the highest embryogenesis was obtained at 1.4% of agar. Somatic embrygenesis

of callus cells were also affected by carbohydrates in the culture medium, the effect of lactose was higher than that of sucrose. The highest somatic embryo formation was observed at 7% of lactose. The embryogenic callus formed cotyledon when cultured on a medium containing adenine, the highest cotyledon formation was observed at 0.7 mg/L among the tested concentrations of adenine 0.3, 0.5, 0.7, or 0.9 mg/L. The shoots could be regenerated when the cotyledon sections were cultured on a medium containing growth regulators. Two levels of NAA, 0.00 and 0.01 mg/L, and four levels of BA, 0.00, 0.10, 0.50, or 1.00 mg/L, were tested. The optimum concentrations of NAA and BA were 0.01 mg/L and 0.10 mg/L, respectively. Shoots were regenerated in 8 ~ 12 weeks from cotyledon sections at this condition. The shoots were micro-grafted onto rootstock of *Poncirus trifoliana* and normal plants were obtained.

Introduction

Sexual hybridization is useful for breeding of many plants, however there is a limitation in application of this method to some species of citrus including satsuma mandarin due to their male sterility, poly-embryony, and long juvenility. Plant transformation seems to be much more convenient method for breeding of these citrus species. For applying this technique to a specific plant species, a proper tissue culture system should be established first. In the development of efficient tissue culture system, optimization of each condition for induction and maintenance of embryogenic callus, and for plant regeneration is important (Carimi *et al*., 1998).

Since Maheswari and Ranganswamy (1958) reported the direct induction of somatic embryos from ovules of *Citrus*. In *Citrus* the regenerations of plant were attempted with tissues of excised nucelli (Rangan *et al*,. 1968), abortive ovules (Bitters *et al*., 1970), unfertilized ovules (Button and Bornman, 1971), undeveloped ovules (Starrantino and Russo, 1980), isolated nucellar embryos (Litz *et al*., 1985) and juice vesicle of satsuma mandarin (Nito and Iwamasa, 1990). And Carimi *et al*. (1994, 1998) and Fiore *et al*. (2002) attempted with stigma and style. Gill *et al*. (1995) obtained regenerated plants using epicotyl, cotyledon, root segments. The abilities to regenerate embryos from callus cells, single cells or protoplast in sweet orange (*C. sinensis*) were relatively high (Kobayashi *et al*., 1983, 1985,

1988; Kochba and Spiegel-Roy, 1973; Vardi and Spiegel-Roy, 1982), but it is low and unstable in satsuma mandarin.

Fortunately, two studies succeeded in inducing embryogenic callus cells by culturing undeveloped ovules excised from mature fruits of satsuma mandarin and in regenerating whole plants from embryogenic callus cells (Ling *et al.,* 1990; Kunitake *et al*., 1991). However, the regeneration efficiency of protoplast culture obtained in these studies was not high enough for experiments on genetic transformation to be undertaken. Kunitake *et al*. (1991) described an efficient protoplast culture system for some cultivars of satsuma mandarin using adenine, and subsequent whole plant regeneration via somatic embryogenesis using lactose. Recently, Han *et al*. (2002) reported a plant regeneration system for citrus in which they used the cells sorted by percoll gradients for the adventitious embryo development.

On the basis of these previous results, we tried to establish an effective system for plant regeneration in 'Miyagawa Wase' satsuma mandarin (*Citrus unshiu* Marc.) which is one of the most important cultivar of citrus in Korea and Japan.

Materials and Methods

Plant materials

Mature fruits of 'Miyagawa Wase', a cultivar of satsuma mandarin (*Citrus unshiu* Marc.), were harvested from greenhouse in Cheju National University. The fruits were surface-sterilized by dipping in 70% ethanol for 5 min. The fruits were cross-sectioned, and the unfertilized and undeveloped seeds of about 1.0 ~ 2.5 mm in length were collected from inner part of juice sac (Figure 1).

Figure 1. Unfertilized and undeveloped seeds of 'Miyagawa Wase', a cultivar of satsuma mandarin (*Citrus unshiu* Marc.). A: Cross-section of mature fruit, B ~ C: An unfertilized and undeveloped seed in a juice sac, C: Scale bar in (C) 0.1 mm.

Tissue culture

MS containing 3% sucrose and 0.8% agar (Murahige and Skoog, 1969), MT containing 3% sucrose and 0.8% agar (Murashige and Tucker, 1969), MTⅠ containing 5% sucrose and 0.8% agar (Grosser and Gmitter, 1990a), MTⅡ containing 5% sucrose and 0.8% agar (Grosser and Gmitter, 1990a) and MTⅢ containing 5% sucrose and 0.2% gelrite (Ling et al., 1990) were used as basal culture media. Compositions of the media were described in table 2. The pH of all media was adjusted to 5.8 with 1.0 M or 0.1 M NaOH and then autoclaved at 1.2 ~ 1.3 kg/cm^2 pressure and 121℃ for 15 min. GA_3 used in callus induction media was filtered separately on 0.45 ㎛ membrane and added to autoclaved media just before solidification.

In callus induction agar media were used for culture of unfertilized and undeveloped seeds. Twenty seeds were placed in a petridish (90 mm in diameter) containing 30 ml of medium and sealed with parafilm (3 M Laboratory film, USA). In callus maintaining and multiplication, 0.2% gelrite media containing sucrose were used instead of agar media. The initial amount of callus cells for subculture was 100 mg per petridish (90 mm in diameter). The callus was subcultured every 4 weeks.

In culture of embryogenic callus cells, MT media containing lactose, sucrose and agar were used. The 50 mg of callus cells were cultured per petridish (90 mm in diameter). For cotyledon development, globular embryo was cultured on a medium containing adenine, 20 embryo were cultured per

petridish (90 mm in diameter). In shoot regeneration, segments of abnormal cotyledon were cultured on a medium supplemented with BA and NAA. In a petridish (90 mm in diameter), 9 segment of abnormal cotyledon were cultured.

All the cultures were maintained under in vitro culture conditions adjusted to 25±2℃ and 18 hr photo period of 45 μmol.m^{-2}.s^{-1} illumination provide by cool white fluorescent lamps.

Table 2. Compositions of basal culture media.

Nutrients	Compounds	MS	MT	MT Ⅰ	MT Ⅱ	MTⅢ
		mg/L	mg/L	mg/L	mg/L	mg/L
Macro nutrients	$CaCl_2 \cdot 2H_2O$	176.00	444.40	444.40	444.40	444.40
	NH_4NO_3	660.00	1650.0	1650.0	825.00	1650.00
	KNO_3	760.00	1900.0	1900.0	950.00	1900.00
	KH_2PO_4	68.00	150.00	150.00	150.00	150.00
	$K_2HPO_4 \cdot 2H_2O$	-	20.00	20.00	20.00	20.00
	$MgSO_4 \cdot 7H_2O$	148.00	370.00	370.00	407.00	370.00
	KCl	-	-	-	750.00	-
Micro nutrients	KI	33.20	0.83	0.83	0.83	0.83
	$CoCl_2 \cdot 6H_2O$	1.00	0.03	0.03	0.03	0.03
	H_3BO_3	248.00	6.20	6.20	6.20	6.20
	$Na_2MoO_4 \cdot 2H_2O$	10.00	0.25	0.25	0.25	0.25
	$MnSO_4 \cdot 4H_2O$	892.00	22.30	22.30	22.30	22.30
	$CuSO_4 \cdot 5H_2O$	1.00	0.03	0.03	0.03	0.03
	$ZnSO_4 \cdot 7H_2O$	344.00	8.60	8.60	8.60	8.60
Iron	$FeSO_4 \cdot 7H_2O$	1112.0	27.80	27.80	27.80	27.80
	Na_2EDTA	1492.0	27.30	27.30	27.30	27.30
Vitamines	Inositol	4000.0	100.00	100.00	100.00	100.00
	Nicotinic acid	20.00	5.00	5.00	5.00	5.00
	Pyridoxine-HCl	20.00	10.00	10.00	10.00	10.00
	Glycine	80.00	2.00	2.00	2.00	2.00
	Thiamine-HCl	20.00	10.00	10.00	10.00	10.00
Other nutrients	Glutamine	-	-	-	1550.00	-
	Malt extract	-	-	500.00	500.00	400.00
	GA_3	-	-	-	-	1.00
	Adenine	-	-	-	-	34.00

The pHs of all media were adjusted to 5.8 with 1.0 M or 0.1 M NaOH and then autoclaved at 1.2 ~ 1.3 kg/cm^2 pressure and 121℃ for 15 min.

Cell fractionation by percoll gradient

Embryogenic callus cells were sorted by percoll gradient according to the method of Han *et al*,. (2002). The callus cells of small pieces were transferred to a flask containing 30 mL liquid MS base medium, and cultured for 4 weeks with shaking at 120 rpm. The medium was replaced with new one every 2 weeks. The cultured callus cells were passed through MINI-Silver^{TM}Micro Sieve set (Rickly Hydrological company, USA) and the passed cells were collected. The callus cells were cultured for 1 week in liquid medium containing 0.1 M galactose and 0.1 M sorbitol. And then, the cells were loaded onto a gradient solution of percoll (colloidal PVP coated with silica, Sigma) in a tube and centrifuged for 5 min at 3000 ×g. The percoll concentrations were 20, 30, 40, 50, or 60 %. The cells were collected from each layer of percoll gradient and cultured for 6 weeks on MT medium containing 500 mg/L malt extract, 7% lactose and 1.2% agar.

Basic procedure for plant regeneration

Unfertilized and undeveloped seeds taken from the mature fruit were cultured for 8 weeks on a MT base medium for callus induction. The induced callus cells were multiplied by two step culture. In the first step newly induced callus was cultured for 8 weeks on a MT medium containing GA and adenine. At this step the cultures were kept in dark and subcultured every 2 weeks for preventing embryo formation. In the second step the callus was transferred onto another MT medium in which both GA and adenine were omitted for maintaining embryogenic capacity of cell. The interval of subculture in the second step was 4 weeks. At these callus multiplication stage, 0.2 % gelrite instead of agar and 5% sucrose were used in both media.

Higher embryogenic callus cells were sorted by Percoll gradient and cultured for 6 weeks on a MT medium containing malt extract and lactose. Green-color embryos were collected and cultured for cotyledon induction on the above medium for 4 weeks, and then transferred onto a MT medium containing sorbitol, galactose, gelrite and adenine for 8 ~ 12 weeks. Deep-green abnormal cotyledons were collected and sectioned into about 5 mm size. Nine segment of cotyledon were cultured in a petridish containing MT medium supplemented with BA and NAA until formation of 2 cm shoot.

Seedlings of *Poncirus trifoliata* Raf. were cultured in soil and used as

rootstocks for grafting. Aerial parts of rootstock, up to 5 ~ 7 cm from underground, were removed and then the regenerated shoot were micro-grafted. Prior to grafting the leaves were removed from the regenerated shoot The two contacting region were wrapped tightly with parafilm and transferred to 20 × 20 cm plastic pots with perlite. Grafted plants were grown in a greenhouse.

All the cultures except greenhouse culture were maintained under culture conditions adjusted to 25±2℃ and 18 hr photo period of 45 μmol.m^{-2}.s^{-1} illumination provided by cool white fluorescent lamps.

Results

Factors affecting embryogenic callus induction

Unfertilized and undeveloped seeds of 'Miyagawa Wase' satsuma mandarin (*Citrus unshiu* Marc.) were developed to embryos as shown in Figure 2 (A) when cultured on a MT medium containing 3% sucrose and 0.8% agar. However, the number of embryo formed from one seed was diverse. Callus cells were induced from the surface of these embryo for 8 weeks after culture (Figure 2 B).

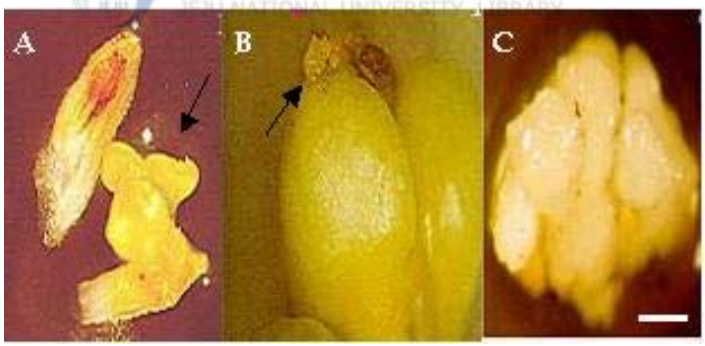

Figure 2. Callus induction from unfertilized and undeveloped seeds of 'Miyagawa Wase' satsuma mandarin (*Citrus unshiu* Marc.). Undeveloped and undeveloped seed was cultured on MT medium. A: An embryo generated from undeveloped seed (indicated by arrow). B: Callus cells formed on surface of embryo (indicated by arrow). C: multiplied callus cells. bar 3.0 mm.

To screen an optimum medium for callus induction, five media (MS, MT containing 3% sucrose and 0.8% agar, modified MTⅠ, MTⅡ and MTⅢ) were evaluated. The efficiency of callus induction was calculated by counting the number of seeds forming callus. Of the above tested media the callus induction efficiency was highest in MT with 11.6% and followed by 7.8% in MTⅡ (Table 3). Callus induction efficiencies grown under other three media, MTⅠ, MS and MTⅢ, were 6.8%, 6.2% and 6.0%, respectively.

Table 3. Effects of culture media on callus induction from undeveloped seeds of satsuma mandarin.

Culture medium[a]	Callus formation (%)[b]
MS	6.2 ± 1.7[c]
MT	11.6 ± 3.1
MT Ⅰ	6.8 ± 1.3
MT Ⅱ	7.8 ± 2.1
MT Ⅲ	6.0 ± 0.9

[a]Compositions of culture media were shown in table 2. [b](Number of seeds forming callus / Total number of tested seeds) × 100. Total number of tested seeds was 20. [c]Mean ± standard deviation of 10 replicates.

After 8 weeks the induced callus cells were taken out and cultured for another 8 weeks on MTⅢ medium at dark condition. Then, friable callus cells were collected to examine the multiplication rate of callus.

In the result of callus cells multiplication in MS, MT, MTⅠ and MTⅡ supplement with various concentration (1, 2, 5, 10, 15, or 20%) of sucrose, the weight of callus cells were increased about 4 folds over initial weight in 5% sucrose, and followed by 10% (3 folds), 5% (2 folds) and 15% (2 folds). On the other hand, in MTⅡ, the weight of callus cells cultured in 10% sucrose were much more increased than those in other concentrations, but multiplication efficiency was lower than those in MS, MT, MTⅠ, or MTⅡ supplemented with 5% sucrose (Table 4).

Table 4. Effects of sucrose concentrations in culture media on the growth of callus cells of satsuma mandarin.

Sucrose concentration (%)	Culture medium			
	MS	MT	MT Ⅰ	MT Ⅱ
1	+	+	+	+
2	+	+	++	+
3	++	++	++	++
5	++++	++++	++++	++
10	+++	+++	+++	+++
15	++	++	++	++
20	+	+	+	+

+, poor (100 ~ 200 mg); ++, fair (200 ~ 300 mg); +++, good (300 ~ 400 mg); ++++, very good (> 400 mg). The initial weight of callus cells was 100 mg per petridish of 5 replicates. Growth rates were estimated by measuring the weight of callus cells after 6 weeks. Although all media were solidified with 0.2% gelrite instead of agar, other compositions of each media were same in the table 2.

Factors affecting embryogenesis

To examine the embryogenic capacity of cells, callus cells were fractionated into 5 types by Percoll gradient (20, 30, 40, 50, or 60 %) as shown in Figure 3. The embryogenic capacity was assessed by the number of callus cells forming green globular embryo. The highest number (14.4) of somatic embryo was observed in culture of 40% percoll fraction. The callus cells in fraction of 30% and 50% percoll formed the number of 8.8 and 8.6 somatic embryo, respectively. However, in case of 20% and 60% percoll fraction, somatic embryos were less than 5 (Figure 4). Somatic embryogenesis from callus cells fractionated with 40% percoll gradation was shown in Figure 5.

The efficiency of somatic embryo formation according to agar concentration (0.8, 1.0, 1.2, 1.4, or 1.6%) was highest in 1.4% agar and the number of somatic embryo was 9.4. The next was in 1.6% agar and followed by 1.2% and 1.0% agar. In 0.8% agar, the number of somatic embryo was lowest with 7.3 (Figure 6).

In case of the efficiency of somatic embryo formation according to lactose concentration (3, 5, 7, 9, or 11%), the number of somatic embryo was highest with 21.0 in 7% lactose and followed by 19.7, 19.3 and 13.7 in 5%, 3% and 9% lactose, respectively. In 11% lactose, the number of somatic embryo was lowest with 8.3 (Figure 7). In contrast, the efficiency of somatic embryo formation in using sucrose (3, 5, 10, 15, or 20%) was lower than in

using lactose as a whole. A great numbers of somatic embryo were observed with 5.4 in 15% sucrose and the next was 1.8 in 10% sucrose. However, In the other concentrations of sucrose, somatic embryo was formed a few or not (Figure 8).

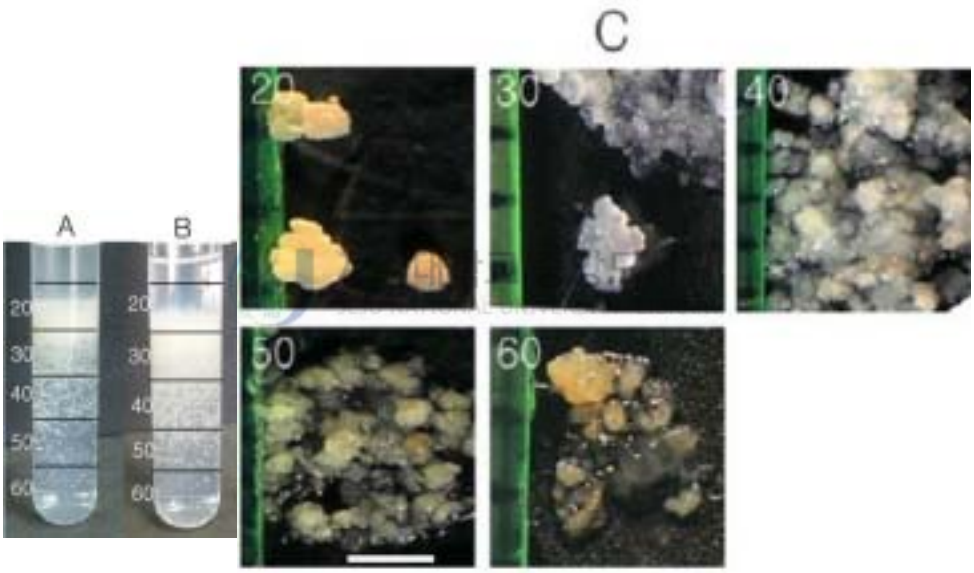

Figure 3. Fractionation of callus cells by centrifuge with Percoll gradient. A: Cell suspensions in a centrifuge tube before gradient application, B: Cell suspensions in a centrifuge tube after gradient application, C: Typical Cells fractionated in each percoll concentration. Scale bar 1.0 mm.

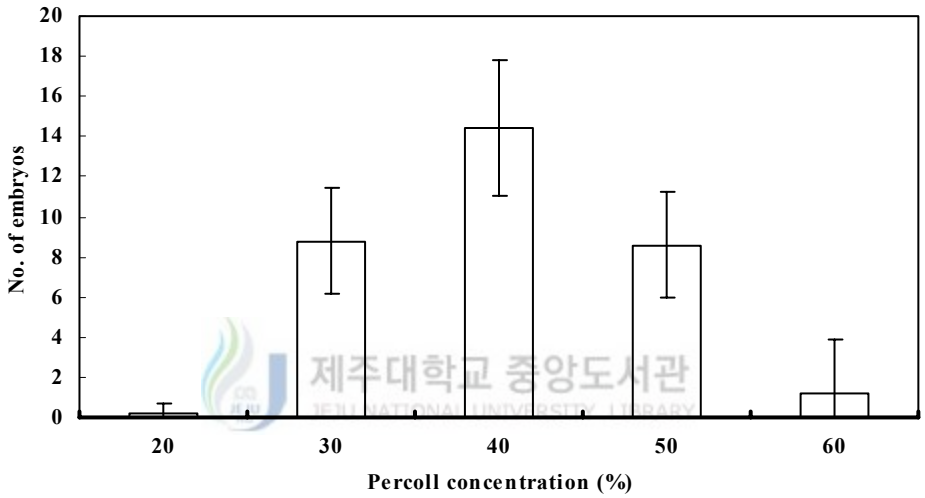

Figure 4. Embryogenic capacity of each type of callus cells fractionated by percoll gradient. The callus cells were cultured on MT medium containing 500 mg/L malt extract, 7% lactose and 1.2% agar. Columns indicate the number of somatic embryo formed from callus cells. Bars refer to standard errors of 5 replicates.

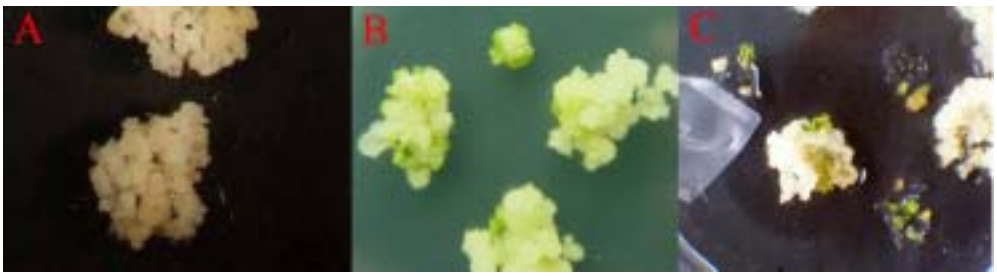

Figure 5. Somatic embryogenesis of callus cells obtained from the layer of 40% percoll after centrifugation. The callus cells were cultured on MT medium containing 500 mg/L malt extract, 7% lactose and 1.2% agar. A: The callus cells fractionated in 40% percoll layer. B: Green somatic embryo induced from callus cells. C: Globular embryo developed from B.

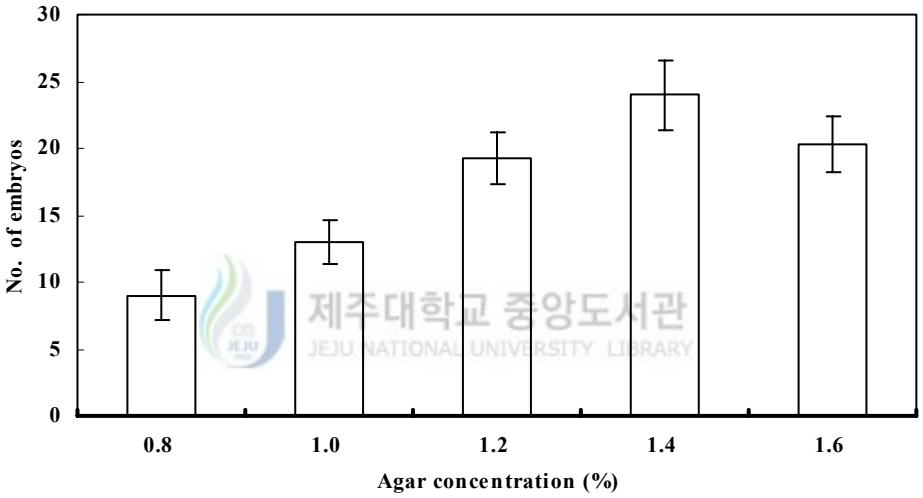

Figure 6. Effect of agar concentration in the medium on somatic embryo formation of callus cells. Columns indicate the number of somatic embryo formed from callus cells. Bars refer to standard errors of 4 replicates.

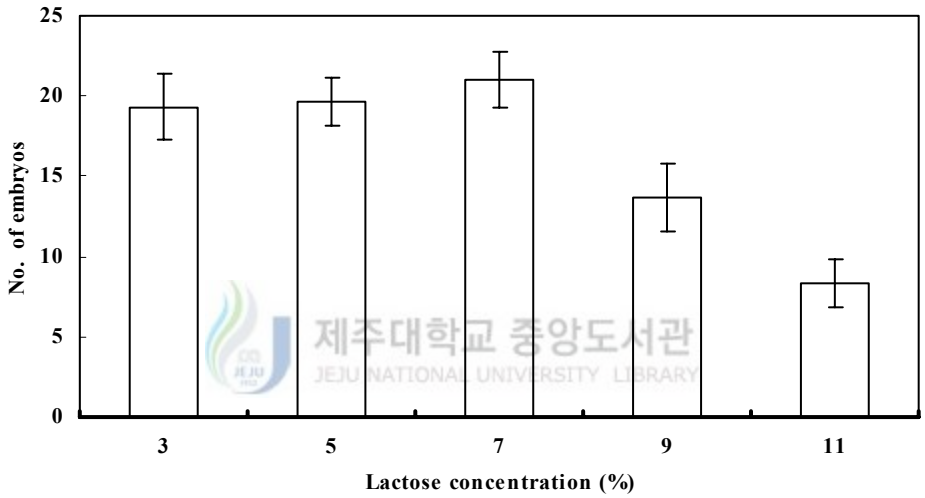

Figure 7. Effect of lactose concentration in the medium on somatic embryo formation of callus cells. Columns indicate the number of somatic embryo formed from callus cells. Bars refer to standard errors of 3 replicates.

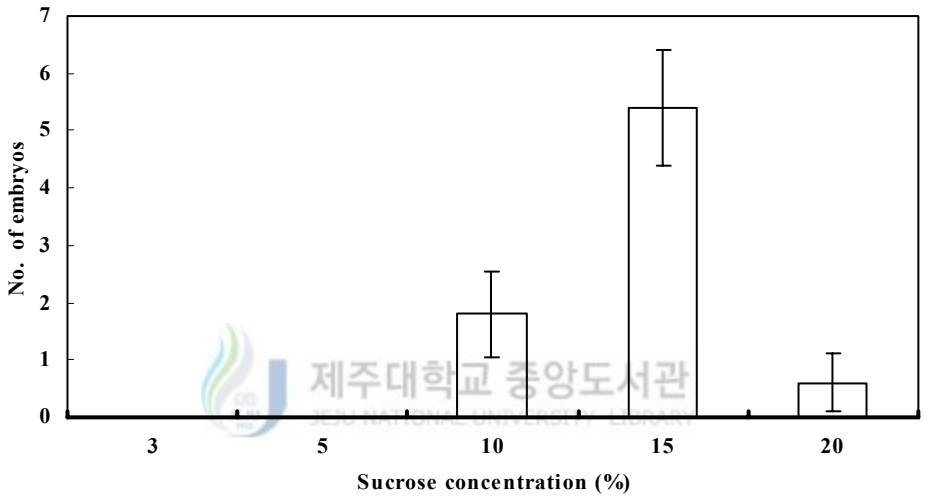

Figure 8. Effect of sucrose concentration in the medium on somatic embryo formation of callus cells. The callus cells were cultured on MT medium containing various concentration of sucrose, 500 mg/L malt extract, 1.4% agar. Columns indicate the number of somatic embryo formed from callus cells. Bars refer to standard errors of 5 replicates.

Factors affecting plant regeneration

Various concentration (0, 0.3, 0.5, 0.7, or 0.9 mg/L) of adenine were tested to investigate the best condition for cotyledon induction from globular embryo of callus. As a results, cotyledon formations were observed within a range of 0.3 to 0.9 mg/L adenine. The cotyledon was not formed in non-treatment of adenine. The highest number (13.0) of cotyledon was observed in culture of 0.7 mg/L adenine concentration (Figure 9).

When the globular embryo was cultured on the medium supplemented with 0.7 mg/L adenine, globular embryo was developed to cotyledon through torpedo-shaped embryo and heart-shaped embryo. The development stage of cotyledon from globular embryo was described in Figure 10. However, the great majority of cotyledon were developed to abnormal cotyledon or weak plant when cotyledon were continually cultured on the same medium (Figure 11).

On the other hand, for induction of abnormal cotyledon to normal plants or normal shoot, sectioned abnormal cotyledon were cultured on medium MT medium supplemented with various combinations of 0.00 or 0.01 mg/L NAA and 0.00 ~ 1.00 mg/L BA. As a results, while the number of normal shoots formed from nine segment of cotyledon was about two in non-treatment of both NAA and BA, a great numbers of normal shoots (5.6) were observed in culture of 0.01 mg/L NAA and 0.10 mg/L BA. However, the shoot regeneration was completely inhibited in 1.00 mg/ L BA or 0.01 mg/L NAA

alone. And a combination of 0.01 mg/L NAA and 1.00 mg/L BA wasn't effective for optimal shoot regeneration from segment of abnormal cotyledon (Table 5 and Figure 12).

Regenerated shoot about 2.0 cm in length was grafted on rootstock *in vitro* and then it was capped with clear bottle (13 × 7 cm ID) for preservation of humidity. After regeneration of new shoots, the clear bottle was removed and grown in green house (Figure 13).

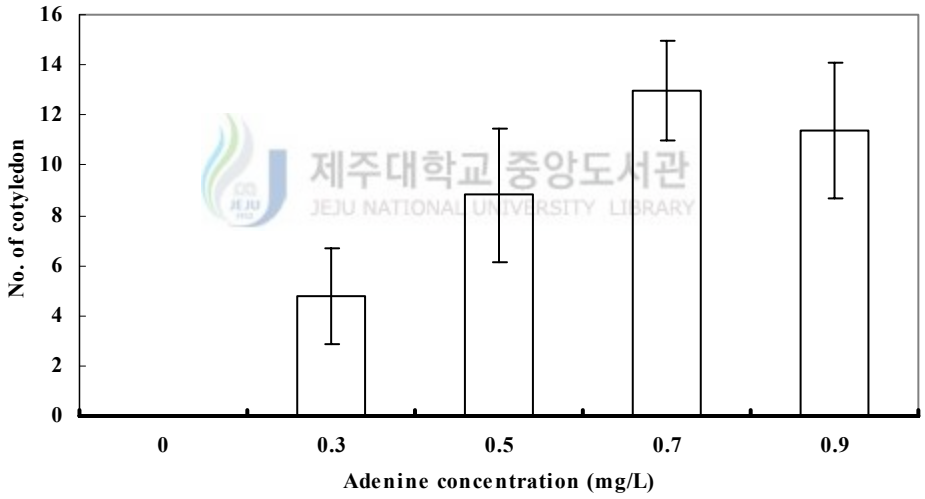

Figure 9. Effects of adenine concentration in the medium on cotyledon formation from globular embryo. Somatic globular embryos were cultured on MT Medium containing various concentration adenine, sorbitol 0.1 M, galactose 0.1 M and gelrite 0.2 % for 4 weeks. Results represent means ± standard deviation of 5 replicates.

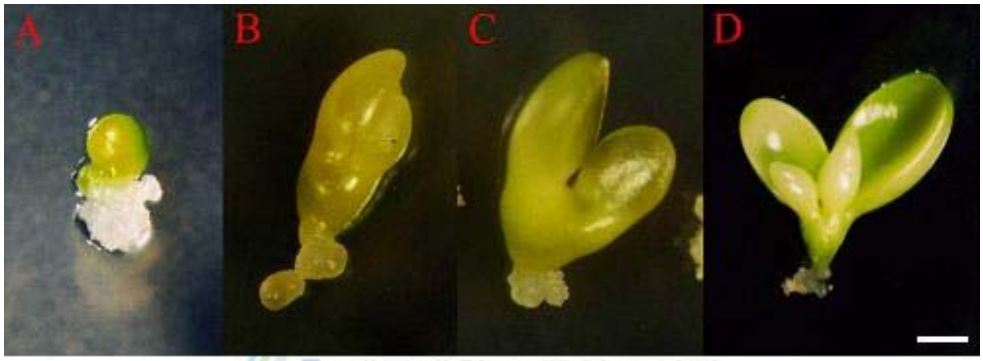

Figure 10. Development of cotyledons from globular stage of somatic embryo of callus cells. For induction of globular embryo (A), callus was cultured on MT medium containing 500 mg/L malt extract, 7% lactose and 1.4% agar for 4 weeks. The induction of cotyledon (D) from globular embryo (A) was carried out on MT medium containing 0.1 M sorbitol, 0.1 M galactose, 0.7 mg/L adenine and 0.2% gelrite for 4 weeks, bar 2 mm.

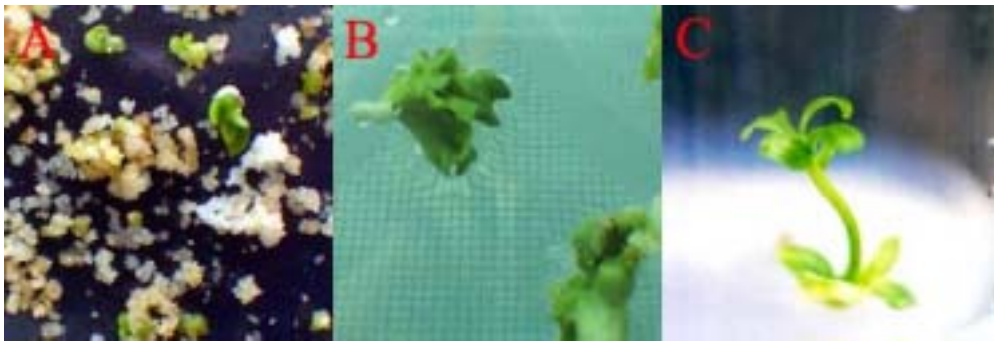

Figure 11. Development of abnormal plants with more than two cotyledons from globular stage of somatic embryos on growth regulator-free medium. Cotyledon was cultured continuously for 8 ~ 12 weeks on cotyledon induction medium, which was MT medium containing 0.1 M sorbitol, 0.1 M galactose, 0.7 mg/L adenine and 0.2% gelrite. A: Cotyledon induced from globular embryo. B: Abnormal cotyledon developed from normal after about 6 weeks culture. C: A weak plant formed from abnormal cotyledon.

Table 5. Effects of BA and NAA on shoot regeneration from segment of abnormal cotyledon in satsuma mandarin.

Growth regulators(mg/L)		No. of shoots
NAA	BA	
0	0.0	1.8 ±0.8c*
	0.1	2.0 ±0.7c
	0.5	2.4 ±0.5c
	1.0	0.0±0.0d
0.01	0	0.0±0.0d
	0.1	5.6 ±1.1a
	0.5	3.2 ±0.8b
	1.0	0.0±0.0d

Abnormal cotyledon elongated from normal cotyledon was cut with knife and segments were cultured on MT medium containing various combination of BA with NAA, 3% sucrose and 0.8% agar for 8 ~ 12 weeks. Result represent means ± standard deviation of 5 replicates. *Means separation within columns by Duncans multiple range test (MRf) at 0.0001 level.

Figure 12. Development of normal shoots from abnormal cotyledon sections cultured on a medium containing growth regulators. The segment of abnormal cotyledon was cultured on MT medium containing 0.01 mg/L NAA, 0.1 mg/L BA, 3% sucrose and 0.8% agar for 8 ~ 12 weeks. bar 2.0 mm. A: Abnormal cotyledon before cut. B ~ D: Growing stage of shoot induced from segment of abnormal cotyledon.

Figure 13. *M*icro-grafting of a regenerated shoot onto a rootstock of *P. trifoliate* and its acclimatization. A: Grafted shoot tightly wrapped with parafilm. Grafted plants were kept humidity at 25℃, 16hr of photoperiod, 45 $\mu mol.m^{-2}.s^{-1}$ illumination for 4 ~ 6 weeks. B: Grafted plant was transferred in greenhouse for acclimatization after about 6 and the photo was taken 12 weeks after grafting. C: A grafted plant after 6 months.

Discussion

In the previous reports using an unfertilized and undeveloped seed of satsuma mandarin (Ling *et al.*, 1990, Kunitake *et al.*, 1991), callus was induced mainly from the hypocotyl region of embryo. In this study, however, callus was induced on the surface of embryo developed from unfertilized and undeveloped seed of mature fruit. The quality of callus obtained from the embryo surface was good enough to regenerate plant via somatic embryogenesis..

Until now most of the researchers have used MTⅢ medium for callus induction in satsuma mandarin and in our result the efficiency (about 6%) was similar to those of the previous reports when used MTⅢ medium. However, when used MT medium containing 3% sucrose and 0.8% agar the callus induction efficiency (about 12 %) was higher than MTⅢ and other media (MS, MTⅠ, or MTⅡ). Therefore, for the callus induction in 'Miyagawa Wase' satsuma mandarin MT medium containing 3% sucrose and 0.8% agar was appeared to be more efficient than previous MTⅢ.

The callus multiplication efficiencies were simillar among medium types. This indicated that composition and level of basic nutrients may be less effective for the callus multiplication. However, multiplication efficiency of callus was significantly different depending on sucrose concentration, and difference among highest (5%) and lowest (1, 2, or 20%) was about 4 folds.

The data suggested that sucrose concentration was one of important factor, and 5% sucrose was optimum in callus muliplication of satsuma mandarin.

Embryogenic capacities of callus cells were different among the cell types fractionated by Percoll gradient. The highest number of somatic embryo was formed from callus in 40% percoll. This result was similar to the previous report that the size, density or specific gravity of cells might be one of important factor in somatic embryogenesis (Han *et al*., 2002).

Formation rate of somatic embryos was best on a medium containing 1.4% agar concentration. This concentration was higher than that of normal in plant tissue culture. Nevertheless, the result was similar to the previous report that the plant regeneration ratio was increased using 1.6% agar (Moon *et al*., 1988), and related with influence of gelling agent at callus growth and embryo development (Zimmerman and Cobb 1989). Therefore, this result suggested that the water stress may be related to embryogenesis.

In general, the somatic embryogenesis of citrus was induced by the change of carbohydrate composition (Kochba *et al*., 1978b, 1982; Ben-Hayyim and Neumann, 1983; Jimenez and Guevara 1996, 2001). Various concentration of sucrose and lactose were investigated as carbohydrate source. The results showed that lactose was more effective than sucrose, and the efficiency in 7% lactose was over 4 folds higher than in 15% sucrose. This was similar to the previous report that lactose was the most efficient factor in inducing somatic embryo from callus cells of satsuma mandarin (Kunitake

et al., 1991). Therefore, somatic embryogenesis of satsuma mandarin can be effectively stimulated by 7% lactose.

Adenine was previously used by Han *et al*. for cotyledon induction in satsuma mandarin. Adenine effectiveness were examined with five different concentration. Our results showed that the highest number of cotyledon was observed in medium containing 0.7 mg/L adenine, that is the same concentration reported by Han *et al*., 2002. However, these cotyledon developed to abnormally weak plants which were different from normal healthy ones.

For normal plant production, new method was required, so many combinations with BA and NAA were tested based on the previous study that BA and NAA were used for induction of shoot from epicotyl in orange. However, when combination with BA and NAA were treated in whole abnormal cotyledon, normal shoot or plant was not generated (results not shown). In contrary, in segment of abnormal cotyledon, normal shoot or plant was generated by treatment of combination with BA and NAA, and the best combination was 0.01 mg/L NAA and 0.10 mg/L BA. Root was generated from the regenerated shoot, but root formation was not favorable and growing rate was very slow. This problems was solved by grafting (Peña *et a*l., 1995; Bond *et al*., 1998; Kayim *et al*., 2004). Grafted shoot on rootstocks of *Poncirus trifoliata* Raf. was sucessfully grown to 10 ~ 15 cm after 6 months.

Whole plants can be obtained according to the following procedure (Figure 14). The callus is induced in 8 weeks from unfertilized and undeveloped seed in mature fruit on a MT medium containing 3% sucrose and 0.8% agar. The induced callus cells are multiplied by two step culture. In the first step newly induced callus is cultured on a MTⅢ medium instead of agar and 5% sucrose for 8 weeks in dark with subculture of 2 weeks interval. In the second step the callus is transferred onto a MT medium containing 5% sucrose and 0.8% agar. In the second step the callus is cultured for 8 weeks in light condition with 4 weeks interval of subculture.

The callus with high embryogenic capacity is isolated from the 40% percoll fraction and green somatic embryos are obtained in 6 weeks by culturing the callus cells on a MT medium containing 500 mg/L malt extract, 7% lactose and 1.4% agar. Abnormal cotyledons are developed from embryo by culturing for 8 weeks on a MT medium containing 0.7 mg/L adenine, 0.1M galactose, 0.1M sorbitol and 0.2% gelrite. Normal shoots are regenerated in 8 ~ 12 weeks from the culture of segment of abnormal cotyledon on a MT medium containing 3% sucrose, 0.8% agar, 0.01 mg/L NAA and 0.10 mg/L BA. Shoots are micro-grafted onto rootstocks of *Poncirus trifoliana* and whole plants are obtained. Using this method we increased the efficiency of callus induction and multiplication, and established the optimal conditions of somatic embyrogenesis and plant regeneration. Therefore, we suggest that this system is the optimal protocol for plant

regeneration from callus in 'Miyagawa Wase' satsuma mandarin.

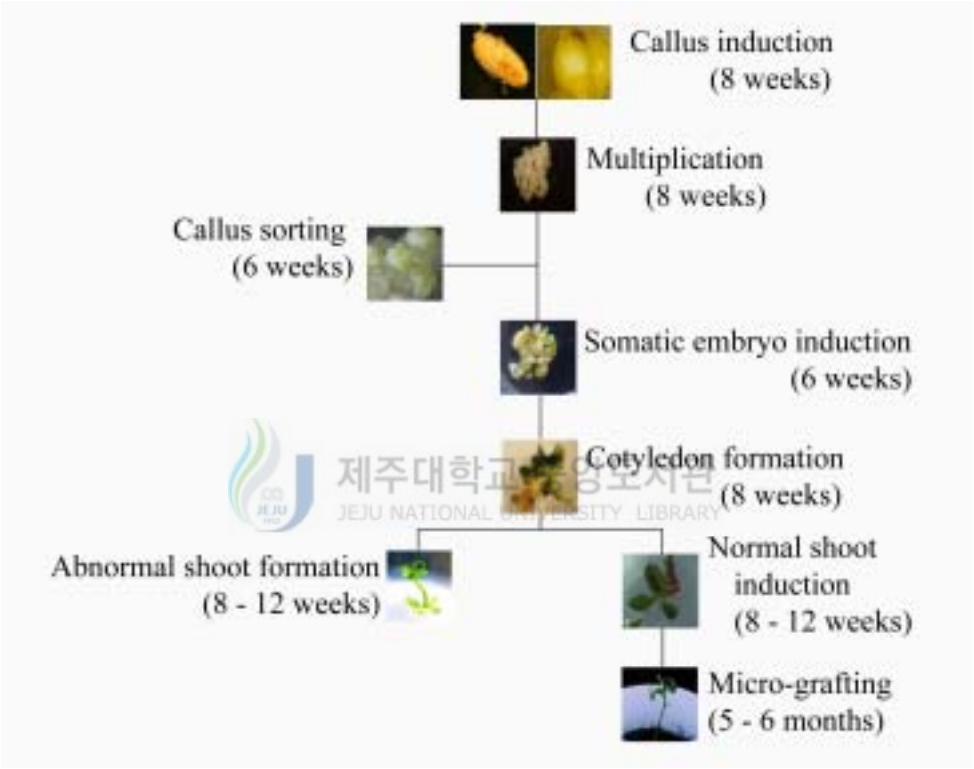

Figure 14. Overall procedure for plant regeneration from callus cells in 'Miyagawa Wase' satsuma mandarin (*Citrus unshiu* Marc.).

PART II. *Agrobacterium*-mediated Transformation in *Citrus unshiu* Marc.

Abstract

To establish an *Agrobacterium*-mediated transformation system for 'Miyagawa Wase' satsuma mandarin (*Citrus unshiu* Marc.), the factors such as bacterial density, inoculation time, co-cultivation period, and AS concentration which known to affect transformation efficiency were examined.

For genetic transformation, embryogenic callus cells induced from undeveloped seeds were infected with *A. tumefaciens* strain LBA4404 carrying pIG121 vector harboring hpt and gus genes. Transformation efficiency was evaluated by transient GUS expression. The highest transformation efficiency was obtained at 0.6 OD_{600} of bacterial density for inoculation, 20 min of inoculation time, 5 days of co-cultivation period, and 100 uM of acetosyringone concentration in the medium for inoculation and co-cultivation.

Co-cultivated callus was cultured for 4 weeks on MT Ⅰ medium and transferred onto a selective medium (MT Ⅰ medium supplemented with 7% lactose, 25 mg/L hygromycin, 250 mg/L cefotaxime and 1.2% agar).

On this medium hygromycin-resistant somatic embryos were obtained in 6 weeks. The selected somatic embryos were transferred again onto a MT

medium containing 0.1 M sorbitol, 0.1 M galactose, 0.7 mg/L adenine, 25 mg/L hygromycin, cefotaxime 250 mg/L and 0.2% gelrite. The cotyledons were formed in 4 ~ 6 weeks. Normal shoots were obtained in 8 ~ 12 weeks by the culture of cotyledon sections on a MT medium containing 0.01 mg/L NAA and 0.10 mg/L BA. The shoots of 2 cm were micro-grafted onto rootstock of *P. trifoliana.*

The putative transgenic plants were subjected to histochemical GUS assay, PCR and Southern blot analysis. The results of histochemical GUS assay showed that the gene was expressed in leaf, petiole as well as stem. The number of plant line which expressed GUS gene was 8 of 10 plant lines. When genomic DNAs of the transgenic plant lines were analyzed for hpt and gus by PCR all of the tested 8 lines showed positive DNA bands corresponding to each gene. The Southern blot analysis of genomic DNAs for gus and hpt revealed that these genes were stably integrated into at least 4 plant lines among the 6 tested lines.

Introduction

Recent developments in molecular biology and transformation method in other plant species have offered the possibility to apply a more convenient technique of molecular breeding for improvement of citrus species. As in other plants several methods can be used in transformation of citrus species, for example, direct uptake of naked DNA by protoplasts, physical delivery of foreign DNA by microprojectile bombardment and biological introduction of foreign gene by *Agrobacterium*-mediated procedure.

Transformation using protoplast were investigated by several investigators (Kobayashi and Uchimiya, 1989; Vardi *et al*., 1990). Uptake of DNA into protoplast could be achieved by treatments polyethylene glycol (PEG) or electroporation (Hidaka and Omura, 1993). These methods dose not have the host range limitations. However, there were several disadvantages due to high toxicity of PEG to protoplast of several species (Constable *et al*., 1975), difficulty in separation and cultivation of protoplasts and low regeneration efficiency (Genga *et al*., 1991; Van *et al*., 1995) which resulted in low transformation efficiency. The transformation efficiency using this technique was estimated to be approximately 1.0 x 10-6 (Kobayashi and Uchimiya, 1989). Micro-projectile bombardment (Sanford *et al*., 1987) is another efficient method for gene dilivery which is very simple, dose not has host range and can be applied to any type of plant cells. But this method has

disadvantages which requires expensive equipments and materials. One of the most critical disadvantage is that transgenic plants obtained by using this method have usually multiple copy numbers.

Compared to the above mechanical or chemical methods ***Ag****robacterium*-mediated method is based on the natural and biological process. Although this method has also several disadvantages of limited host range and low plant regeneration efficiency due to using carbenicillin or cefotaxime for removal of bacteria after infection (Chee and Slighton, 1992), it is very cheap and has an important advantage which produce transgenic plants with single copy number of introduced gene and most widely used.

As in other crops the lots of *Agrobacterium*-mediated transformation protocols have been proposed in citrus and some of them were successful to obtain transgenic plants of several citrus species. Tansgenic plants have been produced from carizo citrange (Yu *et al*., 2002), pineapple sweet orange (Cervera *et al*., 1998), navel orange (Bond & Roose, 1998), mexican lime (Dominguez *et al*., 2000), sour orange (Ghotbel *et al*., 2000), grapefruit (Yang *et al*., 2000), swingle citrumelo (Molinari *et al*., 2004), Valencia sweet orange (Li and Deng 2003), and Ponkan mandarin (Li and Deng, 2002). These previous research were focussed on the citrus species other than mandarin except Ponkan.

In contrast to other citrus species mandarin, especially satsuma mandarin has known to have lower stability of callus cells in embryogenic capacity and

be much more difficult in plant regeneration (Han *et al*., 2005). Until now, no successful transformation system was established in satsuma mandarin and no transgenic plant of satsuma mandarin has been reported. In this study, several key factors related to plant regeneration and *Agrobacterium*-mediated transformation were examined to establish an effiient genetic transformation system in 'Miyagawa Wase', a cultivar of satsuma mandarin (*Citrus unshiu* Marc.).

Materials and Methods

Plant materials and tissue culture

Mature fruits of 'Miyagawa Wase', a cultivar of satsuma mandarin (*Citrus unshiu* Marc.), were used as source of plant material for transformation. The fruits were surface-sterilized by dipping in 70% ethanol for 5 min, then cross-sectioned. Unfertilized and undeveloped seeds with the length of about 1.0 ~ 2.5 mm were isolated from inner part of juice segments, and the isolated seeds were used for callus induction without further surface-sterilization. Embryogenic callus cells were induced on MT medium 8 weeks after initial culture of unfertilized and undeveloped seeds. The multiplication of these embryogenic callus cells was conducted with four times of subculture at intervals of two weeks to obtain plenty of callus cells for genetic transformation on MTⅢ medium. The multiplied embryogenic callus cells were maintained with continuous subculture at intervals of four weeks on MT medium containing 5% sucrose and 0.8% agar.

The selection of embryogenic callus cells with high frequency of somatic embryogenesis was conducted by liquid culture and separation with concentration gradient of percoll. Embryogenic callus cells were cultured for four weeks on liquid MS medium containing 10 mg/L kinetin and 6% sucrose by shaking at 120 rpm, broken into pieces with passing by 1.0 mm MINI-Silver^{TM}Micro Sieve (Rickly Hydrological company, USA), and then

fractionated by percoll gradient at 30, 40, or 50% concentration and centrifugation at 3000 ×**g** for 5 min. Embryogenic callus cells positioned in 40% percoll layer were collected and used as materials for an inoculation of *Agrobacterium*.

Bacterial strain and plasmid vector

Agrobacterium tumefaciens strains, LBA4404, harboring pIG121 vector was used for transformation. The T-DNA region of the binary vector pIG121 consisted of the *neomycin phosphotransferase* II (NPTII) gene under the control of nopaline synthase (NOS) promoter, the *hygromycin phosphotransferase* (HPT) gene under the control of cauliflower mosaic virus (CaMV) 35S promoter, and the *β-glucuronidase* (GUS) gene with an intron (GUS-intron) fused to the CaMV35S promoter (Figure 15).

Bacteria was incubated on solid YEB medium (5 g/L beef extract, 1.0 g/L yeast extract, 5 g/L peptone, 5 g/L sucrose, 0.5 g/L $MgSO_4$, 5 g/L sucrose and 1.5% agar) supplemented with 50 mg/L hygromycin and 50 mg/L kanamycin, for 3 days at 28℃. A single colony was cultured in liquid YEB medium overnight at 28℃ with shaking (150 rpm). Bacterial cells were centrifuged at 3,000 ×g for 10 min in room temperature and pellet was resuspended with co-cultivation medium.

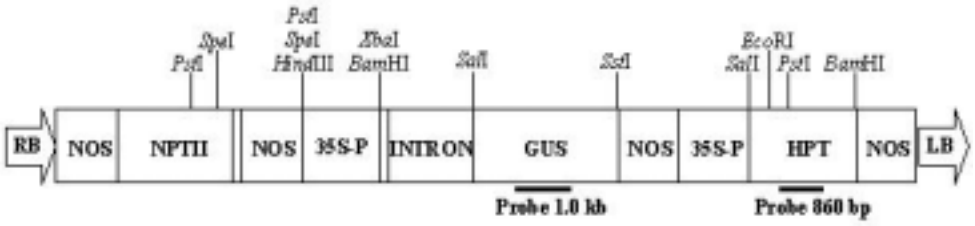

Figure. 15. Schematic diagram of T-DNA region of pIG121 plasmid vector used for transformation. NPT II: neomycin *phosphotransferase* II, 35S-P: CaMV 35S promoter of cauliflower mosaic virus, HPT: hygromycin *phosphotransferase*, GUS: *β-glucuronidase*, NOS: Terminator of nopaline synthase gene.

***Agrobacterium-mediated* transformation**

(1) *A. tumefaciens* LBA 4404 containing pIG121 was grown in 50 ml liquid YEB medium supplemented with 50 mg/L kanamycin and 50 mg/L hygromycin by shaking at 150 rpm in 28℃ until bacteria density reached to 0.2, 0.4, 0.6, 0.8, or 1.0 at OD_{600}.

(2) The overnight culture of *A. tumefaciens* was centrifuged at 3,000 ×g for 10 min, and pellet was resuspended with 50 ml resuspension medium (MTⅠ medium, 100 uM AS and pH 5.2) to the density of 0.2, 0.4, 0.6, 0.8, or 1.0 at OD_{600}.

(3) Pre-cultured embryogenic callus cells were immersed in suspended bacteria culture for 0, 10, 20, 30, 40, 50, or 60 min with shaking at 150 rpm at 28℃. Embryogenic callus cells were then briefly blot-dried on filter

paper, and co-cultivated on the medium (MT Ⅰ medium with 50, 100, 150, 200, or 300 uM AS and pH 5.2) at 25℃ in the dark for 0, 1, 2, 3, 4, 5, or 6 days.

(4) For bacteria elimination, embryogenic callus cells were washed with sterile double distilled water containing 600 mg/L carbenicillin after co-cultivation and was dried on filter paper.

(5) Embryogenic callus cells were cultured on the MT Ⅰ medium supplemented with 250 mg/L cefotaxime for 4 weeks.

(6) For the first selection, embryogenic callus cells were cultured for embryo induction on the MT medium supplemented with 7% lactose, 500 mg/L malt extract, 25 mg/L hygromycin, 250mg/L cefotaxime, and 1.2% agar for 12 weeks at intervals of 6 weeks.

(7) The hygromycin-resistant globular embryos were cultured for cotyledon development on MT medium containing 0.1 M sorbitol, 0.1 M galactose, 7 mg/L adenine, 25 mg/L hygromycin, 250 mg/L cefotaxime, and 0.2% gelrite for 8 weeks.

(8) The segments sectioned from hygromycin-resistant abnormal cotyledons were cultured on the MT medium containing 0.01 mg/L NAA, 0.1 mg/L BA, 3% sucrose, and 0.8% agar for 12 weeks. Then, the normal shoots were grafted onto *trifoliate* orange (*Poncirus trifoliata* Raf.) rootstocks. Grafted plants were acclimatized in greenhouse.

Histochemical analysis for GUS expression

Histochemical GUS staining was performed according to the method of Jefferson (1987). Callus cells or leaves were incubated overnight at 37℃ in X-gluc reaction buffer containing 34 mM phosphate buffered saline, 10mM EDTA (pH8.0), 0.5mM $K_3[Fe(CN_6)]_3H_2O$, 0.5 mM $K_4[Fe(CN_6)]_3H_2O$, 2mM X-gluc, and 0.1% triton X-100. Chlorophyll was removed from the plant tissues by soaking in ethanol 70%. GUS spots were observed under optical microscope.

Isolation of genomic DNA

Total genomic DNA was isolated from leaf tissues of control (non-transgenic plants) and putative transgenic plants by the protocol modified from the method of Shure *et al*., (1983). About 0.2 ~ 0.5 g leaves were frozen in liquid nitrogen and homogenized manually with mortar and pestle. Then, it was mixed with pre-heated 700 ㎕ DNA extraction buffer (7 M urea, 0.35 M NaCl, 50 mM Tris-HCl/pH 8.0, 20 mM EDTA, 1% sarcosine, and 0.6% SDS) and the mixture was transferred to 1.5 ml microtube. It was incubated at 65℃ for 5 min with gentle inversions. After addition of 700 ㎕ of phenol/chloroform solution, it was incubated for 10 min with gentle inversions, and centrifuged at 10000 ×**g** for 10 min. The upper layer was collected, mixed additionally with an equal volume of chloroform, and incubated for 5 min more. Again after centrifugation at

10000 ×g for 10 min, the upper layer was collected. The upper layer was provided with 1/10 volume of 4.4 M NH_4OAc/pH5.2 and equal volume of isopropyl alcohol. Genomic DNA was pooled out, rinsed with 70% ethanol, dried and resuspended with of 100 ㎕ TE buffer (pH8.0).

Polymerase chain reaction analysis

Polymerase chain reactions (PCR) were carried out by amplifying the some regions of transgenes using the following sets of oligonucleotide primers: *gus*, 5'-CAA CGA GCT GAA CTG GCA GA-3' (forward) and 5'-GGC ACA GCA CAT CAA AGA GA-3' (reverse); *hpt*, 5'-GAT GTA GGA GGG CGT GGA TAT GTC-3' (forward) and 5'-CTT CTA CAC AGC CAT CGG TCC AGA-3' (reverse).

Southern blot analysis

For southern blot analysis, about 10 ㎍ of *Hind*III or *Hind*III/*Sac*I-digested genomic DNA samples were electrophoresed through 0.8% (w/v) agarose gel and treated by depurination in 0.25 N HCl for 10 min, denaturation in 0.5 N NaCl for 1 hr, neutralization in 1.0 M Tris (pH 7.5) and 1.5 M NaCl for 1 hr, and homogenization in 10Ⅹ SSC for 1 hr. *gus* probe and *hpt* probes were prepared by PCR using specific internal primers. and the purification of PCR products with PCR Purification kit (Roche, Germany).

Pre-hybridization was conducted at 60℃ for 30 min in ExpressHyb solution (Clontech, Palo Alto, CA, USA). Hybridization was carried out for 24 h at the same temperature with denatured probe. And then, the blots were washed several times in 2X SSC containing 0.05% SDS at room temperature for 40 min and were washed twice in 0.1X SSC containing 0.1% SDS at 50℃ for 40 min. The membranes were exposed to X-ray film (Biomax-MS, Kodak, NY, USA) for an appropriate time.

Results

Selection markers for transformant screening

To investigate the effective selection reagents for citrus transformation, non-transgenic callus was placed on the MT medium containing 7% lactose, 500 mg/L malt extract, 1.2% agar, and antibiotic of kanamycin (0, 25, 50, 100, 150, or 200 mg/L) or hygromycin (0, 5, 10, 15, 20, or 25 mg/L). Also, sorbitol was used as alternation substance of antibiotic. Various concentrations of sorbitol (0, 1, 2.5, 5, or 7.5%) were added to the MT medium containing 3% sucrose and 0.8% agar. The nine clusters of callus were placed in a petridish and cultured with five replications. Callus cells were multiplied well in free antibiotic medium. However, in the medium containing more than 50 mg/L kanamycin or 10 mg/L hygromycin callus growth was suppressed for 6 weeks (Fig. 16 and Fig. 17). Specially, the inhibition of callus growth was observed markedly from hygromycin than kanamycin and the selective medium supplemented with even more than 100 mg/L kanamycin showed the tendency of embryo formation from survived callus (Fig. 16 and Fig. 17).

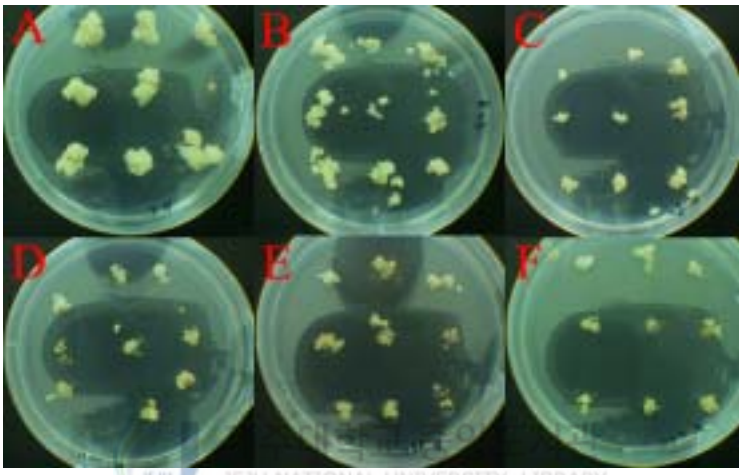

Figure. 16. Growth of non-transformed callus cells on the media containing kanamycin. The callus cells were cultured on MT medium that contained 500 mg/L malt extract, 7% lactose and 1.2% agar for 6 weeks. Concentrations of kanamycin were 0 (A), 25 (B), 50 (C), 100 (D), 150 (E) and 200 mg/L (F). The nine clusters of callus cells were placed in a petridish. A: The callus cells were multiplied well in kanamycin non-treatment. B ~ F: The callus cells were not grown and multiplied.

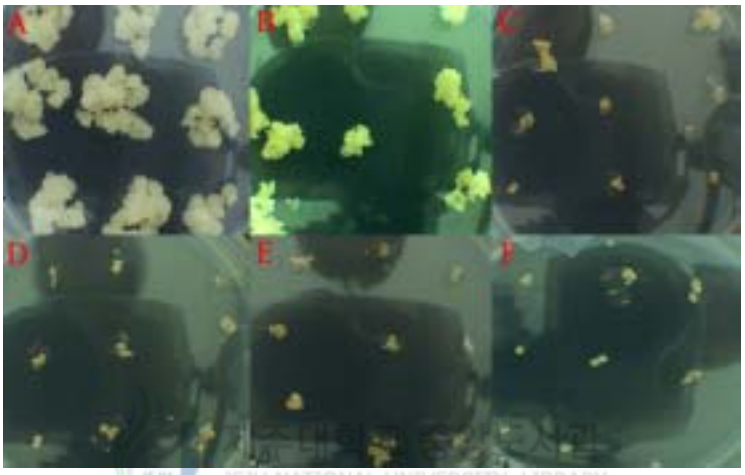

Figure 17. Growth of non-transformed callus cells on the media containing hygromycin. The callus cells were cultured on MT medium containing 500 mg/L malt extract, 7% lactose and 1.2% agar for 6 weeks. Concentrations of hygromycin were 0 (A), 5 (B), 10 (C), 15 (D), 20 (E) and 25 mg/L (F). The nine clusters of callus cells were placed in a petridish. A: The callus cells multiplied well in the medium without hygromycin. B: The multiplication rate of callus cells were lower than that in the Figure A. C ~ F: The callus cells were not grown and multiplied.

From the experiment using sorbitol, callus multiplication and somatic embryo formation decreased according to the increase of sorbitol concentration The callus cells were multiplied well in the medium without sorbitol. The multiplication rate of callus was good in the media containing 1% sorbitol, which is still lower than that in the non-treated medium. The callus was inhibited completely in 5% sorbitol media (Fig. 18).

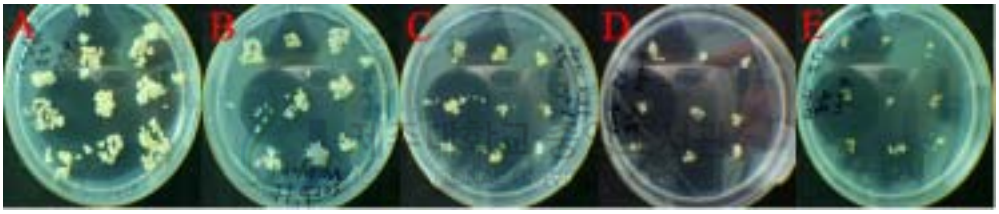

Figure. 18. Growth of non-transformed callus cells on the media containing sorbitol. The callus cells were cultured on MT medium containing 0.8% agar for 6 weeks. Concentrations of sorbitol were 0 (A), 1.0 (B), 2.5 (C), 5.0 (D) and 7.5% (E). The nine clusters of callus cells were placed in a petridish and cultured five replications. A: The callus cells multiplied well in the medium without sorbitol. B: The multiplication rate of callus cells was good, but still lower than that in the Figure A. C ~ E: the callus cells were not grown.

Factors effecting transformation efficiency

To establish an optimum condition for *Agrobacterium*-mediated transformation, the effects of bacterial density (0.2, 0.4, 0.6, 0.8, or 1.0 in OD_{600}), inoculation time (0, 10, 20, 30, 40, 50, or 60 min), co-cultivation period (2, 3, 4, 5, or 6 day) and AS concentration (0, 50, 100, 150, or 200 uM) were examined. The number of GUS spot in callus was counted under optical microscope after co-cultivation and used as criteria of transformation efficiency (Figure 19).

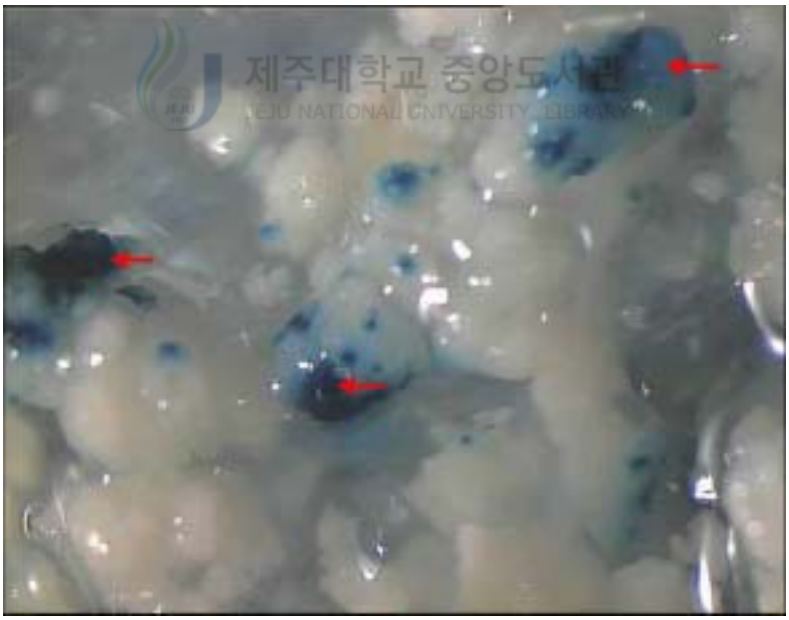

Figure 19. Transient GUS expression of callus cells after co-cultivation with *A. tumefaciens* LBA4404 containing pIG121.

The effect of bacterial density for innoculation on transformation efficiency was examined over the range of 0.2 ~ 1.0 at OD_{600}. The transformation efficiency was increased with bacterial density up to 0.6 of OD_{600} and then decreased. The highest efficiency was obtained in 0.6 of OD_{600} (Figure 20).

The effect of innoculation time on transformation efficiency was examined over the range of 0 ~ 60 min. The transformation efficiency was increased with innoculation time up to 20 min after then decreased. The highest efficiency was obtained in 20 min (Figure 21). Also, the result showed that the more inoculation time is long the more transformation rates is down.

The effect of co-cultivation period on transformation efficiency was examined over the range of 0 ~ 6 days. The transformation efficiency was increased with co-cultivation period over the tested period (Figure 22). However, bacterial growth was observed from 6 days of co-cultivation. Therefore optimum co-cultivation period was 5 days.

The effect of AS concentration in co-cultivation medium on transformation efficiency was examined over the range of 0 ~ 300 uM. The transformation efficiency was increased with AS concentrations up to 100 uM and then decreased (Figure 23).

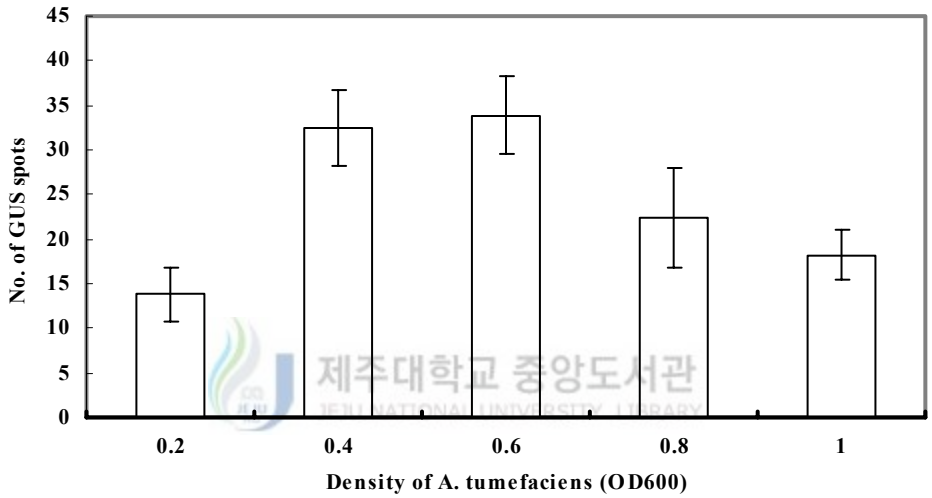

Figure 20. Effect of bacterial density on transformation efficiency. Columns indicate the number of transient GUS expression spots observed under optical microscope. Bar refers to standard error of 5 replicates.

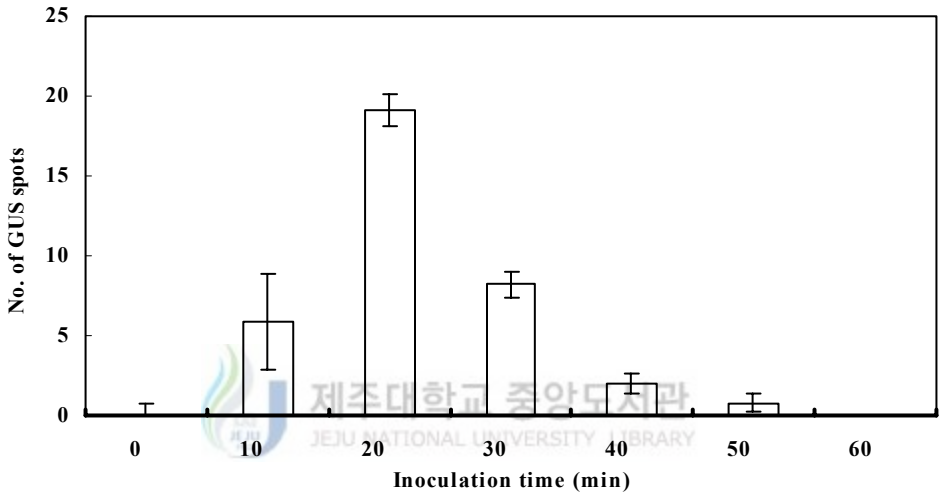

Figure 21. Effect of inoculation time on transformation efficiency. Columns indicate the number of transient GUS expression spots observed under optical microscope. Bar refers to standard error of 5 replicates.

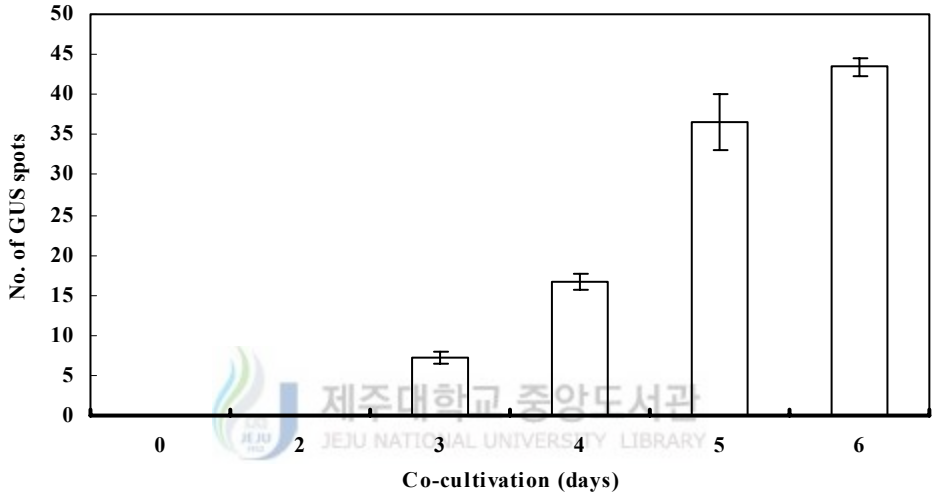

Figure 22. Effect of co-cultivation period on transformation efficiency. Columns indicate the number of transient GUS expression spots observed under optical microscope. Bar refers to standard error of 5 replicates.

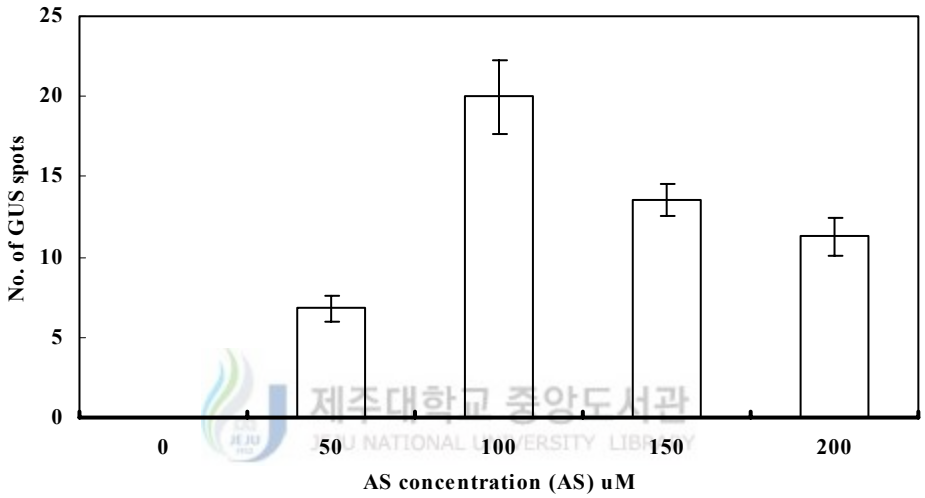

Figure 23. Effect of acetosyringone (AS) concentration in co-cultivation medium on transformation efficiency. Columns indicate the number of transient GUS expression spots observed under optical microscope. Bar refers to standard error of 5 replicates.

Shoot induction from transformed cell

To obtain a whole plant from co-cultivated callus with *Agrobacterium*, the callus obtained from undeveloped seed was innoculated with *Agrobacterium*. Co-cultivated callus was cultured for 12 weeks on selection medium of MT supplemented with 500 mg/L malt extract, 25 mg/L hygromycin, 250 mg/L cefotaxime, 7% lactose, and 1.2% agar (Figure 24 A). On this selection medium, embryogenesis occurs usually in 12 weeks (Figure 24 B).

Embryogenesis callus was cultured for weeks to induce cotyledon on the MT medium containing adenine 0.7 mg/L, 0.1 M galactose, 0.1M sorbitol and gelrite 0.2% (Figure 24 C). The cotyledons were sectioned and cultured on a regeneration medium of MT containing 0.10 mg/L BA and 0.01 mg/L NAA. Shoots could were regenerated in 8 ~ 12 weeks on this medium (Figure 24 D). Also, non-transformed plants were observed a stop of growth on MT medium containing 25 mg/L hygromycin medium after 3 weeks, whereas transformed plants continuously grew well after 4 weeks (Figure 25). The regenerated shoots about 2 cm in length were grafted onto rootstocks in soil and then it was capped with clear bottle (13 × 7 cm ID) for preservation of humidity (Figure 26). After growth of new leaf, the clear bottle was removed and grown in green house (Figure 26).

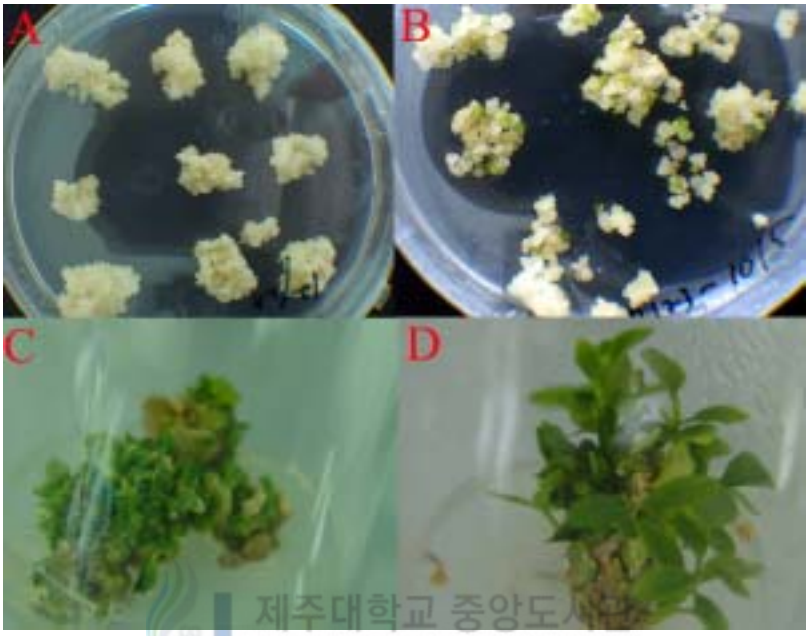

Figure 24. Shoot regenerations from embryogenic callus cells transformed with *A. tumefaciens* LBA4404 containing pIG121. A: The callus cells cultured for 4 weeks on a medium containing cefotaxime after co-cultivation. B: Hygromycin-resistant embryos generated from transformed callus cells after 6 weeks culture on the selective hygromycin medium. C: Abnormal cotyledones induced from transformed somatic embryos after 8 weeks culture on cotyledon induction medium. D: Multi-shoots induced from segment of abnormal cotyledones after 8 weeks culture on a medium containing NAA and BA.

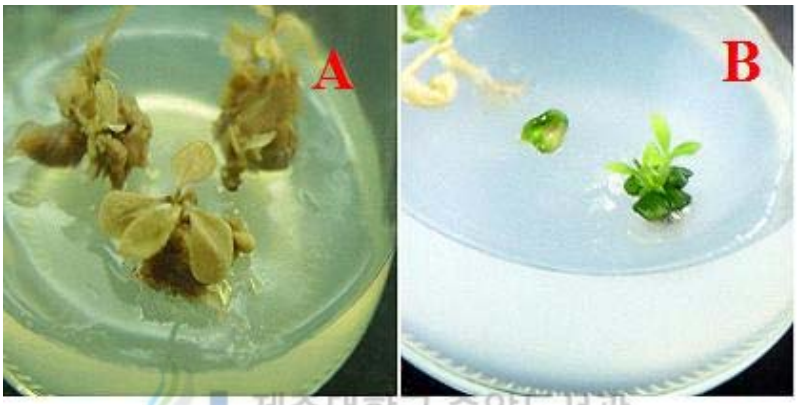

Figure 25. Growth of transgenic plants on the hygromycin medium. A: Non-transformed plants cultured for 3 weeks on the MT medium containing 25 mg/L hygromycin. B: Transformed plants cultured for 4 weeks on the MT medium containing 25 mg/L hygromycin.

Figure 26. Putative transgenic plants micro-grafted onto rootstocks of *P. trifoliate*. Acclimation of grafted transgenic shoots in green house was carried out for about 5 ~ 6 months.

Expression of reporter gene in transgenic plants

For histochemical GUS assay for screening transformants, basal portions of regeneration shoots were incubated overnight at 37℃ in X-gluc reaction buffer. but only 8 out of 10 shoot of analysed was GUS positives. GUS negative shoots only showed white tissues, whereas GUS positive shoots gave strong and uniform blue reactions in all tissues (Figure 27).

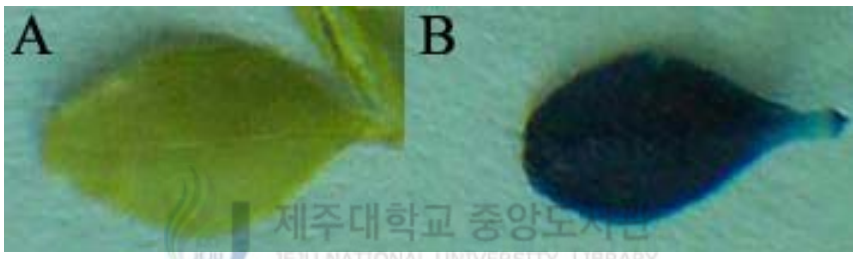

Figure 27. GUS expression in a leaf of transgenic plant. (A) Non-transformed control leaf and (B) Leaf of transformed plant.

Normal shoots were obtained in 8 weeks by the culture of cotyledon sections on a MT medium containing 0.01 mg/L NAA and 0.10 mg/L BA. The shoots of 2 cm were micro-grafted onto rootstock of *P. trifoliana.* The putative transgenic plants were submitted to histochemical GUS assay. The results of histochemical GUS assay showed that the gene was expressed in leaf, petiole as well as stem. The number of plant line which expressed GUS gene was 8 of 10 plant lines (Figure 28).

Figure 28. GUS expression in shoots of transgeneic plants. Blue staining indicates GUS expression.

PCR analysis of introduced genes

To confirm the presence of the *gus* or *hpt* genes in the genome of putative transformants, PCR amplification was performed using each specific gene primers. The 1.0 kb *gus* gene fragments was amplified from transgenic citrus plants. However, no signal was found from sample of non - transformed control plant (Figure 29). Another PCR analysis using the *hpt* gene-specific primers also showed that transgenic plants contain the *hpt* gene (Figure 30).

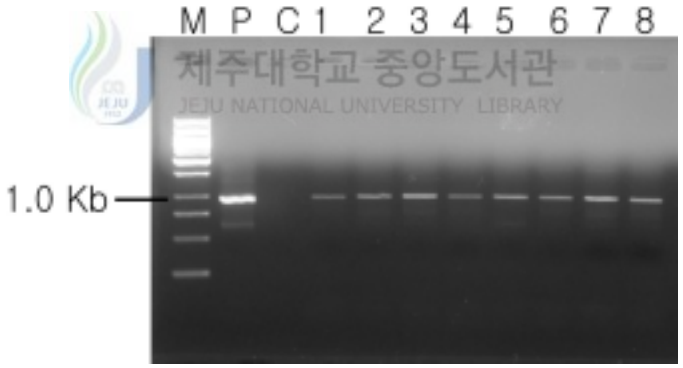

Figure 29. PCR analyses of genomic DNAs of putative transgenic plants with the specific primers for GUS gene. PCR amplification of a 1.0 kb fragment of the GUS gene in putative transgenic citrus plants (line 1 ~ 8). M, DNA size marker; P, positive control (plasmid); C, negative control; lines 1～8; putative transgenic citrus plants lines; B, Blank without DNA.

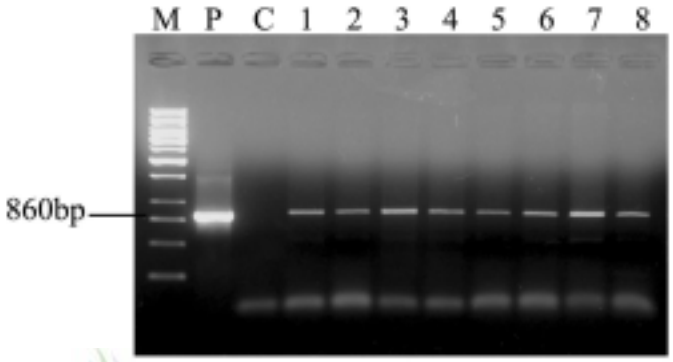

Figure 30 PCR analyses of genomic DNAs of putative transgenic plants with specific primers for HPT gene. PCR amplification of a 860 bp fragment of the hygromycin gene in putative transgenic citrus plants (line 1 ~ 8). M, DNA size marker; P, positive control (plasmid); C, negative control; lines 1～8; putative transgenic citrus plants lines; B, Blank without DNA.

Southern blot analysis of introduced genes

In order to verify stable transformation, six independent PCR-positive lines were analyzed by Southern blot. Southern analysis was performed to confirm the presence of the *gus* and *hpt* genes.

Genomic DNAs were extracted from randomly selected 6 transgenic lines among PCR-positive lines and digested with *Hind*III/*Sac*I to confirm the transgene integration. Radioactively labelled DNA fragment (1.0 kb) corresponding to the GUS coding region was used as probe. Non-transgenic line revealed no band, whereas transgenic lines 1, 2, 4 and 5 showed expected band (3.0 kb). But expected bands were not appeared in transgenic lines 3 and 6 although the *gus* gene was detected by PCR (Figure 31). Also, Genomic DNAs were extracted from randomly selected 6 transgenic lines among PCR-positive lines and digested with *Hind*III to confirm the transgene integration. Radioactively labelled DNA fragment (860 bp) corresponding to the HPT coding region was used as probe. The inserted *hpt* gene was detected in transgenic lines 1, 2, 3, 4 and 5, but not appeared in transgenic line 6 (Figure 32).

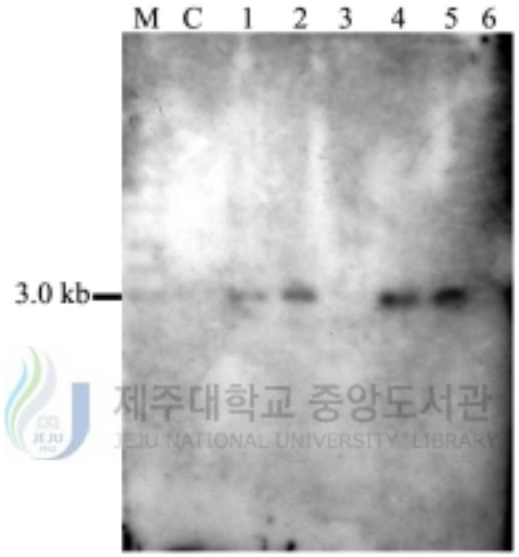

Figure 31. Southern blot analysies of genomic DNAs of putative transgenic plants with ^{32}P-labeled GUS gene probe. About 10 μg of *Hind*III/*Sac*I-digested genomic DNA samples were probed with ^{32}P-labelled GUS probe. Bands corresponding to the 3.0kb fragment. M, DNA size maker; C, non-transgenic citrus plant; Line 1 ~ 6, putative transgenic citrus plants.

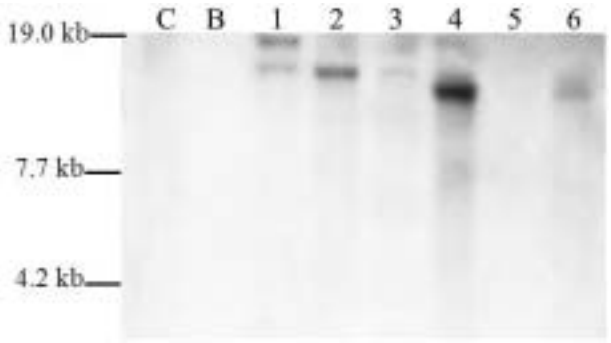

Figure 32. Southern blot analyses of genomic DNAs of putative transgenic plants with ^{32}P-labeled HPT gene probe. About 10 μg of *Hind*III-digested genomic DNA samples were probed with ^{32}P-labeled HPT probe. C, non-transgenic citrus plant; B, blank without genomic DNA; Line 1 ~ 6, putative transgenic citrus plants.

Discussion

We couldn't see effects of kanamycin, although previous researchers used kanamycin as an antibiotic for selection of transgenic citrus embryogenic callus cells (Yao *et al*. 1996). Instead, we observed perfectly holdback of callus growth or embryo formation as using 25 mg/L hygromycin for selection of transformation plants. This result was similar to those using embryogenesis callus of herbaceous plant (Chai *et al*., 2004; Bettany *et al*., 2003).

The highest *gus* transformation efficiency was obtained when 0.6 of bacterial density in OD_{600} was used for innoculation. This result was similar to that of Montoro *et al*. (2000) previously reported. Inoculation for 20 min was the highest *gus* efficiency, which was similar to that of Chai *et al*. (2004).

Generally, co-cultivation of embryogenesis callus cells in herbaceous plant (Wu *et al*., 1998; Ke *et al*., 2001; Khan *et al*., 2001; Chai *et al*., 2004) or a few citrus plants carried out for 3～5 days. Also, Li and Deng (2003) indicated that embryogenic callus cells was caught by 3 days of co-cultivations periods, but at this condition, transient *gus* expression rates was lower when compared with other conditions. Acetosyringone (AS) concentration in 100 μM showed the highest *gus* efficiency. Addition of acetosyringone (100 μM) during co-cultivation was found to be essential for

transformation based on GUS activity (Hiei *et al*., 1994), which was similar to that of Li and Deng (2003).

About 8 of putative transgenic shoots could were obtained from hygromycin-resistant callus cells after *Agrobacterium*-mediated transformation. But, we couldn't induce directly roots from most of transgenic citrus species as reported before (Moor *et al*., 1992). In stead, many researchers could solve a problems of this sort by grafting transgenic shoots onto rootstocks of *Poncirus trifoliata* Raf. (Pena *et al*., 1995; Bond *et al*., 1998; Lu *et al*., 1999; Kayim *et al*., 2004). Ther same method was used to produce transgenic citrus plants.

Eight among transgenic plants were obtained through GUS staining. PCR analysis showed that all the plants contained the GUS gene. However, the inserted GUS or HPT gene were detected in 4 or 5 lines, respectively. Further study is required to answer it. Citrus is very difficult to show posterity because it has long juvenility and seed breeding has not. Therefore, citrus don't need to show copy numbers.

Mandarin species including satsuma mandarin has showed difficulties in obtaining transgenic plants (Li and Deng. 2002). Han *et al*. (2005) reported that satsuma mandarin callus was transformed with *gfp* gene by *Agrobacterium-mediation*, but it was not successful to develop embryos. Embryogenic callus is favored over epicotyl segments for transformation procedures because it has the same genetic background and has a

characteristic of easy mass multiplication. Embryogenic callus has been widely used as materials for genetic transformation in crops such as rice (Gil *et al*., 1999) or maize (Negrotto *et al*., 2000). However, there has not been any successful report to establish an *Agrobacterium*-mediation transformation via somatic embryogenesis, yet.

In conclusion, this study indicated that the *Agrobacterium*-mediated transformation via somatic embryogenesis and transgenic plant regeneration was successfully established in satsuma mandarin.

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