A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Functional expression of miraculin, a taste modifying protein, in *Citrus unshiu* Marc. and characterization of three novel citrus ERF genes

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CONTENTS

DEDICATION

To my wife **Shahida**

To my beloved son **Zinan** whose smile gives me a reason to wake up everyday.

국문 요약

Satsuma mandarin의 일반적으로 잘 알려진 품종인 Miyagawa Wase는 부드러운 껍질과 씨가없는 특성을 가지고 있다. 대부분의 다른 품종과 다르게 Satsuma mandarin은 긴 juvenility, 높은 이형 접합성, 그리고 polyembryony, gamete sterility, nucellar embryony같은 몇몇 특유 생식상의 특징때문에 통상적인 육종 방법으로 육종시키기 어렵다.

본 연구에서는 수정되지 않은 ovules로부터 유도된 embryogenic calluse를 이용하여 *Agrobacterium*을 매개로 하는 Miyagawa Wase의 형질전환을 수행하였다.

Agrobacterium tumefaciens strain EHA105가 miraculin 유전자를 포함하는 binary vector pCAMBIA1300을 harboring 해주었고 선별 마커로서 hygromycin이 사용되었다. 100 μM acetosyringone을 포함하는 배지에서 공동배양 5일 후 calluse는 15 mg/L hygromycin 과 250 mg/L cefotaxime을 포함하는 액체 half EME배지로 옮겨져 2주 배양되었다. 이어서 calluse는 20 mg/L hygromycin을 포함하는 고체 선별 배지에서 4주간 배양되었고 25 mg/L hygromycin을 포함하는 배지에서 4주 간 배양하여 선별하였다.

총 168개의 resistant한 embryo가 선별되어 embryo elongation배지로 옮겨졌다.

3주 배양 후, heart모양의 embryo는 embryo germination을 위해 1 mg/L GA3, 20 ml/L coconut water, 20 μg/L NAA, 그리고 14.6 μg/L coumarin을 포함하는 MT배지로 옮겨졌다.

마지막으로 135개의 germinated된 embryo는 30 g/L sucrose 와 8 g/L agar를 포함하는 MT배지에서 배양되어 115개의 normal한 plant를 얻을 수 있었다.

PCR을 통해 miraculin 유전자를 포함하고 있음을 확인하여 37개의 형질전환된

Miyagawa wase식물을 얻었다.

무작위적으로 선별한 5개 식물의 southern blot 분석을 통해 miraculin transgene이 Miyagawa Wase genome상에 안정적으로 통합되었음이 확인되었다.

Miraculin은 신 맛을 단 맛으로 변화시키는 특징을 갖는 taste modifying proteins이다.

이 특이한 특징이 이 단백질에 대해 관심을 갖게하였다.

Miraculin의 유전적으로 안정하고 높은 발현이 Western blot 분석을 통해 확인었 형질전환된Miyagawa Wase에서 이 단백질의 발현은 잎에서 recombinant miraculin단질이 significant하게 축적 된 결과 이다.형질전환 식물에서의 recombinant miraculin의 발현은 자연적인 miraculin과 유사하게 단맛을 유도하는 특성을 강하게 나타내었다.

transgenic Miyagawa Wase Satsuma Mandarin에서 recombinant miraculin의 dimerization과 *N*-glycosylation은 형질전환 식물에서 recombinant miraculin이 정확하게 processed되고 있음을 설명한다. 이 결과는 citrus같은 woody 식물에서의 발현시스템의 새로운 길을 여는 것이고 recombinant miraculin protein을 생산하기위한 적당한 대체 방법이 될수 있음을 설명한다.

AP2 / EREBP 전사인자의 큰 그룹을 구성하는 ERF는 식물의 발생과 physiological 과정에서 다양한 기능이 있고 식물의 biotic하고 abiotic stress 저항성을 조절하는데 큰 역할을 한다. 그러나 감귤에서 이 그룹에 속하는 많은 유전자는 아직 기능적으로 알려져 있지 않다. 본 연구에서 Ueno Wase의 cDNA library에서 3가지 novel ERF gene이 분리되었고 CuERF1, CuERF5, CuERF6로 명명되었다.

이 CuERF gene의 추정되는 아미노산 서열은 알려진 다른 AP2/ERF domain과 매우 유사한 AP2/ERF domain을 포함하고 있다. 그 기능을 특징화하기위해 이 3가

지 CuERF gene이 다른 식물에 비해 gene regulation이 매우 간단한 애기장대로 형질전환되었다.

분리된 CuERF gene은 stress에 중요한 영향을 끼친다고 보였다. 이 결과는 CuERF가 식물이 salt tolerance같은 abiotic stress에 반응하는데 중요한 factor로서 기능을 가질 것이라고 설명하고 citrus의 다양한 stress 반응을 조절하는 기작을 연구하는데 중요한 단서를 제공할 것이다.

SUMMARY

Miyagawa Wase, a common cultivar of the Satsuma mandarin (*Citrus unshiu* Marc.), is characterized by its tender peel and seedless nature. Unlike most fruit species, Satsuma mandarins are difficult to breed using conventional breeding methods due to long juvenility, high heterozygosity, and several unique reproductive characteristics such as polyembryony, male sterility, and nucellar embryony. In this study, *Agrobacterium*-mediated transformation of Miyagawa Wase was performed using embryogenic calluses from unfertilized ovules. *Agrobacterium tumefaciens* strain EHA105 harboring the binary vector pCAMBIA1300 that contained the miraculin gene and hygromycin as a selection marker were used. After 5 days of co-culture in a medium containing 100 μM acetosyringone, calluses were transferred to the liquid half EME medium with 15 mg/L hygromycin and 250 mg/L cefotaxime and then cultured for 2 weeks. Subsequently, the calluses were grown on a solid selection medium with 20 mg/L hygromycin for 4 weeks, followed by selection with 25 mg/L hygromycin for 4 more weeks. A Total 168 resistant embryo were then selected and transferred to the embryo maturation medium. After 3 weeks of culture, the heart-shaped embryos were transferred to MT medium containing 1 mg/L GA_3, 20 ml/L coconut water, 20 μg/L NAA, and 14.6 μg/L coumarin for embryo germination. Finally, 135 germinated embryos were cultured on MT medium containing 30 g/L sucrose and 8 g/L agar and 115 normal plants recovered. The transformation procedure yielded 37 transgenic Miyagawa Wase plants containing the miraculin genes as verified by PCR amplification. Southern blot analyses of randomly selected 5 plants further confirmed that the miraculin transgene was stably integrated into the Miyagawa Wase genome.

Miraculin, a taste modifying proteins which has the unusual property of being able to modify a sour taste into a sweet taste. This unique property has led to increasing interest in this

protein. High and genetically stable expression of miraculin was confirmed by western blot analysis and expression of this protein in transgenic Miyagawa Wase plants resulted in the accumulation of significant amount of recombinant miraculin protein in the leaves. Recombinant miraculin expressed in transgenic plants showed strong sweetness-inducing activity, similar to that of native miraculin. Dimerization and *N*-glycosylation of recombinant miraculin in transgenic Miyagawa Wase Satsuma mandarin plants demonstrate that recombinant miraculin was correctly processed in transgenic plants. These results open up a new way of expression system in woody plants like citrus and can be a suitable alternative for producing recombinant miraculin protein.

Ethylene responsive factors (ERFs), composing the largest group of AP2/EREBP transcription factors are involved in a variety of functions in the developmental and physiological process in plants and play important role in regulating plant biotic and abiotic stress tolerance. However, in citrus, most of the genes in this group are functionally unknown yet. In this study, three novel ERF genes were isolated, designated as CuERF1, CuERF5 and CuERF6 from cDNA library of the citrus cultivar, Ueno Wase (*Citrus unshiu* Marc.). The deduced amino acid sequences of these CuERFs genes contained an AP2/ERF domain which shared high similarity with other reported AP2/ERF domains. To characterize their functions, these three CuERF genes were transformed to the Arabidopsis since network of gene regulation in Arabidopsis is far clear than in any other plants species. The isolated CuERF genes showed positive effect on salt stress. These results indicate that CuERF genes might have function as positive factors in the plant responses to abiotic stresses like salt stress and provide useful clues for further research into the mechanism of them in regulating citrus multiple stress responses.

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ABBREVIATIONS

AP2	:	APETALA 2
AS	:	Acetosyringone
℃	:	Degree Celsius
CaMV	:	Cauliflower mosaic virus
CBF	:	C-repeat binding factor
CTV	:	Citrus tristeza virus
CuERF	:	*Citrus unshiu* ethylene responsive factor
DDW	:	Double distilled water
DREB	:	Dehydration responsive element binding
DTT	:	Dithiothreitol
EDTA	:	Ethylene diamine tetra acetic acid
EREBP	:	Ethylene responsive element binding protein
ERF	:	Ethylene responsive factor
EST	:	Expressed sequence tag
GA_3	:	Gibberellic acid
GUS	:	β-Glucuronidase
HCl	:	Hydrochloric acid
HPT	:	Hygromycin phosphotransferase
IPTG	:	Isopropyl-β-D-1-thiogalactopyranoside
LB	:	Luria Bertani medium
MT	:	Murashige and Tucker basal medium
MW	:	Molecular weight
NaCl	:	Sodium chloride
NOS	:	Nopaline synthase

NPTII II	:	Neomycin phosphotransferase II
OD	:	Optical density
ORF	:	Open reading frame
PCR	:	Polymerase chain reaction
PEG	:	Polyethylene glycol
PVPP	:	Polyvinylpolypyrrolidone
SDS	:	Sodium dodecyl sulfate
SDS-PAGE	:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SSC	:	Saline sodium citrate
TBS	:	Tris-buffered saline
TBST	:	Tris buffered Saline tween-20
TE	:	Tris-EDTA
T-DNA	:	Transfer DNA
YEP	:	Yeast extract peptone medium

RESEARCH BACKGROUND

Citrus is one of the most important commercial and nutritional fruit in the world and are the first fruit crop in international trade in terms of value. The genus *Citrus* belongs to the family Rutaceae, which comprises 33 genera and 203 species throughout the world (Spegel-Roy, 1996). There are five groups of C*itrus* species. They are i) mandarin (*C. reticulata* Blanco and *C. unshiu* Marc.), ii) sweet orange (*C. sinensis* [L.] Osb.), iii) lemon (*C. limon* Burm. f.), iv) lime (*C. aurantifolia* L.), and v) grapefruit (*C. paradisi* Macf.). Out of these five groups the mandarin is easily peeled with the fingers, starting at the thick rind covering the depression at the top of the fruit, and can be easily split into even segments without squirting juice which makes it convenient to eat. Satsuma mandarins are major citrus crops that occupy greater than 90% of the total acreage used for crops on Jeju Island. In addition, they have been the only species harvested before winter for several decades in this region. As a result, they have become an important breeding resource in South Korea; hence it needs to be improved to cater to the diverse needs of consumers as well as crop breeders. Genetic manipulation through conventional techniques in this genus is invariably a difficult task for plant breeders as it possesses various biological limitations (Singh and Rajam, 2009).

Conventional breeding in citrus has been practiced for decades and is vastly hampered by varieties of unavoidable factors such as apomixis, nucellar polyembryony, pollen or ovule sterility, high heterozygosity, and a very large plant size making controlled crosses a difficult task (Vardi *et al.*, 1975; Vardi, 1981; Martin-Trillo and Martinez-Zapater, 2002). Added to these are instances of cross or self-incompatibility that jeopardizes breeding efforts even further. Most of the species are apomictic where in the development of embryo initiates directly from the nucellar tissue, resulting the suppression of the growth of zygotic

embryo (Kultunow *et al.,* 1995). Furthermore, citrus species also exhibit a very long juvenile period, which may extend from 5 to 21 years to enter the reproductive stage. Even, in the nature, propagation and cultivation of citrus is limited to a particular season and favourable climatic conditions. Above all these factors combined together greatly hinder breeding efforts in citrus cultivars including Satsuma mandarin cv Miyagawa Wase. Therefore, genetic transformation of these citrus species seems to be much more convenient method for citrus breeding.

In recent years, there has been a major thrust in citrus improvement as competition from international citrus markets, pest and disease pressure, and other biotic and abiotic conditions stimulate worldwide interest (Grosser *et al.,* 2000). Citrus transformation has been successfully performed on many species and hybrids (Table 1.) In fact, several methods available for the genetic transformation of citrus and the most popular method to transform a wide range of citrus cultivars is *Agrobacterium*-mediated transformation using epicotyl explants as target cells for incorporation of the T-DNA (Dutt and Grosser, 2009). However, this method is not suitable for the transformation of any seedless cultivar; specialty cultivars in the mandarin group remain difficult to transform using this method (Khawale *et al.,* 2006; Dutt *et al.,* 2009). Genetic transformation using embryogenic callus offers a practical alternative to the transformation of epicotyl explants. Furthermore, in contrast to orange species Satsuma mandarin species had been known to be especially difficult not only to obtain embryogenic callus from nucellar tissue but also to obtain hybrid because of poly-embryony and male sterility and thus much more difficult in plant regeneration (Han *et al.,* 2005). Until present, there is no successful transformation system was established in Satsuma mandarin. In this study, *Agrobacterium*-mediated transformations of embryogenic calluses were studied, and a genetic transformation system was established in 'Miyagawa Wase', a cultivar of Satsuma mandarin (*Citrus unshiu* Marc.) containing miraculin gene.

Miraculin is a taste-modifying protein isolated from the red berries of *Richadella dulcifica* , a shrub native to West Africa. Miraculin by itself is not sweet, but it is able to turn a sour taste into a sweet taste. Owing to this unique property, the berry has been called the miracle fruit. West Africans people traditionally used this fruit for improving flavor and suppress bitterness in food and drink. For example, they are used to improve the flavor of maize dishes, such as agidi, and beverages, such as palm wine or tea. Now a days, these proteins have been used in the food-processing industry as sweetening agents, flavor enhancers, and animal fodder supplements (Sun *et al.,* 2006).

Miraculin gene was first isolated by Kurihara and Beidler (1968). The complete amino acid sequence of miraculin has been determined (Theerasilp *et al.,* 1989), and the cDNA corresponding to miraculin has been cloned and sequenced (Masuda *et al.,* 1995). This proteins can act at extremely low concentrations, and because of this low effective dose, they are effectively non-carcinogenic and acceptable for diabetics in flavor and sweetening formulations. The sweetness induced by citric acid after exposure to miraculin is estimated to be about 3000 times that of sucrose on a per weight basis (Kurihara and Beidler, 1969; Theerasilp and Kurihara, 1988; Gibbs *et al.,* 1996; Kurihara and Nirasawa, 1997), and interest has been increasing in miraculin due to this amazing property. Miracle fruit is available fresh and in powdered or tablet form in Japan, where it is particularly popular among people with diabetes or on diets.

Therefore the taste-modifying protein, miraculin, is obviously an attractive alternative to some of the more traditional sweeteners, such as sucrose. However, the commercial feasibility of miraculin is very low because the natural source of this protein is a tropical plant that is difficult to cultivate outside of its natural environment. To overcome the barriers standing in the way of miraculin mass production, the miraculin gene has been introduced into other organisms to produce recombinant miraculin. Miraculin production in *Escherichia coli* (Kurihara, 1992), yeast, and tobacco (Kurihara and Nirasawa, 1997) has

been attempted but failed to express miraculin with taste-modifying activity. Recently, the production of miraculin in *E. coli* was reattempted. This study showed that the taste modifying activity, without glycosylation, was no more than 72% that of native miraculin even with 1.6 time's higher density than the refined native glycoprotein (Matsuyama *et al.,* 2009).

However, when *Aspergillus oryzae* was used as a host for expressing miraculin, the recombinant miraculin protein exhibited taste-modifying properties (Ito *et al.,* 2007). From the literature studied in miraculin transformation, only a few crop species including lettuce (Sun *et al.,* 2006), tomato (Sun *et al.,* 2007) and strawberry (Sugaya *et. al.,* 2008) successfully expressed recombinant miraculin, but in subsequent generations, stable miraculin expression was observed in tomato and strawberry but silencing of the transgene occurred in lettuce, although the recombinant protein was correctly folded in all cases. In this study, recombinant miraculin has also successfully expressed in perennial woody plants like citrus and expressed recombinant miraculin in Miyagawa Wase transgenic plants possesses strong sweetness-inducing activity.

The ethylene-responsive factor (ERF) proteins, which make up a large family of transcription factors in plants, are excellent candidates for the study of how plants adapt to various stresses. This AP2/ethylene-responsive element-binding proteins exist extensively in plants and microorganisms which characterized by highly conserved novel plant-specific DNA binding domain containing of 57-66 amino acids in size (Okamura *et al.,* 1997; Magnani *et al.,* 2004; Nakano *et al.,* 2006). The AP2/ERF genes constitute a large multigene family divided into four subfamilies named AP2, CBF/DREB, ERF, and RAV based on their sequence similarities and numbers of AP2/ERF domains (Sakuma *et al.,* 2002). AP2 subfamily proteins contain two AP2/ERF domains, and genes in this subfamily participate in the regulation of developmental processes (Elliott *et al.,* 1996; Chuck *et al.,* 1998; Boutilier *et al.,* 2002). In contrast to the AP2 and RAV subfamily members, the CBF/DREB and ERF

subfamily proteins contain single AP2/ERF domains. The genes in the CBF/DREB subfamily play a crucial role in the resistance of plants to abiotic stresses by recognizing the dehydration responsive or cold-repeat element (DRE/CRT) with a core motif of A/GCCGAC (Yamaguchi-Shinozaki and Shinozaki, 1994; Thomashow, 1999). Many of the ERF subfamily members also bind DRE/CRT elements (Lee *et al.,* 2004; Xu *et al.,* 2007).

ERF transcription factors have been identified in various plant species, such as *Arabidopsis thaliana* (Onate-Sanchez and Singh, 2002), tobacco (*Nicotiana tabacum*) (Fischer and Droge-Laser, 2004), and tomato (*Lycopersicon esculentum*) (Tournier *et al.,* 2003). Although ERF subfamily transcription factors have been identified in different plant species, only a few have been characterized (Onate-Sanchez and Singh, 2002; Sakuma *et al*., 2002) including citrus ERF gene family (Cevik and Moore, 2006; Champ *et al.,* 2007).

Considering the above information, the present study was undertaken with the following specific objectives:

- To develop an efficient *Agrobacterium*-mediated transformation protocol for Miyagawa Wase, Satsuma mandarin
- To investigate the functional expression of recombinant miraculin protein in transgenic Miyagawa Wase Satsuma mandarin plants
- To clone the novel ERF genes from citrus (Ueno Wase, *Citrus unshiu* Marc.) and
- To investigate the function of these novel ERF genes by overexpression in *Arabidopsis*.

Table 1. Major crop species for which genetic transformation system has been applied to produce transgenic citrus plants having genes of agronomic interest

Common name	Scientific name	Gene introduced	References
Kiwifruit and Trifoliate orange	*Actinidia chinensis Poncirus trifoliata*	Gene encoding human epidermal growth factor (hEGF)	Kobayashi *et al.,* (1996)
Sour orange	*C. aurantium*	Coat protein gene of CTV	Gutierrez *et al.,* (1997)
Trifoliate orange	*Poncirus trifoliata*	rolC gene	Kaneyoshi and Kobayashi, (1999)
Sour orange	*C. aurantium*	Coat protein gene of CTV	Ghorbel *et al.,* (2000)
West Indian lime	*C. aurantifolia*	Genes for decreased seed set	Kultunow *et al.,* (2000)
Grapefruit	*C. paradisi*	Coat protein gene of CTV	Moore *et al.,* (2000)
Carrizo citrange	*C. sinensis* × *P. trifoliata*	HAL2 gene	Cervera *et al.,* (2000)
Troyer citrange	*C. sinensis* × *P. trifoliata*	Truncated version of CTV and Bar gene	Piestun *et al.,* (2000)
Mexican Lime	*C. aurantifolia*	Coat protein gene of CTV	Dominguez *et al.,* (2000)
Grapefruit	*C. paradisi*	Gna and uncp genes	Yang *et al.,* (2000)
Carrizo citrange	*C. sinensis* × *P. trifoliata*	LEAFY and APETALA1	Pena *et al.* (2001)
Carrizo citrange, and Poncirus trifoliate	*C. sinensis (L.) Osbeck and P. trifoliata*	antisense CS-ACS1	Wong *et al.,* (2001)
Grapefruit	*C. paradisi*	Carotenoid Biosynthetic genes	Costa *et al.,* (2002)
Ponkan mandarin	*Citrus reticulata. Blanco*	Chimeric ribonuclease gene	Li *et al.,* (2002)
Troyer citrange	*C. sinensis* × *P. trifoliata*	rolABC genes	Gentile *et al.,* (2002)
Grapefruit	*C. paradisi*	CTV genes	Febres *et al.,* (2003)
Valencia sweet orange	*C. sinensis*	pTA29-barnase gene	Li *et al.,* (2003)
Carrizo citrange	*C. sinensis* × *P. trifoliata*	Citrus blight-associated gene	Kayim *et al.,* (2004)
Trifoliate orange	*P. trifoliata*	Capsid polyprotein gene (pCP	Iwanami and Shimizu, (2004)
Trifoliate orange	*P. trifoliata*	Citrus FT (CiFT)	Endo *et al.,* (2005)
Valencia orange	*C. sinensis*	Pectin methylesterasegene	Guo *et al.,* (2005)
Rangpur Lime	*C. limonia*	bO (bacterio-opsin)	Azevedo *et al.,* (2006)
Hamlin	*C. sinensis*	Attacin A gene	Boscariol *et al.,* (2006)
Hamlin	*C. sinensis*	Xa21	Omar *et al.,* (2007)
Pineapple Sour orange	*Citrus sinensis L. Osbeck*	Coat protein gene of CPsV	Zanek *et al.,* (2008)
Hamlin	*C. sinensis*	hrpN gene	Barbosa-Mendes *et al.,* (2009)

Part I

Agrobacterium-mediated transformation of embryogenic calluses of Miyagawa Wase Satsuma mandarin (*Citrus unshiu* Marc.) and the regeneration of plants containing the miraculin gene

ABSTRACT

Miyagawa Wase, a common cultivar of the Satsuma mandarin (*Citrus unshiu* Marc.), is characterized by its tender peel and seedless nature. In this study, *Agrobacterium*-mediated transformation of Miyagawa Wase was performd using embryogenic calluses from unfertilized ovules. *Agrobacterium tumefaciens* strain EHA105 harboring the binary vector pCAMBIA1300 that contained the miraculin gene (a taste-modifying protein) and hygromycin as a selection marker were used. After 5 days of co-culture in a medium containing 100 μM acetosyringone, calluses were transferred to the liquid half EME medium with 15 mg/L hygromycin and 250 mg/L cefotaxime and then cultured for 2 weeks. Subsequently, the calluses were grown on a solid selection medium with 20 mg/L hygromycin for 4 weeks, followed by selection with 25 mg/L hygromycin for 4 more weeks. Total 168 resistant embryos were selected and transferred to the embryo maturation medium. After 3 weeks of culture, the heart-shaped embryos were transferred to MT medium containing 1 mg/L GA_3, 20 ml/L coconut water, 20 μg/L NAA, and 14.6 μg/L coumarin for embryo germination. Finally, 135 germinated embryos were cultured on MT medium containing 30 g/L sucrose and 8 g/L agar and recovered 115 normal plants. This transformation procedure yielded 37 trangenic plants containing miraculin genes as verified by PCR amplification. Southern blot analyse of 5 randomly selected plants further confirmed the miraculin transgene was stably integrated into the Miyagawa Wase genome.

1. INTRODUCTION

Citrus fruits are economically valuable crops throughout the world due to their high nutritional value, large volume of production, and use in diverse processed products (Chaturvedi *et al.,* 2001). Miyagawa Wase, a common cultivar of the Satsuma mandarin (*Citrus unshiu* Marc.), is grown in Korea, China, and Japan. Unlike most fruit species, Satsuma mandarins are difficult to breed using conventional breeding methods due to long juvenility, high heterozygosity, and several unique reproductive characteristics such as polyembryony, generative sterility, and parthenocarpy (Grosser and Gmitter, 1990). As a result, most cultivars of the Satsuma mandarin have arisen from bud mutations or clonal selections despite efforts to develop new cultivars by sexual hybridization (Moore *et al.,* 2005).

Therefore, genetic engineering is the most promising method for improving and developing new cultivars of the Satsuma mandarin, such as Miyagawa Wase. Several different methods can be used to introduce transgenes into citrus. For example, *Agrobacterium*-mediated transformation has been performed using juvenile epicotyl segments (Moore *et al.,* 1992; Luth and Moore, 1999; Ananthakrishnan *et al.,* 2007; Dutt and Grosser, 2009; Dutt *et al.,* 2010; Cardoso *et al.,* 2010), mature internode segments from greenhouse-grown plants (Cervera *et al.,* 1998; Almeida *et al.,* 2003), and embryogenic calluses from unfertilized ovules (Li *et al.,* 2002, 2003). DNA can also be directly incorporated into protoplasts by electroporation (Niedz *et al.,* 2003) or PEG-mediated transformation (Fleming *et al.,* 2000; Olivares-Fuster *et al.,* 2000; Guo *et al.,* 2005; Omar *et al.,* 2007). Although transformation has been used for several citrus species, there have not been any reports of transgenic Miyagawa Wase. This may be due to the low frequency of embryogenesis and instability of embryos from calluses of Satsuma mandarin (Ling *et al.,*

1990; Han *et al.,* 2002).

In this study, we used *Agrobacterium* to transform embryogenic calluses of Miyagawa Wase with a binary vector containing the miraculin gene. Miraculin is a taste-modifying protein found in the fruit of the miracle plant (*Richadella dulcifica*). Miraculin is not sweet, but it has the unusual property of converting a sour taste into a sweet taste. This is the first report of a transgenic Miyagawa Wase Satsuma mandarin (*Citrus unshiu* Marc.) via somatic embryogenesis.

2. MATERIALS AND METHODS

2.1 Initiation and maintenance of embryogenic calluses

Embryogenic calluses were obtained from unfertilized ovules of Miyagawa Wase. Somatic embryogenesis was initiated and embryogenic calluses were maintained as described previously (Jin *et al.,* 2007). Briefly, embryogenic calluses were maintained on solid EME medium (MT-based medium supplemented with 500 mg/L malt extract, 50 g/L sucrose and 8 g/L agar) in a growth chamber at 25 ± 2ºC with cool white fluorescent light (16 hrs photoperiod at an intensity of 60 μmol·m^{-2} s^{-1}) and a relative humidity of 60%. The calluses were subcultured every 3 weeks.

2.2 Plasmid construction and bacterial strain

The pCAMBIA1300 binary vector (CAMBIA, Canberra, Australia) includes the selectable marker gene neomycin phosphotransferase II and the pUC18 polylinker without the promoter site. For plasmid construction, pBI121 vector were digested with *Eco*RI and *Hin*dIII, and then isolated the DNA fragment containing the GUS reporter gene, CaMV35S promoter, and NOS terminator. Subsequently, this fragment was ligated into the *Eco*RI–*Hin*dIII site of the pCAMBIA1300 binary vector. The GUS gene was digested with *Xba*I and *Sac*I, and then replaced with the miraculin gene. Finally, the constructed plasmid (Fig. 1) was introduced into *A. tumefaciens* strain EHA105 for the plant transformation experiments.

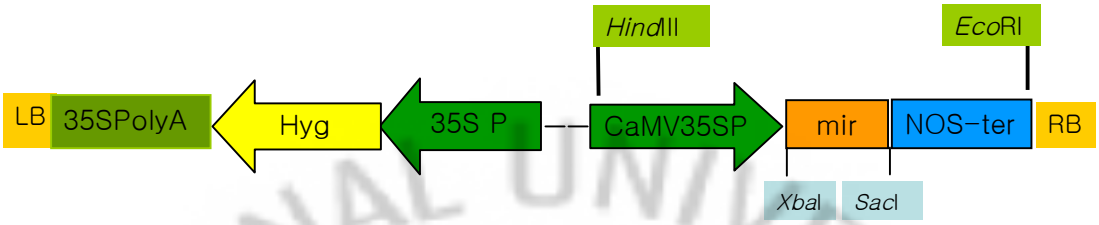

Fig. 1. Schematic representation of the binary vector pCAMBIA1300 with its position of different enzymes of citrus genetic transformation. RB: Right border, LB: Left border, NOS-ter: Terminator of nopaline synthase gene, Mir: Miraculin gene, CaMV 35SP: 35S promoter of cauliflower mosaic virus, Hyg: Hygromycin, PolyA: Poly tail A.

2.3 Transformation and selection

Agrobacterium-mediated transformation was performed as described by Duan *et al.* (2007) with a few minor modifications. Four days before the inoculation, *Agrobacterium* cells harboring the pCAMBIA1300 vector containing the miraculin gene were streaked onto solid LB medium supplemented with 50 mg/L rifampicin and 50 mg/L kanamycin, and then incubated at 28ºC. After 2 days, a fresh single colony was subcultured in 5 ml YEP liquid medium overnight at 28ºC in a shaking incubator at 250 rpm, followed by the addition of 45 ml fresh YEP liquid medium and incubation for additional 5 hrs. When the OD_{600} of the culture was approximately 0.6, it was centrifuged at 5000 rpm for 10 min, and the pellet was then resuspended to OD_{600} = 0.6 with MT liquid medium containing 500 mg/L malt extract, 50 g/L sucrose, and 100 μM acetosyringone.

For inoculation, embryogenic calluses were immersed in this *Agrobacterium* suspension in a shaking incubator at 150 rpm for 20 min. at 28ºC. They were then blotted with sterile filter paper and transferred to solid MT medium supplemented with 50 g/L sucrose, 500 mg/L malt extract, and 100 μM acetosyringone. After 5 days of co-culture in darkness at 24ºC, the calluses were washed with 600 mg/L carbenicillin and then blotted on a sterile filter paper. The dried calluses were transferred to liquid half EME medium supplemented with 50 g/L sucrose, 500 mg/L malt extract, 1.55 g/L glutamine, 15 mg/L hygromycin, and 250 mg/L cefotaxime in a shaking incubator at 70 rpm for 2 weeks at 26ºC. Subsequently, the calluses were transferred to solid MT medium containing 7% lactose, 1.2% agar, 20 mg/L hygromycin, and 250 mg/L cefotaxime. After 4 weeks of culture, most of the calluses had become necrotic and died. The calluses that proliferated on the selection medium were transferred to and selected on the same medium with higher concentrations of agar (1.6%), hygromycin (25 mg/L), and cefotaxime (250 mg/L). After 4 more weeks of culture, the surviving calluses that formed green, globular embryos were used for regeneration.

2.4 Regeneration

Regeneration was performed according to the method of Jin *et al.,* (2007) with minor modification. The hygromycin-resistant embryos were transferred to the embryo maturation medium (MT-based medium containing 0.1 M sorbitol, 0.1 M galactose, 0.7 mg/L adenine, and 0.2% Gelrite). After 3 weeks, the heart-shaped embryos were transferred to the embryo germination medium (MT-based medium supplemented with 20 ml/L coconut water, 14.6 μg/L coumarin, 20 μg/L NAA, 1 mg/L GA_3, and 2 g/L Gelrite). Four weeks later, the embryos having shoots and roots were transferred onto MT medium containing 30 g/L sucrose and 8 g/L agar for normal plant growth.

2.5 Polymerase chain reaction

Polymerase chain reaction (PCR) analysis was used to detect the miraculin gene in the transformed Miyagawa Wase plants. Genomic DNA was extracted from the leaves (100 mg) of both transgenic and non-transgenic plants using the G-Spin ™ IIP genomic DNA extraction kit (iNtRON Biotechnology, Inc. South Korea).The forward and reverse miraculin gene specific PCR primers were 5′–TTTTCTAGAATGAAGGAATTAACAATGCT–3′ and 5′–TTTGAGCTCTTAGAAGTATACGGTTTTGT–3′), respectively. The forward and reverse hygromycin gene specific PCR primers were 5′–CTTCTACACAGCCATCGGTCCAGA–3′ and 5′–GATGTAGGAGGGCGTGGATATGTC–3′), respectively. PCR was performed with 100 ng of genomic DNA as the template and i-Taq™ DNA polymerase (iNtRON Biotechnology, Inc. South Korea). The PCR conditions were: pre-heating at 94ºC for 5 min; 35 cycles at 94ºC for 1 min, 55ºC for 1 min, and 72ºC for 1 min; and finally 72ºC for 10 min. After amplification, the PCR products were electrophoresed on 1% agarose gels at 100 V for 30 min and then visualized with ethidium bromide staining.

2.6 Southern blot analysis

A Southern blot analysis was performed to confirm the stable integration of the miraculin gene and determine its copy number in transgenic plants. First, genomic DNA was isolated from the fresh leaves (100 mg) of transgenic and non-transgenic Miyagawa Wase plants using the G-Spin ™ IIP genomic DNA extraction kit (iNtRON Biotechnology, Inc. South Korea). About 10 μg of genomic DNA was digested with the *Xba*I restriction enzyme that cuts at a single site in the transfer DNA (T-DNA). The digested DNA was electrophoresed on a 1.0% agarose gel at 50 V for 4 hrs in 0.5X TBE buffer. After depurination, denaturation, and neutralization, the DNA was transferred to Hybond-N^+ nylon

membrane (GE Healthcare UK Ltd., Buckinghamshire, UK). The membrane was hybridized overnight at 45℃ with DIG-labeled miraculin probes (PCR DIG Probe Synthesis Kit; Roche Diagnostics GmbH, Mannheim, Germany). Subsequently, CDP-Star chemiluminescent substrate (GE Healthcare UK Ltd., Buckinghamshire, UK) was used to detect hybridization signals on an X-ray film. Genomic DNA extracted from an untransformed plant was used as the negative control.

Transformation method

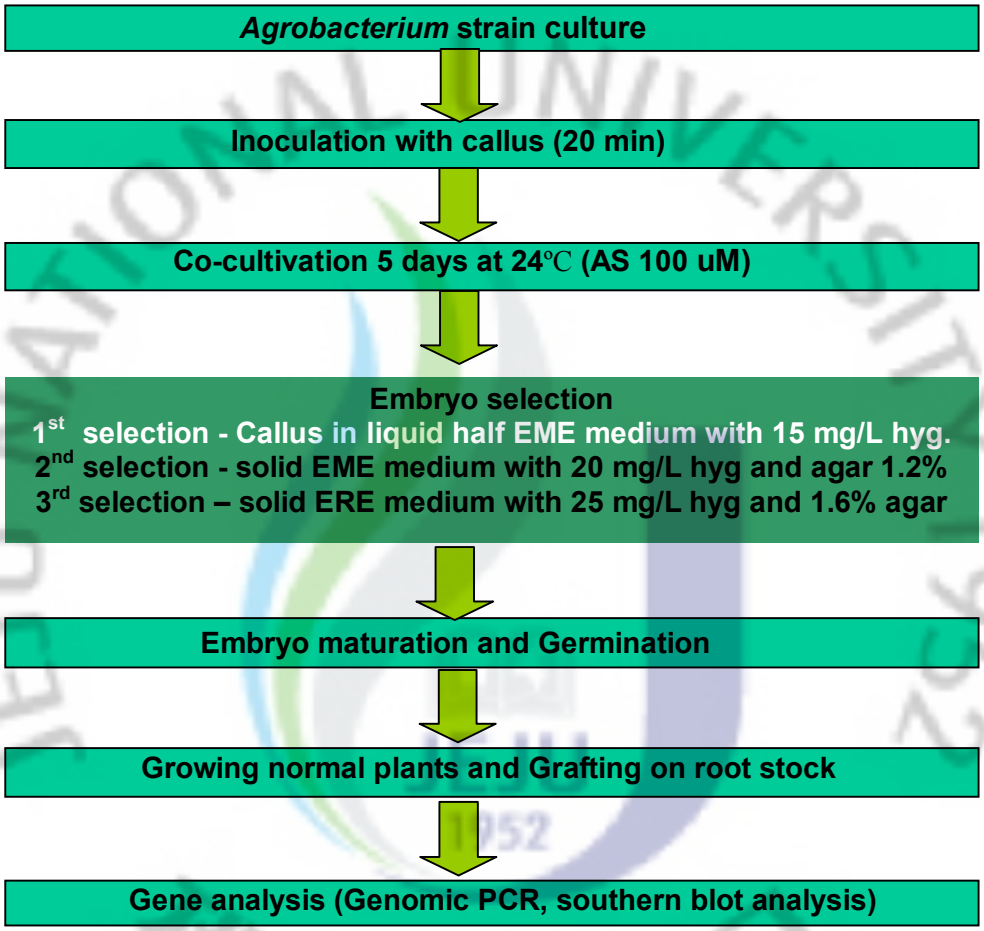

Fig. 2. Overall procedure for *Agrobacterium*-mediated transformation of the gene of interest of the embryogenic callus and regeneration of plants in 'Miyagawa Wase' satsuma mandarin (*Citrus unshiu* Marc.).

3. RESULTS

3.1 Determination of the optimum hygromycin concentration in the selection medium

Before transforming Miyagawa Wase calluses, the optimum hygromycin concentration were determined in the selection medium. Calluses were cultured on both antibiotic-free medium and on the same medium containing hygromycin at different concentrations (5, 10, 15, 20, and 25 mg/L). After 6 weeks, most of the calluses grown on medium containing 5–15 mg/L hygromycin were brown and stopped growing, but there were a few that grew slowly. All the calluses cultured on medium containing 20–25 mg/L hygromycin became necrotic and died (fig. 3). Therefore, we concluded that medium containing 15–25 mg/L hygromycin was optimal for selecting transformants.

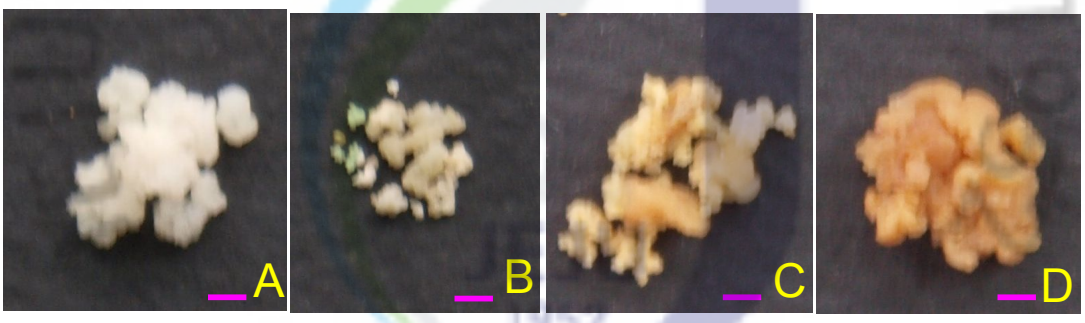

Fig. 3. Representative calluses showing growth of non-transformed calluses on the media containing different concentration of hygromycin. The calluses were cultured on MT medium containing 500 mg/L malt extract, 7% lactose and 1.2% agar for 6 weeks. Concentrations of hygromycin were 0 (A), 15(B), 20 (C) and 25 (D) mg/L. The calluses were multiplied in control medium (A) whereas a very few multiplication occurred in 15 mg/L hygromycin (B) but no multiplication occure and calluses were necrotic in the medium containing 20 and 25 mg/L hygromycin (C and D). Scale bars represent 0.5 cm.

3.2 Regeneration of hygromycin-resistant plants

Three different concentrations of hygromycin (15, 20, and 25 mg/L) were used to select hygromycin-resistant embryos. First, the co-cultured calluses were incubated in the liquid half EME medium containing 15 mg/L hygromycin for 2 weeks. A second culture was on solid MT medium containing 20 mg/L hygromycin for 4 weeks. Finally, a third culture was selected with 25 mg/L hygromycin for 4 more weeks. Hygromycin-resistant embryos proliferated and turned green, while others became necrotic and died (Fig. 4A).

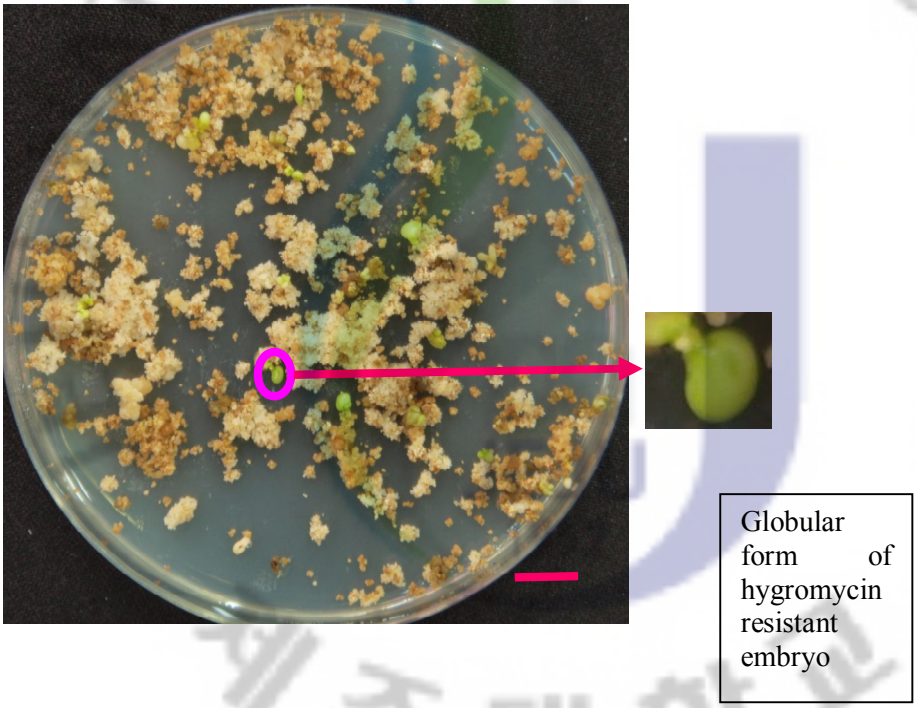

Fig. 4A. Regeneration of Miyagawa Wase embryogenic calluses after *Agrobacterium*-mediated transformation. The hygromycin resistant embryos (green) growing on hygromycin selection medium during the third selection. Scale bar represent 1 cm.

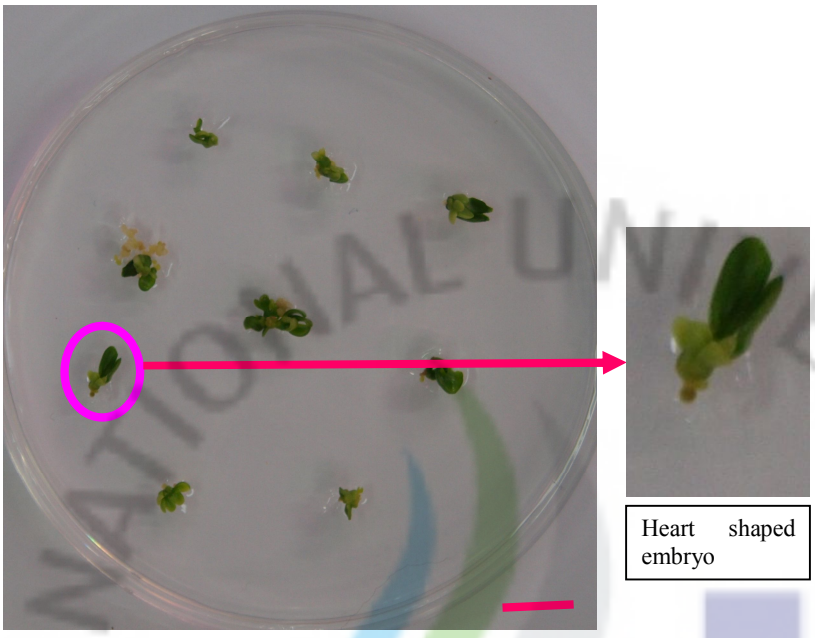

Fig. 4B. Regeneration of Miyagawa Wase embryogenic calluses after *Agrobacterium*-mediated transformation. The heart shaped embryos after 3 weeks culture on embryo maturation medium. Scale bar represent 1cm.

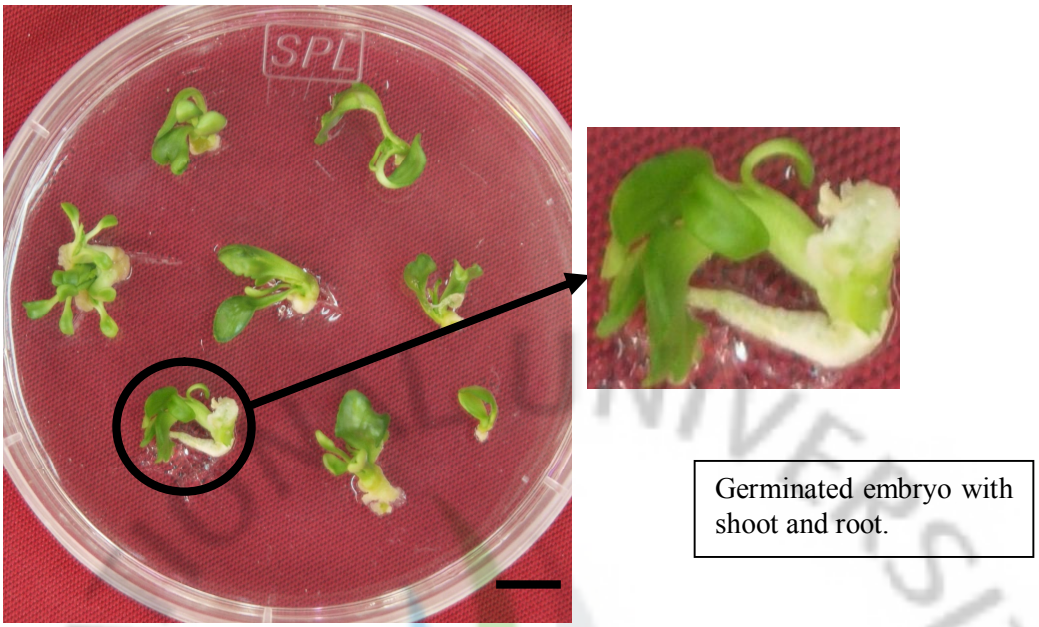

Fig. 4C. Regeneration of Miyagawa Wase embryogenic calluses after *Agrobacterium*-mediated transformation. The germinated embryos after 4 weeks of culture on the germination medium. Scale bar represent 1 cm

A total of 168 resistant embryos from 10 Petri dishes were transferred to the embryo maturation medium. Three weeks later, heart-shaped embryos were observed (Fig. 4B) and these heart shaped embryos were transferred to the germination medium. After 4 weeks of culture, 135 germinated embryos (Fig. 4C) were transferred to MT medium supplemented with 0.8% agar and 3% sucrose for normal plant growth. A total of 115 normal plants, which had well-developed shoots and roots, were regenerated from different embryos. Finally, these putative transgenic plants were *in vitro* grafted onto the rootstock plants (Fig. 5).

Fig. 5. Transgenic plants containing miraculin gene: Transgenic plant showing well-developed shoots and roots (left) and shoot-tip *in vitro* grafting onto rootstock plants. Scale bars represent 1 cm

In vitro well developed grafted plants further grafted on the citrus root stock in soil pot (Fig. 6).

Fig. 6. Transgenic plants containing miraculin gene grafting onto the citrus root stock in soil pot. Scale bar represent 1 cm

3.3 PCR and Southern blot analysis

Subsequently, genomic DNA was extracted from each of the putative transgenic plants and analyzed with PCR. These analyses showed that the plasmid control and 37 of 115 plants had the 662 bp fragment corresponding to the miraculin gene, but none of the untransformed plants contained this fragment. PCR analyses also confirmed the presence of the 850-bp hygromycin gene in the genomic DNA of the transgenic plants. Representative PCR analyses of 7 randomly selected transgenic plants are shown in Figure 7a, b. The absence of the miraculin gene in the other 78 plants may be due to the growth of escapes, regeneration of untransformed calluses, or loss of the miraculin gene during subculturing due to unstable integration into the genome.

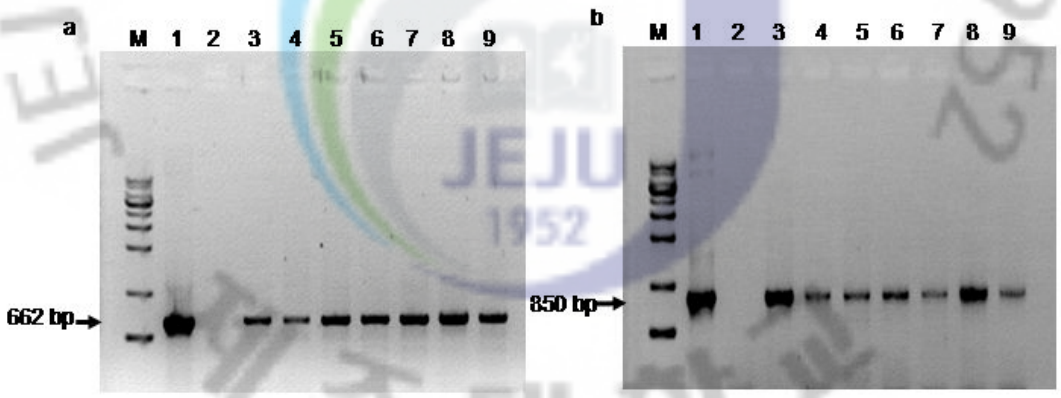

Fig. 7. PCR analyses of transgenic Miyagawa Wase plantlets. **a,** 662-bp miraculin gene fragment, **b,** 850-bp hygromycin gene fragment. M: 1 kb DNA molecular weight marker, lane 1: plasmid DNA (positive control), lane 2: untransformed plant genomic DNA (negative control), lanes 3–9: transformed transgenic plant genomic DNA

For Southern blot analysis, genomic DNA was extracted from 5 randomly selected PCR-positive plants and 1 untransformed plant and digested with *Xba*1. DIG-labeled miraculin probes were hybridized with the digested genomic DNA. Different patterns of bands were observed in the 5 transgenic plants, but no bands were detected in the untransformed plant (Fig. 8). Among the 5 transgenic plants, 2 lines (1 and 5) had 2 copies of the miraculin gene, while the other 3 lines had 1 copy. On the basis of the differences in the patterns of bands, all 5 transgenic plants were determined to be independent transformants.

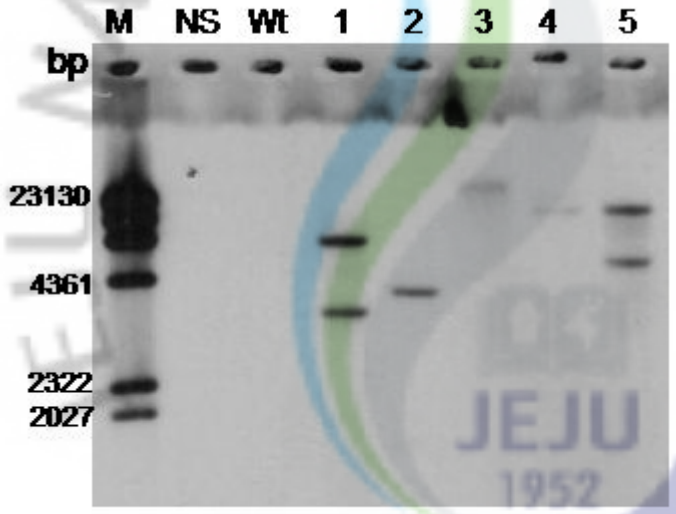

Fig. 8. **Southern blot analysis of transgenic Miyagawa Wase plants.** M: DNA molecular weight marker II, NS: no sample, Wt: wild type (negative control), lanes 1–5: transgenic Miyagawa Wase plants.

4. DISCUSSIONS

Among the three different concentrations of hygromycin (15, 20, and 25 mg/L) used in this study, first, the calluses were incubated in the liquid half EME medium containing 15 mg/L hygromycin for 2 weeks after co-culture. Cells in a liquid medium undergo a conditioning phase and resulting the occurance of changes in the endogenous hormone levels delays in response to the carbohydrate treatments (Jimenez *et al*. 2001). Cabasson *et al.* (1997) found that liquid medium improves and facilitates somatic embryo development. For embryogenic cell suspension culture, liquid medium rapidly proliferate cells and subsequently subculture on a 2 weeks transfer cycles (Grosser and Gmitter, 1990).

Hygromycin 25 mg/L was effective to select transformed callus of Miyagawa Wase. Hygromycin B efficiently discriminated between the transgenic and non-transgenic cells by suppressing the non-transgenic cells and allowing efficient proliferation of transformed cells. Necrosis and cell death was observed in the non-transformed calluses, which made identification and selection of transgenic tissues effectively and easier.

In the second and third selections, the agar concentrations (1.2% and 1.6%, respectively) were gradually increased. After the third selection, the hygromycin-resistant calluses became green and globular. Yun *et al.* (2006) and Jin *et al.* (2007) also found that agar concentrations greater than 1.2.0% were effective for callus induction and embryogenesis in Miyagawa Wase.

Table 2. Transgenic Miyagawa Wase (*Citrus unshiu* Marc.) plants containing miraculin genes *in Aagrobacterium*-mediated transformation system.

Total Number of petridish	Number of hygromycin resistant embryos	Number of selected germinated embryos	Number of selected normal plants	Number of genomic PCR confirmed plants
10	168	135	115	37

Plant regeneration from a single protoplast or callus mainly depends on the potential for somatic embryo formation. However, the potential for cell division, embryo formation, and plant regeneration from protoplasts or calluses is very low in some citrus species and cultivars, especially in mandarins due to browning and poor growth of calluses and embryoids (Han *et al.,* 2002). Since the transformation efficiency of citrus is affected by many factors, many transformation protocols are species- or even cultivar-dependent (Pena *et al.,* 1995; Gutierrez-E *et al.,* 1997; Bond and Roose, 1998; Domínguez *et al.,* 2000). Previous studies have reported that the transformation efficiency of Ponkan mandarin is about 20% (Li *et al.,* 2002) and that of Valencia sweet orange is 30% (Li *et al.,* 2003). The difference in transformation efficiencies may be due to cultivar differences. Dutt and Grosser, (2009) also found wide variation in transformation efficiencies in different cultivars, ranging from 47% in Carrizo and 40% in Duncan to 25% in Hamlin and 8% in Mexican lime while use epicotyle segments. The efficiency of *Agrobacterium*-mediated transformation system in this study is about 22%, based on the proportion of the transgenic plantlets to hygromycin-resistant calluses of Satsuma mandarin cv. Miyagawa Wase (Table 2.).

This study is the first successful transfer of a new gene into Miyagawa Wase. We have shown that our transformation system is efficient and reliable. In addition, this system has other useful advantages, such as genetically identical source material, production of

many transgenic plants, and straightforward regeneration procedures. Furthermore, transgenic calli can also be preserved over the long-term and induced to regenerate transgenic plants when necessary. We also proved it by regenerating the transgenic plants and confirmed by PCR and Southern analysis. The results presented here will be made an important contribution to the genetic transformation of Miyagawa Wase, an important citrus cultivar of Satsuma mandarin, demonstrating the possibilities for introduction of foreign genes for horticultural interest.

Part II

Functional expression of miraculin, a taste-modifying protein, in transgenic Miyagawa Wase Satsuma mandarin (*Citrus unshiu* Marc.)

ABSTRACT

Miraculin is a taste modifying plant proteins which possesses the peculiar property of being able to modify a sour taste into a sweet taste and interest has been increasing in miraculin due to this amazing property. In this study, miraculin protein was introduced into the Miyagawa Wase Satsuma mandarin (*Citrus unshiu* Marc.) calluses by *Agrobacterum*-mediated transformation to produce citrus transgenic plant under the control of 35S promoter. Satsuma mandarin is one of the choicest citrus varieties grown widely and commercially in Korea (especially in Jeju Island) and Japan, But Satsuma mandarin remains recalcitrant to transformation. Expression of the recombinant miraculin protein in transgenic plants resulted in the accumulation of significant amount of miraculin protein in the leaves. To investigate the expressed protein is correctly modified; dimerization and *N*-glycosylation of recombinant miraculin were analyzed in transgenic Miyagawa Wase Satsuma mandarin plants. Recombinant miraculin expressed in transgenic plants showed strong sweetness-inducing activity, similar to that of native miraculin. These results open up a new way of expression system in woody plants like citrus and can be a suitable alternative for producing recombinant miraculin protein.

1. **INTRODUCTION**

Miraculin is a taste-modifying plant protein isolated from miraculin fruit, the red berries of *Richadella dulcifica*, a shrub native to tropical West Africa (Therasilp and Kurihara, 1988). Miraculin is not sweet by itself, but it can modify a sour taste ino a sweet taste. For instance, once the human tongue is exposed to miraculin protein, its taste modifying unique property causes a sour taste to be perceived as sweet, and the modification effect can last as much as 1-2 h (Kurihara and Biidler, 1968; Brouwer *et al.,* 1968) even for a long period after consumption. Indigenous peoples of West Africa often use this berries of this shrub to improve the palatability of acidic foods and beverages and sometimes used to improve flavor and suppress bitterness in food and drink. For example, they are used to improve the flavor of maize dishes, such as agidi, and beverages, such as palm wine or tea. Now a days, these proteins have been used as sweetening agents, flavor enhancers, and animal fodder supplements in the food-processing industry (Sun *et al.,* 2006). As miraculin is readily soluble protein and relatively heat stable, it can also be a potential sweetner in acidic food (e.g. soft drinks).

Miraculin protein can act at extremely low concentrations, The sweetness induced by citric acid after exposure to miraculin is estimated by many researcher and it is to be about 3000 times that of sucrose on a per weight basis (Kurihara and Beidler, 1969; Theerasilp and Kurihara, 1988; Gibbs *et al.,* 1996; Kurihara and Nirasawa, 1997), and presently interest has been increasing in miraculin due to this amazing property.

Miraculin has great value as a diet and low calorie sweetener, but the commercial feasibility of miraculin is very low because this protein is still dependent on its natural source; thus, it is extracted from the miracle fruit and sold in tablets, or its seedlings and fruits are sold at an expense by importing the plant from its origin. To overcome the barriers

standing in the way of its mass production, the miraculin gene has been introduced into the foreign host to produce recombinant miraculin. To date, many attempt has been made to produce miraculin in foreign host like, *Escherichia coli* (Kurihara, 1992), yeast, and tobacco (Kurihara and Nirasawa, 1997) but failed to express miraculin with taste-modifying activity. Very recently, the production of miraculin in *E. coli* was reattempted and they showed that the taste modifying activity, without glycosylation, was no more than 72% that of native miraculin even with 1.6 time's higher density than the refined native glycoprotein (Matsuyama *et al.,* 2009).

However, when *Aspergillus oryzae* was used as a host for expressing miraculin, the recombinant miraculin protein exhibited taste-modifying properties (Ito *et al.,* 2007). From the literature studied in miraculin transformation, only a few crop species including lettuce (Sun *et al.,* 2006), tomato (Sun *et al.,* 2007) and strawberry (Sugaya *et. al.,* 2008) have been successfully expressed recombinant miraculin, In subsequent generations, stable miraculin expression was observed in tomato and strawberry but silencing of the transgene occurred in lettuce, although the recombinant protein was correctly folded in all cases.

In this study, recombinant miraculin have been successfully expressed in perennial woody plants like citrus. Worldwide one of the most demanded studied and consumed fruit has been the citrus concurrently its production has been increasing considerably for the few years. Citrus fruits are the highest value crop in terms of international trade. Among the citrus cultivar Miyagawa Wase Satsuma mandarin has unique characteristics for its seedless nature and growing in popularity as a fresh fruit for its ease of consumption. These results suggest that transgenic Miyagawa Wase plants can be good alternative vehicles for producing a biologically active form of miraculin protein.

2. MATERIALS AND METHODS

2.1 Construction of plasmid for its expression

The binary vector pCAMBIA1300 (CAMBIA, Canberra, Australia) carrying the plant selectable marker neomycin phosphotransferase II (nptII) gene and polylinker side pUC18 but absence promoter site. For construction, firstly GUS with CaMV35S promoter and nos terminal from the vector, pBI121 digested with *Eco*RI and *Hin*dIII were replaced. After that miraculin gene (Kindly provided by Dr. Sun) replaced to the GUS site digested to *Xba*1/*Sac*1. The constructed plasmid (Fig. 1) was then introduced into *A. tumefaciens* strain EHA105 for plant transformation experiments.

2.2 Transformation of Miyagawa Wase plants

Embryogenic callus from the unfertilized and undeveloped seeds of mature fruit of Miyagawa Wase, a cultivar from the Satsuma mandarin (*Citrus unshiu* Marc) was used in this study. The calluses were transformed by infection with *A. tumefaciens* EHA105 harboring the binary vectors pCAMBIA1300 with 35S promoter which contains miraculin coding region (GenBank accession number D38598). The *Agrobacterium* cells was streaked onto the LB solid medium supplemented with 50 mg/L rifampicin and 50 mg/L kanamycin at 28ºC at 250 rpm. After 2 days of growth, a fresh single colony was sub-cultured in 5 mL YEP liquid medium overnight at 28ºC in a shaking incubator at 250 rpm, followed by the addition of 45 mL of fresh YEP liquid medium and culture for 5 hrs more. When the resultant bacterial culture with an OD_{600} value of around 0.6 then collected by centrifugation at 5000 rpm for 10 mins and adjusted to an OD_{600}=0.6 with MT liquid medium containing 500 mg/L malt extract, 50 g/L sucrose and 100 μM acetosyringone (Sigma-Aldrich).

Embryogenic calluses were immersed in this *Agrobacterium* suspension for inoculation in a shaking incubator at 150 rpm for 20 mins. Then blotted dry on sterile filter paper and transferred to solidified MT medium supplemented with 50 g/L sucrose, 500 mg/L malt extract and 100 μM acetosyringone and co-culture in darkness at 24ºC for 5 days. After culture on hygromycin (15, 20 and 25 mg/L) selection medium, the hygromycin resistant calluses were used for regeneration. Regeneration was carried out according to the method described by Jin *et al.,* (2007). The resistant embryos were transferred to the embryo maturation medium and cultured for 3 weeks followed by germination medium. Four weeks culture on germination medium, the embryo containing shoot and root were transferred into MT medium (MT basal medium supplemented with 3% sucrose and 0.8% agar) for normal plant growth. After transgenic confirm by genomic PCR and southern blot analysis, the normal plants were then in vitro grafting on trifoliate orange and transferred to the green house.

2.3 Preparation of anti-miraculin antibody

Anti-miraculin antibody was prepared as follows: recombinant protein containing 1-191 amino acids of mature miraculin was produced in *E.coli* using pET-15b (Novagen) expression vector and used to raise antiserum in rabbits in assistance with Ab Frontier (Young In Frontier Co. Ltd. Seoul, South Korea).

2.4 Protein extraction and Western blot analysis

The expression level of accumulated recombinant miraculin protein in transgenic Miyagawa Wase plants were determined using western blot analysis. Frozen leaves sample were use to extract the soluble protein. Fully expanded young leaves were collected from the transgenic and non-transgenic Miyagawa Wase plants and ground in liquid nitrogen to a fine

powder. The powder (0.1 g) was resuspended in 200 µl of exraction buffers containing 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 2 mM EDTA and 2% Polyvinylpolypyrrolidone (PVPP). The leaf extract was centrifuged at 15000 rpm for 20 min at 4ºC, and the resulting supernatant was subjected to western blot analysis. The protein concentrations in the extracts were determined using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Inc., USA). The extracted proteins (2 µg per lane) were separated by SDS–PAGE and electrophoreically transferred onto Hybond ^{TM} membrane (GE Healthcare UK Ltd, Buckinghamshire, UK). The membrane then was blocking with 5% skim milk overnight at 4ºC. The blots were reacted with anti-miraculin antibody followed by incubation with anti-rabbit immunoglobulin G (IgG) coupled to horseradish peroxidase. Immunoreactive signals were detected using WEST-ZOL plus western blot detection system (iNtRON Biotechnology Inc.) and the membrane was expose to X-Ray film (Agfa Health care, Germany).

2.5 Analysis of dimerization and *N*-glycosylation of recombinant miraculin expressed in Miyagawa Wase transgenic plants

To confirm whether the recombinant miraculin expressed in transgenic Miyagawa Wase formed a disulphide linked dimer, extracted protein from transgenic Miyagawa Wase leaf were subjected to SDS-PAGE under non-reducing and reducing conditions. The extracts (2 µg per lane) from transgenic Miyagawa Wase plants were separated by SDS–PAGE under non-reducing and reducing conditions and analyzed by western blotting as described above.

The method of *N*-glycosylation of recombinant miraculin expressed in Miyagawa Wase transgenic plants performed according to describe Sun *et al.,* (2006). In brief, The total soluble proteins (20 µg) extracted from transgenic Miyagawa Wase plants were prepared in 0.01M sodium acetate buffer (pH 5.13) and boiled for 5 min, and then 2.5 mU *N*-glycosidase A (Roche Diagnostics GmbH, Germany) were added. Enzyme digests were conducted at

37ºC for 24 h, and aliquots of the proteins were subjected to SDS-PAGE in the presence of dithiothreitol (DTT) and western blotting with the anti-miraculin antibody.

2.6 Expression of recombinant miraculin protein in *E. coli*

A method of expression of recombinant miraculin in *E. coli* was as follows: recombinant protein containing amino acids 1-191 of mature miraculin was produced in *E. coli* strain BL21 using expression vector pET-21a-d (+) (Novagen). These bacterial cells transformed with pET21a-miraculin were grown at 37ºC in 5 mL of LB medium with 100 mg/L ampicillin in shaking incubator at 225 rpm overnight (Pre-culture) after that 1 mL of this pre-cultured was added to 100 mL of LB medium with 100 mg/L ampicillin and culture to an OD_{600} of ~0.5–0.8, followed by grown at 18ºC for 16 hrs after the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation with 8000 rpm 10 min at 4ºC and washing with 100 mL DDW. After washing resuspended the pellet with 1X PBS buffer (127 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4) and sonicated on ice for 20 times each of 15 sec and I mins interval of each sonication . The lysed cells were centrifuged (13,000 rpm 10 min at 4ºC), and the resultant supernatant was used for SDS-PAGE.

2.7 Measurement of taste-modifying activity

The taste-modifying activity of miraculin was evaluated as described previously (Sun *et al.,* 2006). Prior to evaluation, subjects repeatedly tasted a series of standard sucrose solutions (0.1, 0.2, 0.3, 0.4, and 0.5 M) and remembered their sweetness intensity. Miyagawa Wase transgenic plants leaves were washed in water and chewed 1 g and held in the mouth (tongue) for 3 min. Then the subjects spat out the material, rinsed the mouth with water and sipped 5 ml of 0.02 M citric acid and finally evaluated the sweetness intensity by choosing a

standard sucrose solution (0.1, 0.2, 0.3, 0.4, and 0.5 M) with an equivalent intensity of sweetness.

3. RESULTS

3.1 Transgenic plant production containing miraculin gene

Miraculin accumulates specifically in the pulp of the miracle fruit (Masuda *et al.,* 1995). To determine if miraculin could be produced in other plant tissues, we constructed chimeric genes using the miraculin gene with 35S promoters (Fig. 1). The transgenic plants were regenerated from embryogenic calluses inoculated with *A. tumefaciens* strain EHA 105 harboring the binary vector pCAMBIA1300 containing the miraculin gene and hygromycin as a selection marker. Only hygromycin (25 mg/L) resistant embryos were selected for regeneration and the morphologically normal plants were selected as candidate transgenic plants for further studies. The presence of the transgene was verified by genomic PCR for miraculin (fig. 7a) and hygromycin gene specific primer (fig. 7b). Out of 115 normal plants 37 were transgenic plants as verified by genomic PCR. Finally, integration of the transgene was confirmed by genomic southern blotting analysis (fig. 8). In this article the functionally expression of the taste-modifying protein miraculin have described in woody plants like citrus. Citrus is easy to manipulate in vitro and amenable to cell culture techniques (Grosser *et al*., 2000). This ability to quickly incorporate a gene of interest into a desirable cultivar makes genetic transformation a favorable alternative to produce recombinant gene.

3.2 Expression of the miraculin protein in transgenic Miyagawa Wase plants

To confirm the expression of miraculin protein in transgenic Miyagawa Wase plants, total soluble protein was extracted from the leaves of transgenic plants and analyzed by western bloting. As expected, miraculin was not detected in leaves harvested from untransformed control (Wild type) Miyagawa Wase plants. On the other hand, miraculin was detected in all independent line. Representative western blot analyses of 7 randomly selected

transgenic plants are shown in Fig. 9. The molecular mass of miraculin in these lines was very similar to that of native miraculin under reducing (28 kDa) conditions. Expression level of recombinant miraculin gene was markedly varied among the different transgenic lines. Similar results have been found in transgenic lettuce, tomato and straw berry (Sun *et al.,* 2006; Sugaya *et al.,* 2008; Kim *et al.,* 2010) suggesting that recombinant miraculin correctly expressed in transgenic Miyagawa Wase plants.

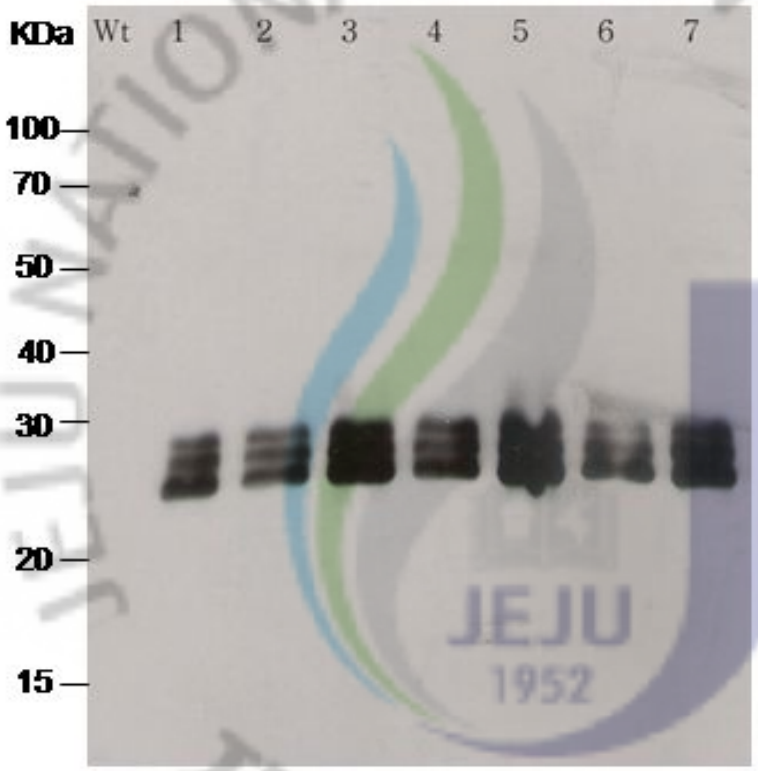

Fig. 9. Detection and expression of the recombinant miraculin protein in transgenic Miyagawa Wase plants. SDS-PAGE was performed under reducng conditions. Wt: Wild type (untransformed Miyagawa Wase) plants, lane 1-7: Transgenic Miyagawa Wase plants. The size of the protein standard is shown in KDa on left.

3.3 Analysis of dimerization and *N*-glycosylation of recombinant miraculin expressed in Miyagawa Wase transgenic plants

Naturally miraculin is a basic glycoprotein and it has been reported to be a disulphide-linked dimer with two *N*-linked oligosaccharide chains attached to asparagine-42 (Asn42) and Asn186 (Theerasilp *et al*., 1989; Kurihara, 1992). The molecular mass of the miraculin dimer was 43 kDa on SDS–PAGE (Kurihara, 1992). The molecular mass of the recombinant miraculin expressed in transgenic Miyagawa Wase plants was about 45 kDa on non-reduced SDS–PAGE (Fig. 10). This is possible that the recombinant miraculin forms a disulfide-linked dimer and is glycosylated.

To confirm whether miraculin formed a disulphide-linked dimer during post-translational modifications, soluble protein extracts from the randomly selected two transgenic plants were subjected to non-reducing and reducing SDS-PAGE. As shown in Fig. 10, under non-reducing (lanes 1 and 3) and reducing (lanes 2 and 4) conditions, miraculin migrated at about 45 and 28 kDa respectively. These results suggest that the miraculin expressed in transgenic Miyagawa Wase is a dimer and that the dimerization results from the formation of interchain disulfide linkages between the miraculin subunit.

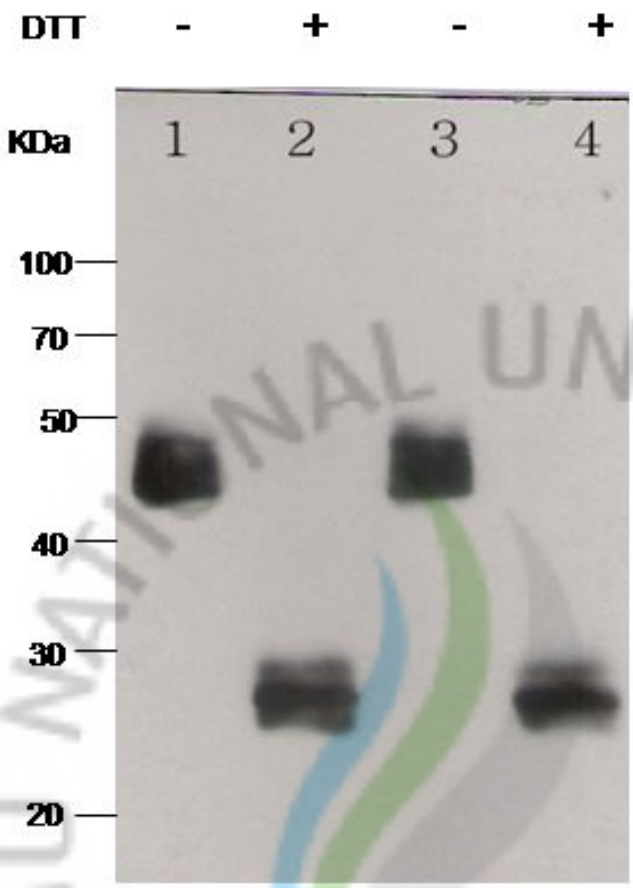

Fig. 10. Analysis of the dimerization of recombinant miraculin expressed in transgenic Miyagawa Wase plants. Lanes 1, 3: SDS-PAGE was performed under non-reducing condition without DTT. Lanes 2, 4: SDS-PAGE was performed under reducing condition with DTT. The sizes of protein standards are shown in kilodaltons (KDa) to the left.

Theerasilp *et al.* (1989) showed that miraculin contains two *N*-glycosylation sites. To confirm whether the miraculin in transgenic Miyagawa Wase is glycosylated, soluble protein extracted from the transgenic plants were treated with peptide *N*-glycosidase A (PNGase A, Roche), a glycoamidase that liberates *N*-linked oligosaccharides from glycopeptides and glycoproteins (Tarentino *et al.,* 1982). After *N*-glycosidase-A treatment, smaller bands with a molecular mass of approximately 23 kDa were detected in transgnic Miyagawa Wase (Fig. 11). This result suggested that the recombinant miraculin in transgenic Miyagawa wase is an *N*-glycosylated protein. Recombinant miraculin was expressed in transgenic lettuce (Sun *et al.,* 2006), transgenic tomato (Sun *et al.*, 2007) and transgenic straw berry (Sugaya *et al.,* 2008) and in all cases miraculin formed a disulfide bond dimmer and was glycosylated similar to native miraculin.

In addition, *Escherichia coli* functionally expressed miraculin protein but were unable to glycosylate (Matsuyama *et al.,* 2009). We functionally expressed the recombinant miraculin in pET21a vector. As shown in fig. 11 (right). *E. coli* expressed miraculin shows single band and lower molecular mass compare to the recombinant miraculin expressed in Miyagawa Wase plants. This also evidence that recombinant miraculin expressed in Miyagawa Wase transgenic plants were glycosylated.

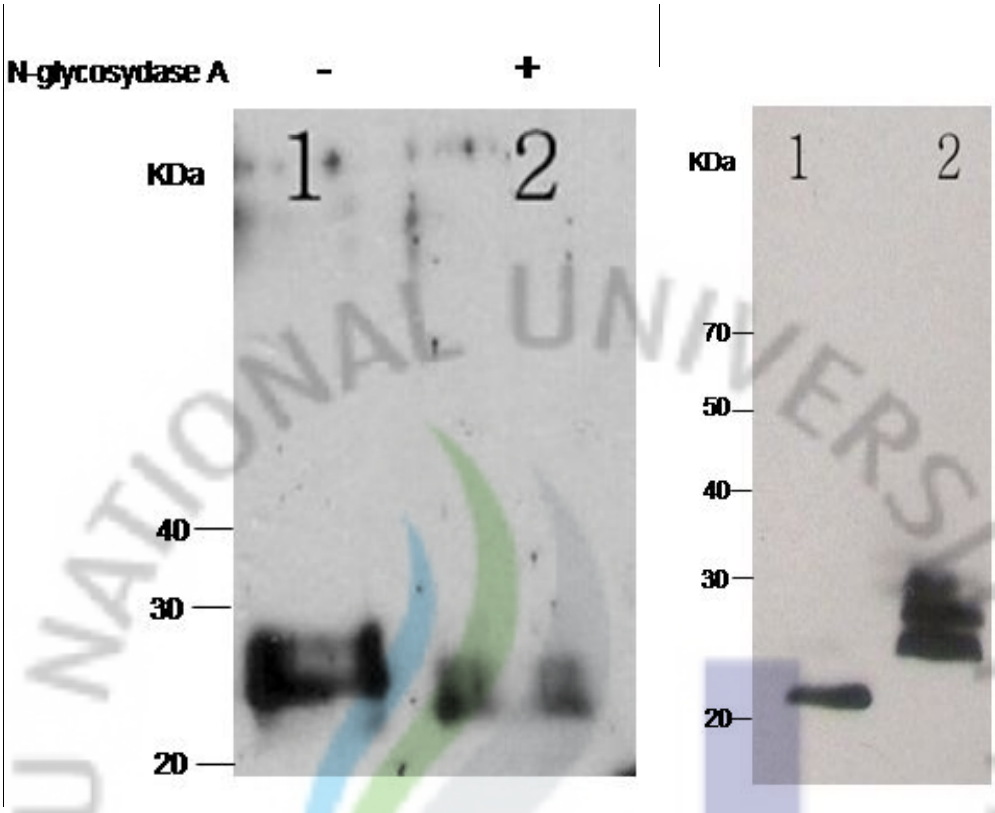

Fig. 11. Analysis of *N*-glycosylation of the recombinant miraculin expressed in transgenic Miyagawa Wase plants and *E. coli*.

Left figure - SDS-PAGE was performed under reducing conditions. Lane 1: Without *N*-glycosidase treated of the recombinant miraculin expressed in Miyagawa Wase and lane 2: *N*-glycosidase treated of the recombinant miraculin expressed in Miyagawa Wase plants. The sizes of protein standards are shown in kDa to the left. Right figure - SDS-PAGE was performed under reducing conditions. Lane 1: Recombinant miraculin protein expressed in *E. coli* (vector PET21a), lane 2: Recombinant miraculin protein expressed in transgenic Miyagawa Wase plant. The size of the protein standard is shown in KDa on the left.

3.4 Assay of taste-modifying activity

Miraculin protein has unique character, able to convert a sour taste into a sweet taste. To acertain whether the miraculin protein with citrus leaves tissue possesses taste-modifying activity, citrus leaves were subjected to sensory evaluation. The taste-modifying activity of recombinant miraculin expressed in transgenic Miyagawa Wase plants was estimated according to the previously reported method (Sun *et al.,* 2006). The sweetness intensity induced by 0.02 M citric acid after 1 g citrus leaves tissue was held in the mouth was equivalent to that of about 0.28 M sucrose (Table 3.). This value is higher than that of sweetness induced by lettuce and tomato leaves tissue and almost similar to that of one miraculin fruit (0.31M) (Sun *et al.,* 2006; Kurihara and Nirasawa, 1997). Sun *et al.* (2006) showed that the sweetness intensity of 1 g lettuce leaves tissue was 0.19 M sucrose. No sweetness was induced under the conditions of this analysis with non-transgenic Miyagawa Wase. These results clearly demonstrate that the recombinant miraculin expressed in Miyagawa Wase transgenic plants possesses strong sweetness-inducing activity.

Table 3. Sweetness intensities of recombinant miraculin expressed in transgenic Miyagawa wase plants

Source materials	Weight of Leaves tissue (g)	Induced sweetness (SEV) (M)
Miyagawa Wase Leaves tissue	1.0	0.28 ± 0.02

The sucrose equivalence value (SEV) corresponds to the sweetness intensity induced by 0.02 M citric acid evaluated by comparing its sweetness to that of a series of standard sucrose solutions (0.1 - 0.5 M). Data represent the means of five subjects' ±S.E.

4. DISCUSSIONS

In this study, we have described the functionally active and genetically stable expression of the taste modifying protein miraculin in transgenic Miyagawa wase, a woody plant. Transgenic plants have been developed as recombinant protein production systems (Howard and Hood, 2005), and the utility of these systems as bioreactors has been reviewed (Hood and Jilka, 1999; Yoshida and Shinmyo, 2000; Daniell *et al.*, 2001; Hood *et al*., 2002; Horn *et al*., 2004; Streatfield, 2007). In brief, several advantages in plant expression systems such as, low production costs, lack of animal pathogens and ease of increasing production to an agricultural scale. Moreover, plant cells are capable of complex post-translational modifications, like glycosylation, that cannot be performed in bacteria.

In this article, a plant expression system for the production of the taste-modifying protein miraculin as a potential alternative sweetner for diabetic and dietetic purposes was reported. However, the use of this protein is restricted by the limited availability of its natural source (Witty, 1998). Therefore, focused has been made on transgenic plants as an alternative production system to increase the availability of the protein. In the present study, recombinant miraculin produced in transgenic citrus plants formed disulfide-linked dimer, was *N*-glycosylated and showed strong sweetness-inducing activity. This suggests that using 35S promoters, it is possible to produce miraculin in transgenic plants. In addition, these results imply that the cleavable *N*-terminal signal peptide of the precursor of miraculin (Masuda *et al.,* 1995) may regulate folding of the precursor miraculin, induce translocation, and play a role in secretion of the protein. The sweetness after exposure to 1 g of transgenic Miyagawa Wase leaf tissue induced by 0.02 M citric acid was equivalent to that of the sweetness of 0.28 M sucrose. On a molar basis, the sweetness of miraculin is 3000 times that of sucrose. This suggests that the recombinant miraculin expressed in transgenic citrus is

biologically active and that the expression level is sufficient for modifying taste.

Worldwide one of the most demanded studied and consumed fruit has been the citrus concurrently its production has been increasing considerably for the few years. Citrus fruits are the highest value crop in terms of international trade. Among the citrus cultivar Miyagawa Wase Satsuma mandarin has unique characteristics for its seedless nature and growing in popularity as a fresh fruit for its ease of consumption. Citrus is easy to manipulate in vitro and amenable to cell culture techniques (Grosser *et al*., 2000). This ability to quickly incorporate a gene of interest into a desirable cultivar makes genetic transformation a favorable alternative to produce recombinant gene. For these reasons, citrus were used to produce miraculin.

In conclusions, we reported in this article the expression system for the production of the taste-modifying protein miraculin as a potential alternative sweetener in important woody plants like citrus which is commercially cultivated world wide. Recombinant miraculin produced in transgenic Miyagawa Wase plants formed disulfide-linked dimer, and was *N*-glycosylated. Therefore, the production of recombinant miraculin in transgenic Miyagawa Wase citrus plants will open up new ways to study taste-modifying proteins and the commercial application of miraculin as a low-calorie sweetener.

Part III

Cloning and characterization of three novel citrus ERF genes

ABSTRACT

Ethylene responsive factors (ERFs), composing the largest group of AP2 / EREBP transcription factors are involved in a variety of functions in the developmental and physiological process in plants and play an important role in regulating plant biotic and abiotic stresses tolerance. However, most of the genes in this group are functionally unknown yet, even in model plants Arabidopsis. In this study, three novel ERF genes were isolated, designated as CuERF1, CuERF5 and CuERF6 from cDNA library of the citrus cultivar Ueno Wase (*Citrus unshiu* Marc.). The CuERF1, CuERF5 and CuERF6 genes encoded proteins of 168, 187 and 268 amino acids residues with the predicted molecular mass of 18.5, 20.4 and 29.4 KDa respectively. The deduced amino acid sequences of these CuERF genes contained an AP2/ERF domain which shared high similarity with other reported AP2/ERF domains. To characterize their functions these CuERF genes were transformed in Arabidopsis since network of gene regulation in Arabidopsis is far clear than in any other plants species. The isolated CuERF genes have showed positive effect to salt stress. These results indicate that CuERF genes might have function as positive factors in the plant responses to abiotic stresses and provide useful clues for further research into the mechanism of them in regulating citrus multiple stress responses.

1. INTRODUCTION

Plants are often exposed to various biotic and abiotic environmental stresses during their life cycles, for instance, drought, high salinity, low temperature, and pathogen attack are commonly considered as plant stress factors that seriously affect plant growth and productivity (Abeles *et al.,* 1992; Wang *et al.,* 2002; Valliyodan and Nguyen, 2006). Stress-related responses are ultimately conferred by the transcriptional regulation of numerous stress-inducible genes. Previous many studies have shown that a lot of genes are induced under drought, salinity or low temperature stress in plants (Seki *et al.,* 2002). ERE binding factor (ERF) proteins (formerly known as ERE binding proteins [EREBPs]) contain a highly conserved, plant specific DNA binding domain (Hao *et al.,* 1998) consisting of 58–59 amino acids that binds to DNA as a monomer with high affinity. The ERF domain is composed of three ß-sheets and one α-helix, the former interacting monomerically with the GCC box of the target DNA (Allen *et al.,* 1998).

The AP2/ERF domain was first identified in tobacco as a novel 59 amino acids. DNA binding motif shared by four ethylene-responsive element binding proteins (EREBPs) which could specifically bind to the GCC box, an 11-bp sequence conserved in the 5' upstream region of ethylene-inducible pathogenesis-related protein genes (Ohme-Takagi and Shinshi, 1995).

The AP2/ERF gene super family has been divided in three families according to sequence similarity and number of AP2/ERF domains: the ERF family is the most numerous and contains proteins with a single AP2/ERF domain, the AP2 family is characterized by protein sequences with two AP2/ERF domains and the RAV family contains proteins with one AP2/ERF domain and an additional DNA-binding domain named B3 domain (Goremykin and Moser 2009; Nakano *et al.,* 2006). Goremykin and Moser, (2009) classify

Arabidopsis gene family and showed that the total number of AP2/ERF members present in the plant genomes is between 150 and 200 (with the exception of tobacco). By the largest part these proteins belong to the ERF family. However, few members of this huge ERF domain family have been characterized, such as Pti4, Pti5 and Pti6 from tomato (Gu *et al.,* 2000), NtERF1, NtERF2 and NtERF3 (Ohta *et al.,* 2000) from tobacco, AtERF1 to AtERF5 from Arabidopsis (Fujimoto *et al.,* 2000). Among these members, some have been shown to participate in stress responses. For example, Pti6 from tomato and Tsi1 from tobacco are involved in biotic and abiotic stress-responsive gene expression (Park *et al.,* 2001), AtERF4 from Arabidopsis is a negative regulator in the expression of ethylene, jasmonate (JA), and abscisic acid (ABA) responsive genes (McGrah *et al.,* 2005; Yang *et al.,* 2005).

The role of ERF proteins in plants have been studied extensively but in citrus a very few study has been conducted (Cevik and Moore, 2006; champ *et al.,* 2007). Citrus is one of the most commercially important crop species in the world. Ueno Wase Satsuma (*Citrus unshiu* Marc.) an early cultivar are grown widely in Korea and Japan. However, the growth and productivity of citrus are adversely affected by many biotic and abiotic stresses. Because of the prominent roles of AP2/ERF transcription factors in plant stress tolerance, I focused my attention on the isolation and initial characterization of these transcription factors in citrus. In order to characterize a putative regulatory molecular response to environmental stress tolerance, three CuERF genes from *Citrus unshiu* have been cloned and investigated with salt treatments. But further studies should be carried out to prove these hypotheses.

2. MATERIALS AND METHODS

2.1 Cloning and sequencing of CuERF gene

The cDNAs from mRNA of mature fruit of Ueno Wase (*Citrus unshiu* Marc) was used as a template for cloning CuERF genes. First of all, we searched the sequences containing the region of AP2/ERF domain genes by the tools of micro array 300k (www.ggbio.com) followed by homology searched on citrus EST (expressed sequence tag records) database from NCBI (The National centre for biotechnology information, tblastn program). Using the amino acid sequence of AP2/ERF domain from the citrus micro array chips sequence, several citrus ESTs containing putative AP2/ ERF domain have been found. Among them two ESTs from *Citrus sinensis* (Genbank accession number CX076686 and CK938535) and one EST from *Citrus clementina* (Genbank accession number FC909763) were used for further study. Based on the ESTs sequence three pair of specific primers (forward primer: 5'CCG CGT TCT AGA ATG GCG TCC TCA CGT G -3', and reverse primer: 5'- GCT CGA GAG CTC TCA AAG CCA GAG CGG T -3' for CuERF1, forward primer: 5' GCT CTC TCT AGA ATG TCT GTA ACG ACA CAA ACA-3', and reverse primer: 5'- GAG AAA GAG CTC TTA CAA TTC TAA ACA AGA CCC-3' for CuERF5 and forward primer: 5'- GAA CTC TCT AGA ATG GTC GCA GCG CTC GAA AAC-3', and reverse primer: 5'- GAG TTT GAG CTC TCA TGA AGT AAG CCC AGT GGC-3' for CuERF6) were designed and used to amplify the coding region of CuERF genes. The forward primer contained the recognition sequence for *Xba*I, and the reverse primer contained the recognition sequence for *Sac*I. The amplification reaction consisted of 95℃ for 5 min, 35cycles of amplification (95ºC for 1 min, 55ºC for 1 min, and 72ºC for 1 min), and a final extension at 72ºC for 10 min. The resulting polymerase chain reaction (PCR)

fragments were purified and subcloned into the *Xba*I / *Sac*I sites of the plasmid pUC18 in pCAMBIA1300 vector. The sequences of this clone were confirmed by DNA sequencing in EST database.

2.2 Sequence alignment and phylogenetic analysis

A multiple alignment analysis was performed with ClustalW (Thompson *et al.,* 1994) using bioinformatics tools at the website (http://workbench.sdsc.edu) version 3.2. Phylogenetic trees were constructed using the Phylips Drawgram methods (Felsenstein 1989). Database searches were performed with the micro array 300k (www.ggbio.com) program and the National Center for Biotechnology Information (BLAST) search program. Alignment of the structurally related AP2/ERF proteins was performed using Clustal W software (Thompson *et al.,* 1994).

2.3 Plant material and grown conditions

Arabidopsis seeds (ecotype Columbia-0) were washed in 70% ethanol with 0.03% TRITON X-100, rinsed three times with 70% ethanol and finally rinsed three times with sterile water and spread on half MS medium plates. The plates were vernalized for 3 days in darkness at 4℃ and then grown under standard conditions at 22±1℃ under 70 % humidity with a light/dark cycle of 16/8 hrs and after germinated transferred to the soil pot.

2.4 Gene Construction for plant transformation

The pCAMBIA1300 binary vector (CAMBIA, Canberra, Australia) includes the selectable marker gene neomycin phosphotransferase II and the pUC18 polylinker without the promoter site. For plasmid construction, we digested the pBI121 vector with *Eco*RI and

*Hind*III, and then isolated the DNA fragment containing the GUS reporter gene, CaMV35S promoter, and NOS terminator. Subsequently, this fragment was ligated into the *Eco*RI–*Hind*III site of the pCAMBIA1300 vector. The GUS gene was digested with *Xba*I and *Sac*I, and then replaced with the CuERF1, CuERF5 and CuERF6 genes respectively. Finally, the constructed plasmids (Fig. 12) were introduced into *A. tumefaciens* strain EHA105 for the plant transformation experiments.

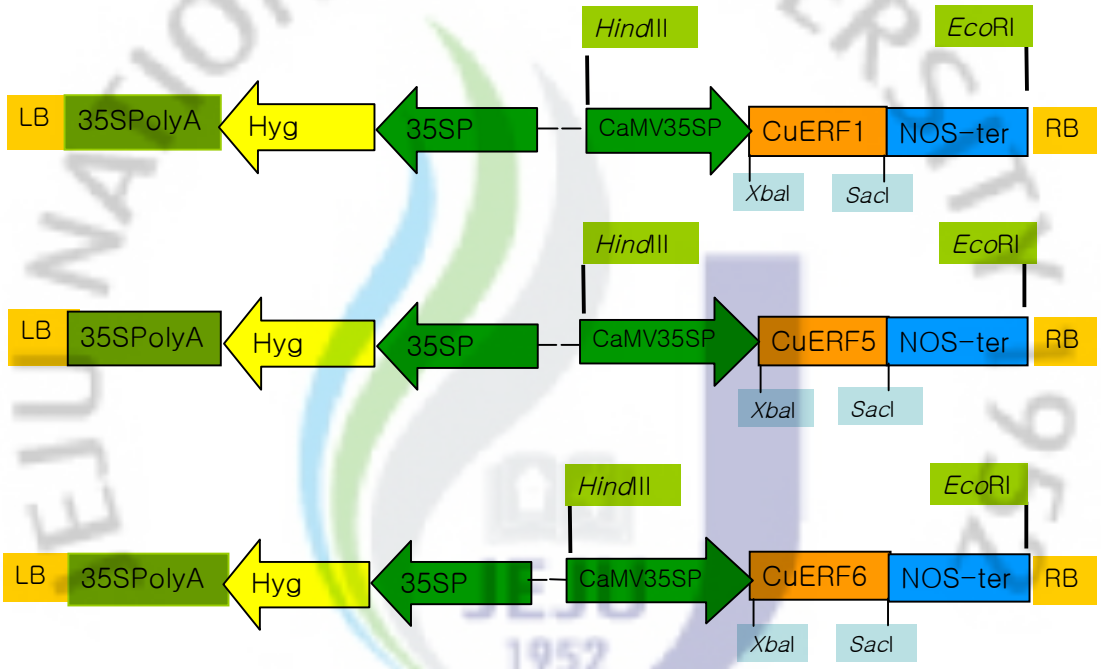

Fig. 12. Schematic representation of the binary vector pCAMBIA1300 with its position of different enzymes of plants genetic transformation. RB: Right border, LB: Left border, NOS-ter: Terminator of nopaline synthase gene, CuERF1: *Citrus unshiu* ethylene responsive factors gene 1, CuERF5: *Citrus unshiu* ethylene responsive factors gene 5, CuERF6: *Citrus unshiu* ethylene responsive factors gene 6, CaMV35SP: 35S promoter of cauliflower mosaic virus, Hyg: Hygromycin, PolyA: Poly tail A.

2.5 Transformation and selection of Arabidopsis plants

Transformation of *A. thaliana* (ecotype Columbia-0) was performed as described by Zhang *et al.* (2006) with a few minor modifications. Briefly, *A. tumefaciens* cells harboring the pCAMBIA1300 vector containing the CuERF genes were streaked onto solid LB medium supplemented with 50 µg/mL rifampicin and 50 µg/mL kanamycin, and then incubated at 28ºC. After 2 days, a fresh single colony was sub cultured in 5 ml LB liquid medium overnight at 28ºC in a shaking incubator at 250 rpm, followed by the addition of 45 ml fresh LB liquid medium and incubation to an OD_{600} of 1.5 to 2.0. Following centrifugation, the cells were suspended in 5 % sucrose (w/v) to an OD_{600} of 1.0 to 1.3 and before inoculation 0.05% silwet L-77 (Momentive Performance materials Inc. North America, USA) were added in the suspended solutions. Plants were inoculated (when most stem were 5-10 cm tall and carried several young floral buds) by slowly pepeting on the bud such a way that a film will be found around the bud. After infiltration the plants were covered with plastic film for 3 days. Hygromycin-resistant T_1 plants were selected by planting seeds on half Murashige and Skoog medium supplemented with 20 µg/mL of hygromycin and transferred the hygromycin resistant seedlings to the soil. Homozygous lines for the transgene were identified in the T_2 generation by segregation for hygromycin resistance. The vector pCAMBIA1300 were also transformation without containing CuERF genes for control.

2.6 Stress treatments

For salt treatments, seeds from transgenic lines and vector control were plated together on MS agar medium with 120 mM NaCl, 250mM sorbitol and 250 mM mannitol and incubated at 4ºC for 3 days before germination. Then the plates were placed vertically at 22±1ºC under long day condition (16 h light/8 h dark), in growth chamber. Root lengths

were measured with a ruler after 10 days to transfer the growth chamber. Photographs were also taken in this time. Three replicates were run for each treatment.

3. RESULTS

3.1 Isolation and sequence analysis of the CuERF genes

To isolate genes from the ERF family of proteins from citrus (*Citrus unshiu* Marc.), microarray chips 300K (www.ggbio.com) tools were used. The known AP2 / EREBP DNA binding domain sequence of citrus EREBP as a query, the tblastn search were performed against the expressed sequence tag (EST) records database and found several sequences encoding putative ERF genes. Three of them designated as CuERF1, CuERF5 and CuERF6 were selected for further investigation since sequence analysis indicated that CuERF1, CuERF5 and CuERF6 were potential ethylene responsive factor genes. Sequence analysis shows that CuERF1 has an open reading frame (ORF) of 507 bp capable of encoding a 168 amino acids long protein (Fig. 13), CuERF5 has an open reading frame (ORF) of 564 bp capable of encoding a 187 amino acids long protein (Fig. 14) and CuERF6 has an open reading frame (ORF) of 804 bp capable of encoding a 268 amino acids long protein (Fig. 15). The predicted protein of CuERF1, CuERF5 and CuERF6 has a calculated molecular mass of 18.5 KDa, 20.4 KDa and 29.4 KDa and p*I* of 4.29, 4.30 and 4.21 respectively.

The multiple sequence alignment of the CuERF genes and other known AP2/EREBP protein showed that similarity restricted intensively to the AP2/EREBP DNA-binding domain region (fig. 16). To determine the relationship between these three novel CuERF genes with other ERF binding proteins, a phylogenetic analysis based on the amino acid sequence of the AP2/ERF domain was carried out and the results are shown in figure 17. CuERF1 and CuERF6 were most closely related to the B-1 and B-3 subgroups of the ERF subfamily respectively. On the contrary, CuERF5 was classified into A-4 subgroups of DREB subfamily. Furthermore, CuERF1 and CuERF6 were evolutionary close to AtERF12,

RRTF1 from Arabidopsis in ERF subfamily whereas CuERF5 was evolutionary close to AtERF29 from Arabidopsis in DREB subfamily. Therefore, it might have similar function with corresponding Arabidopsis ERF genes.

Fig. 13. Gene sequence and deduced amino acid sequence of CuERF1. The ERF domain is indicated by grey boxes. The nucleotide and deduced amino acid residues are numbered in left margin. The termination cordon is marked by an asterisk. The CuERF1 gene database accession number is CX076686.

Fig.14. Gene sequence and deduced amino acid sequence of CuERF5. The ERF domain is indicated by grey boxes. The nucleotide and deduced amino acid residues are numbered in left margin. The termination cordon is marked by an asterisk. The CuERF5 gene database accession number is CK938535.

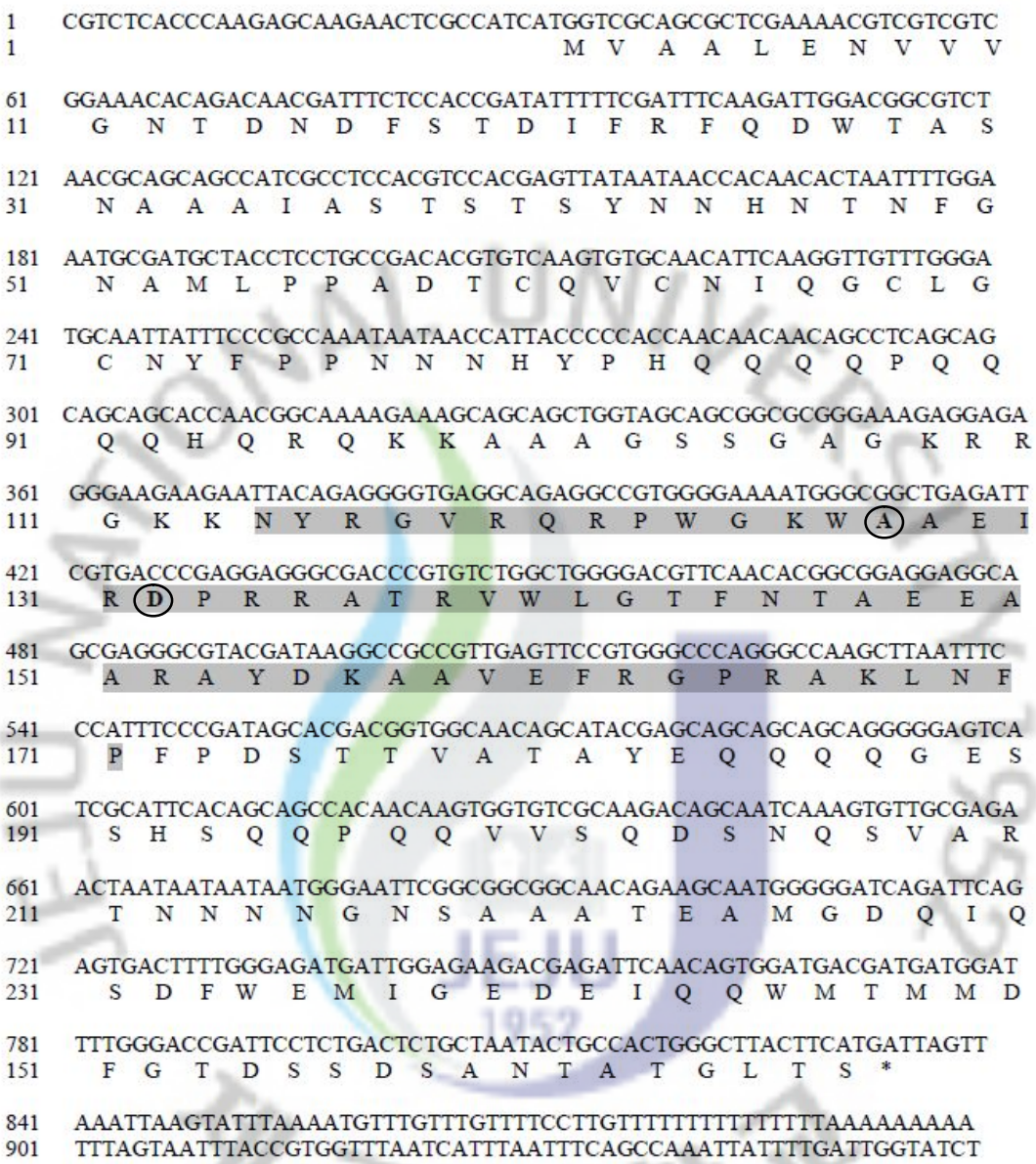

Fig. 15. Gene sequence and deduced amino acid sequence of CuERF6. The ERF domain is indicated by grey boxes. The nucleotide and deduced amino acid residues are numbered in left margin. The termination cordon is marked by an asterisk. The CuERF6 gene database accession number is FC909763.

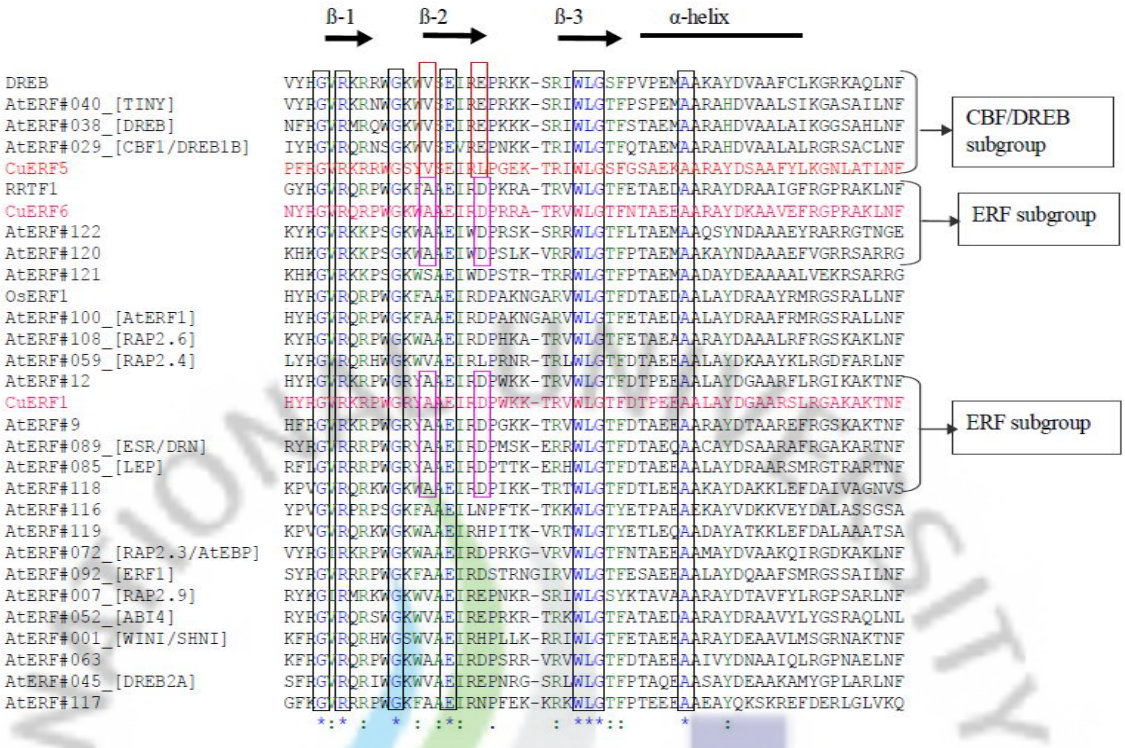

Fig. 16. Multiple sequence alignments of deduced amino acid sequences of CuERF1, CuERF5, and CuERF6 with other selected AP2/ERF domains from AP2/ERF -related proteins. Sequences are aligned using Clustal W program. The GenBank accession numbers of all proteins used here are: AtERF#001 (WINI/SHNI) (NM_101405), AtERF#007 (RAP2.9) (NM_179009), AtERF#009 (NM_123788), AtERF#012 (NM_102602), AtERF#029 (CBF1/DREB1B) (NM_118681), AtERF#038 (DREB) (NM_129125), AtERF#040 (TINY) (NM_122482), AtERF#045 (DREB2A) (NM_111939), AtERF#052 (ABI4) (NM_129580), AtERF#059 (RAP2.4) (NM_106457), AtERF#063 (AY560849), AtERF#072 (RAP2.3/AtEBP) (AY142562), AtERF#085 (LEP) (NM_121394), AtERF#089 (ESR/RDN) (NM_101169), AtERF#092 (ERF1) (NM_113225), AtERF#100 (AtERF1) (NM_117855), AtERF#108 (RAP2.6) (NM_103468), AtERF#116 (NM_102358), AtERF#117 (AY560840), AtERF#118 (AY059782), AtERF#119 (NM_113492), AtERF#120 (NM_127594), AtERF#121 (NM_126101), AtERF#122 (AY560859), OsERF (EF061888), CuERF1 (CX076686), CuERF5 (CK938535), CuERF6 (FC909763), DREB (NM_100008), RRTF1 (NM_119606)

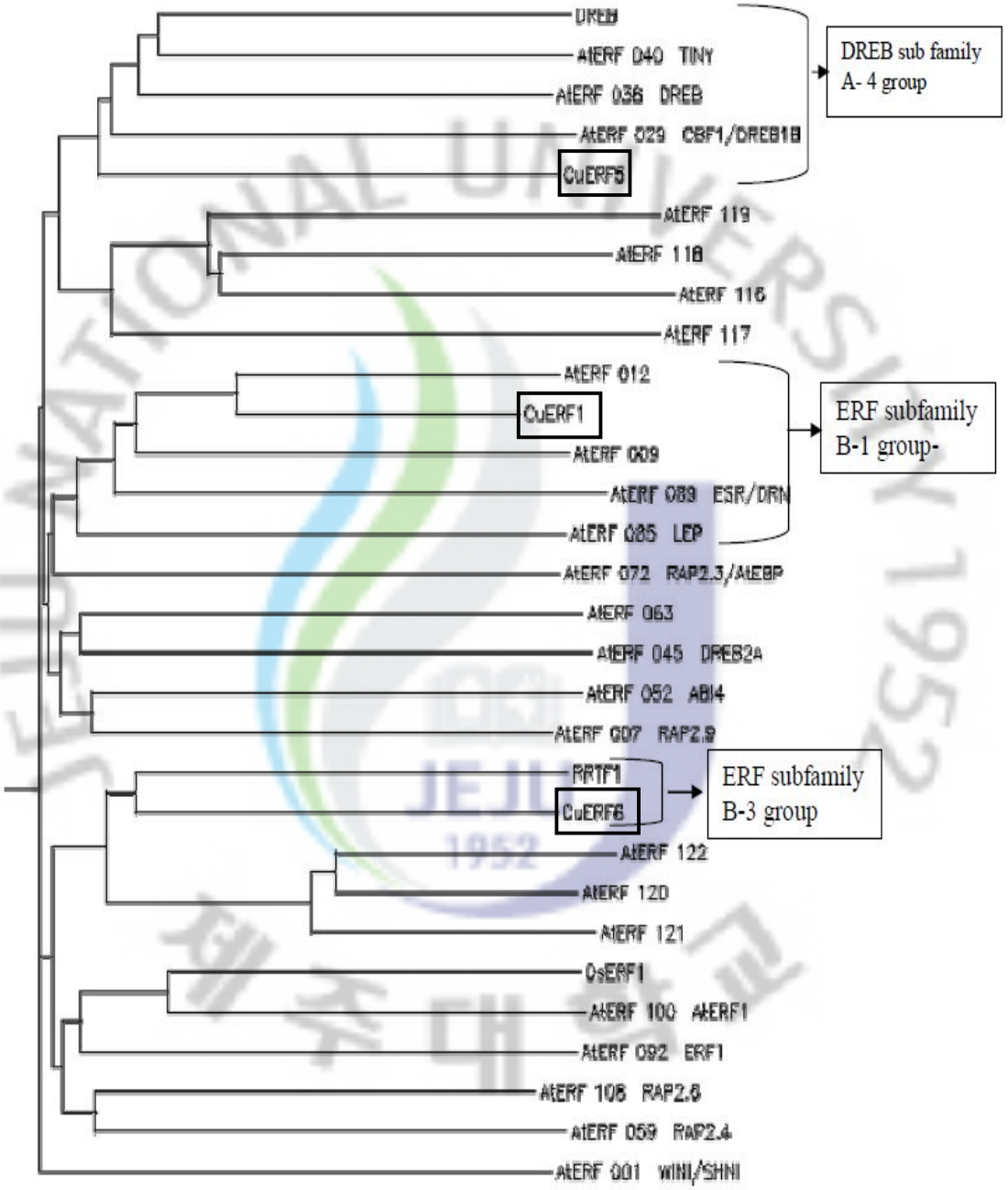

Fig. 17. Phylogenic relationships of CuERF1, CuERF5 and CuERF6 with other selected AP2/ERF domains from AP2/ERF -related proteins. Phylogenic relationships of CuERF genes withsome selected AP2/ERF domains from AP2/ERF-related protein are performed

using the Phylips drawgram methods. The GenBank accession numbers of all proteins used here are: AtERF#001 (WINI/SHNI) (NM_101405), AtERF#007 (RAP2.9) (NM_179009), AtERF#009 (NM_123788), AtERF#012 (NM_102602), AtERF#029 (CBF1/DREB1B) (NM_118681), AtERF#038 (DREB) (NM_129125), AtERF#040 (TINY) (NM_122482), AtERF#045 (DREB2A) (NM_111939), AtERF#052 (ABI4) (NM_129580), AtERF#059 (RAP2.4) (NM_106457), AtERF#063 (AY560849), AtERF#072 (RAP2.3/AtEBP) (AY142562), AtERF#085 (LEP) (NM_121394), AtERF#089 (ESR/RDN) (NM_101169), AtERF#092 (ERF1) (NM_113225), AtERF#100 (AtERF1) (NM_117855), AtERF#108 (RAP2.6) (NM_103468), AtERF#116 (NM_102358), AtERF#117 (AY560840), AtERF#118 (AY059782), AtERF#119 (NM_113492), AtERF#120 (NM_127594), AtERF#121 (NM_126101), AtERF#122 (AY560859), OsERF (EF061888), CuERF1 (CX076686), CuERF5 (CK938535), CuERF6 (FC909763), DREB (NM_100008), RRTF1 (NM_119606)

3.2 Phenotype analysis of the transgenic plants

The overexpression construct of three novel CuERF genes and the pCAMBIA1300 vector (control) were introduced into *Arabidopsis*. Among the three genes (CuERF1, CuERF5 and CuERF6) transferred Arabidopsis plants (T_3) CuERF6 was dwarf size and had more tillers (multishoot) than CuERF5 and vector control Arabidopsis plants (Fig. 18). From the Arabidopsis ERF gene family it has been found that subgroup of B-1 and B-3 ERF genes are involved in the differentiation and developmental of organs (Nakano *et al*., 2006).

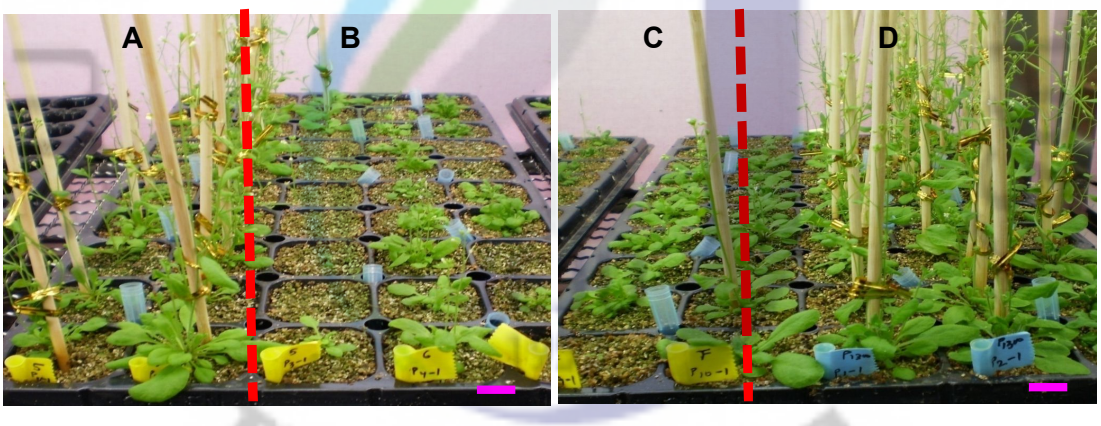

Fig. 18. Phenotype of the CuERF gene transferred *Arabidopsis* (T3) plants. A: CuERF5, B&C: CuERF6, D: Pcambia1300 vector, Scale bars represent 1 cm.

3.3 Effect of CuERF genes on plants abiotic stress (salts) treatments

Transgnic and wild type plants were grown on MS medium containing 120 mM NaCl, 250mM sorbitol and 250 mM mannitol. Root growth was measured on vertical plates. Almost same root growths were showed in CuERF1, CuERF5 and CuERF6 as that of vector control without salt treatments. However, root growth of transgenic Arabidopsis showed higher compare to vector control by the treatments of NaCl, sorbitol and mannitol. Root growth of transgenic Arabidopsis was higher in CuERF1, CuERF5 and CuERF6 more than 3, 12 and 23 fold over that of vector control by the treatments of 120mM NaCl (fig. 19). When treated with 250 mM sorbitol and 250 mM mannitol all three genes showed higher root growth compare to vector control. Among the three genes CuERF6 showed more root growth than other two CuERF gens (fig. 20). From phylogenic tree it has been found that CuERF6 is under B-3 subgroup Arabidopsis genes and many of the ERF genes in this subgroup has the salt tolerance function. TERF1 genes are in this subgroup and it has been found that tomato TERF1 modulates ethylene response and enhances osmotic stress tolerance by activating expression of downstream genes (Huang *et al.,* 2004) and expression of TERF1 in rice regulate expression of stress-responsive genes and enhances tolerance to dropught and high salinity (Gao *et al.*, 2008). CuERF1, CuERF5 and CuERF6 genes has also found to be positive effect on salt stress. Among the three CuERF genes, CuERF6 showed to be more root growth against the 120 mM NaCl, 250 mM sorbioml and 250 mM mannitol. This is due to CuERFgene specific function as similar results have been found in the subgroup-X of Arabidopsis ERF family (Nakano *et al.,* 2006).

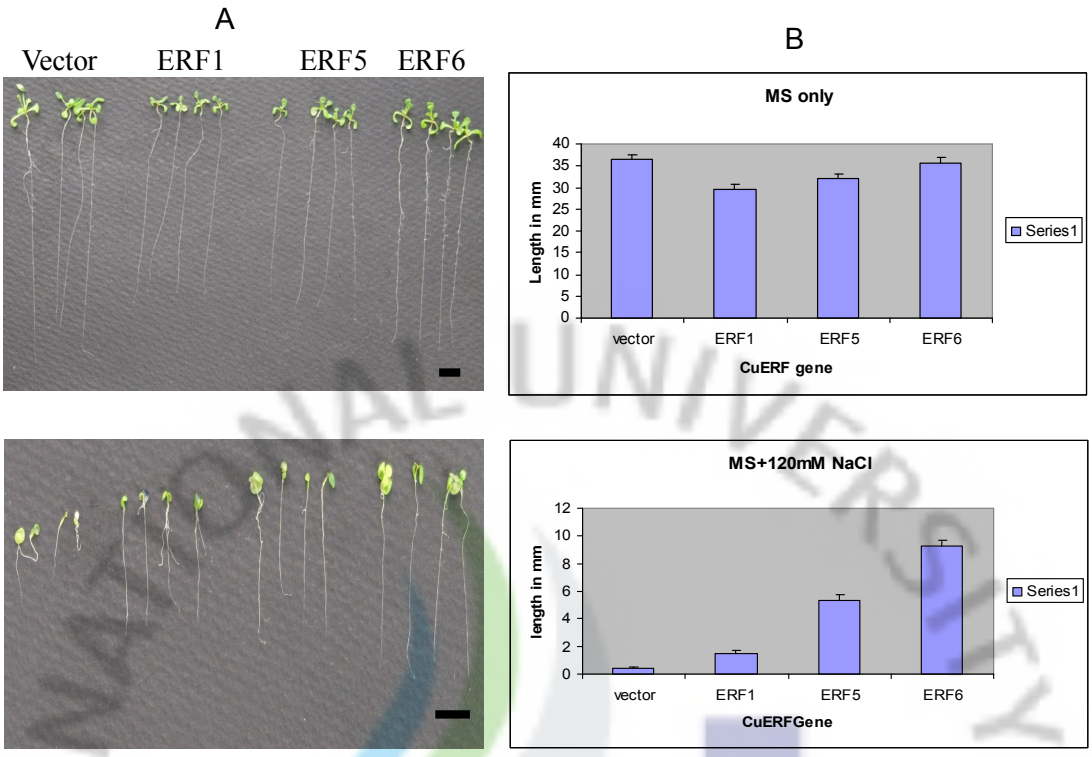

Fig. 19. Effects of salts stress (NaCl) on transgenic and vector control plants. A) Plants were grown vertically on a MS plate containing 120 mM NaCl for 10 days. Photos were taken on day 10 after stratification. Scale bars represent 0.5 cm. B) Root length statistic analysis of plate grown seedlings. Results are presented as means and standard error from more than three independent experiments. For each experiment, the root lengh of more than 10 seedlings was measured.

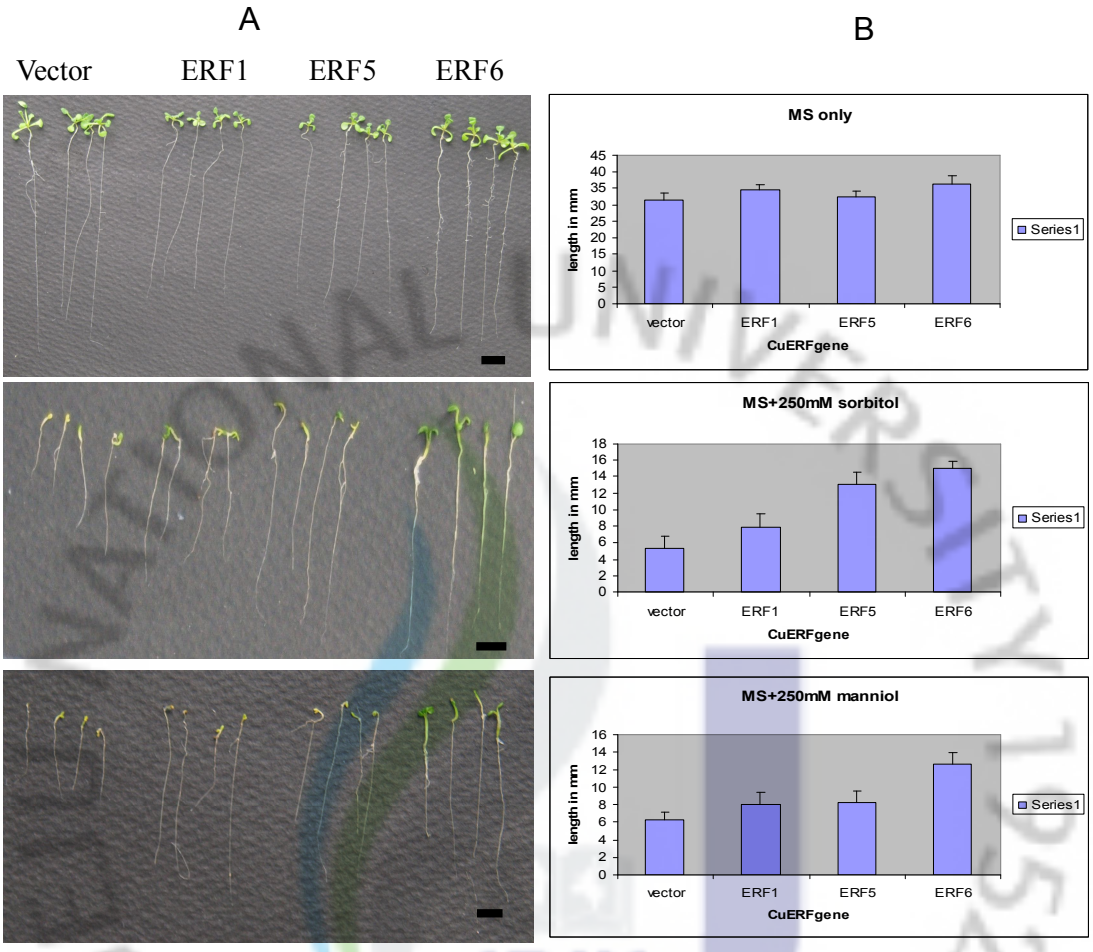

Fig. 20. Effects of salts stresses (sorbiol and manniol) on transgenic and vector control plants. A) Plants were grown vertically on a MS plate containing 250 mM sorbitol (Middle) and 250 mM mannitol (bottom) for 10 days. Photos were taken on day 10 after stratification. Scale bars represent 0.5 cm. B) Root length statistic analysis of plate grown seedlings. Results are presented as means and standard error from more than three independent experiments. For each experiment, the root lengh of more than 10 seedlings was measured.

4. DIS CUSSIONS

The ERF family is a large family of plant transcription factors and is part of the AP2/ERF superfamily, which also contains the AP2, RAV and DREB families (Riechmann and Meyerowitz, 1998). These AP2/ERF families are well-characterized proteins that are involved in a variety of cellular process such as hormonal signal transduction (Ohme-Takagi *et al.,* 1995) response to biotic (Yamamoto *et al.,* 1999; Gu *et al.,* 2000) and abiotic stresses (Liu *et al.,* 1998; Dubouzet *et al.,* 2003), regulation of metabolism (van der and Memelink 2000; Zhang *et al.,* 2005) and in developmental processes (Banno *et al.,* 2001; Chuck *et al.,* 2002). However, our understanding of the plant ERF proteins is still limited as most members of the ERF family have yet to be studied; even in the model plants Arabidopsis and rice, despite the likelihood that these genes play important roles in the regulation of many physiological processes (Nakano *et al.,* 2006; Hu *et al.,* 2008).

A great deal of experimental work will be required to determine the specific biological function of each of these genes. In the present study, three novel CuERF genes from woody plants like *Citrus unshiu* (Marc.) named CuERF1, CuERF5 and CuERF6 were isolated. Sequence alignment reveals that the deduced proteins has a central 58 amino acids AP2/ERF domain and the residues Gly-4, Arg-6, Gly-11, Glu-16, Trp-28, Leu-29, Gly-30, and Ala-38 are completely conserved among the sequences presented in the figure 16. In addition, more than 95% of the ERF family members contain Val-5, Arg-8, Ala-15, Ile-17, Arg-18, Arg-26, Phe-32, Ala-39, Tyr-42, Asp-43, and Asn-57 residues (Fig.16). These observations are generally consistent with earlier reports (Nakano *et al.,* 2006; Sakuma *et al.,* 2002). However, this same alignment indicated that the CuERF1, CuERF5 and CuERF6 are a novel member of the ERF family proteins. Furthermore, the AP2 domain of CuERF1 and CuERF6 have two conserved amino acid residues, alanine in position 14 and aspartic acid in

position 19 (fig. 13 and fig. 15) which is a typical characteristic of the ERF binding domain (Sakuma *et al.,* 2002). Sequence alignment together with phylogenetic relationship (fig. 17) with other AP2/ERF protein suggesting that our CuERF1 and CuERF6 is a member of the ERF subfamily proteins whereas CuERF5 is a member of the DREB sub-family protein.

Several reports have demonstrated that the 14th valine (V) and the 19th glutamic acid (E) may be decisive for DNA-binding specificity of DREB proteins in the AP2 domain (Sakuma *et al.,* 2002; Hao *et al.,* 2002). Particularly, the 14th valine (V) was a key to determining the specific binding activity between this protein and the DRE sequence. Li *et al.* (2005) concluded that three DREB-like proteins from soybean could bind to the DRE element regardless of glutamic (E) or leucine acid (L) at the 19th residue. At the 19th position, OsDREB1A protein containing valine (V) but not glutamic (E) acid, could bind to GCCGAC more preferentially than to ACCGAC, while OsDREB2A protein containing valine and glutamic acid at the 14th and 19th positions respectively bound to GCCGAC and ACCGAC at the same efficiency (Dubouzet *et al.,* 2003). However, recent research of several ERF proteins showed that the 14th and 19th amino acid in the ERF domain might be not significant for DNA-binding as was previously reported (Tang *et al.,* 2007).

ERF family genes play various roles in plant growth, development, and response to different environmental stress factors (Okamuro *et al.,* 1997). A number of environmental conditions including drought, cold, and high salinity subject plants osmotic stress (Luan 2002), which adversely affect the growth of plants and the productivity of crops (Yamaguchi-Shinozaki and Shinozaki, 2006). However, there are increasing reports that some genes regulate not only stress responses but also plant growth. For instance, tobacco ethylene receptor NTHK1 has been found to function in plant leaf growth and salt stress responses (Zhou *et al.,* 2006). Moreover, some ERF/AP2 proteins play a crucial role in growth and stress response. For example, expression of CAP2 from Chickpea (*Cicer arietinum*) enhances growth and tolerance to dehydration and salt stress in transgenic

tobacco (Shukla *et al.,* 2006), GmERF3 genes showed increased resistant to and enhanced tolerance to high salinity and drought stresses (Zhang *et al.,* 2009). In our study, we also found that CuERF1, CuERF5 and CuERF6 have positive effect against salt stress compared to vector control.

In conclusion, we have cloned and characterized three ERF genes namely CuERF1, CuERF5 and CuERF6 from woody plants citrus. Our results on CuERF1, CuERF5 and CuERF6 genes and other ERF genes in other species showed that Citrus ERF genes are largely conserved both in sequence and in functions in abiotic stress as in Arabidopsis. As alteration of CuERF1, CuERF5 and CuERF6 expression can significantly modify the positive effect on root growh against salt stress shown in this study, CuERF1, CuERF5 and CuERF6 may be considered as a candidate target for improvement of crop traits be genetic engineering approach in future.

CONCLUSION

The significance of the potential benefits biotechnology-based science offers can be appreciated by considering the tremendous progress in plant improvement that has been made in recent years. Recent advances in molecular genetics, informatics, and genomics research have created many new possibilities for applying biotechnology in agriculture. The findings of this study are as follows:

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- ❖ The transformation procedure yielded 37 transgenic Miyagawa Wase plants from 168 hygromycin resisant embryos containing the miraculin genes as verified by PCR amplification. Southern blot analyses of randomly selected 5 plants further confirmed that the miraculin transgene was stably integrated into the Miyagawa Wase genome.

- ❖ High and genetically stable expression of miraculin was confirmed by western blot analysis and expression of this protein in transgenic Miyagawa Wase plants resulted in the accumulation of significant amount of recombinant miraculin protein in the leaves. Recombinant miraculin expressed in transgenic plants showed strong sweetness-inducing activity, similar to that of native miraculin. Dimerization and *N*-glycosylation of recombinant miraculin in transgenic Miyagawa Wase Satsuma mandarin plants demonstrate that recombinant miraculin was correctly processed in transgenic plants.

❖ Three novel ERF genes were isolated, designated as CuERF1, CuERF5 and CuERF6 from cDNA library of the citrus cultivar, Ueno Wase (*Citrus unshiu* Marc.). The deduced amino acid sequences of these CuERFs genes contained an AP2/ERF domain which shared high similarity with other reported AP2/ERF domains. Transgenic Arabidopsis containing these CuERF genes showed a positive effect on the plant root growth against abiotic stress like salt stress.

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APPENDIX

Citrus transformation stock solution and media composition

Table A-1. *Agrobacteroium* strain culture medium composition: YEP medium (Liquid)

Components	g/L
Beef extract	5.0
Yeast extract	1.0
Peptone or Tryptone	5.0
Sucrose	5.0
$MgSO_4$	0.5

Table A-2. LB medium composition (1L)

Components	g/L
Tryptone	10.0
Yeast extract	5.0
Sodium clhloride	10.0
Agar (for solid medium)	15.0

Table A-3. Co-culture medium (Liquid and solid)

Components	**mg/L**
NH_4NO_3	1650
KNO_3	1900
$MgSO_4.7H_2O$	370
KH_2PO_4 (monobasic)	150
K_2HPO_4 (dibasic)	20
H_3BO_3	6.20
$MnSO_4.H_2O$	16.80
$ZnSO_4.7H_2O$	8.60
KI (Potassium Iodite)	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Na_2EDTA	37.25
$FeSO_4.7H_2O$	27.85
$CaCl_2.2H_2O$	439.95
Myo inositol	100
Thiamine HCL (hydrochloride)	10
Pyridoxine HCL (hydrochloride)	10
Nicotinic acid	5
Glycine	2
Malt extract	500
Sucrose	50000
Acetosyringone	100μM
Agar	8000
pH	5.2

For liquid medium above medium without agar powder. Autoclave at 121ºC 15 minutes. Acetosyringone were used after autoclave when temperature below 50ºC.

Table A-4. Preparation of EME medium for callus sub culture

Components	mg/L
NH_4NO_3	1650
KNO_3	1900
$MgSO_4.7H_2O$	370
KH_2PO_4 (monobasic)	150
K_2HPO_4 (dibasic)	20
H_3BO_3	6.20
$MnSO_4.H_2O$	16.80
$ZnSO_4.7H_2O$	8.60
KI (Potassium Iodite)	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.\ 5H_2O$	0.025
$CoCl_2.\ 6H_2O$	0.025
Na_2EDTA	37.25
$FeSO_4.\ 7H_2O$	27.85
$CaCl_2\ .2H_2O$	439.95
Myo inositol	100
Thiamine HCL (hydrochloride)	10
Pyridoxine HCL (hydrochloride)	10
Nicotinic acid	5
Glycine	2
Malt extract	500
Sucrose	50000
Agar	8000
pH	5.7-5.8

Autoclave at 121℃ 15 minutes

Table A-5. Preparation of different selecion medium

Components	First slection medium(1/2 EME)	Second slection medium	third slection medium
	mg/L	mg/L	mg/L
NH_4NO_3	825	1650	1650
KNO_3	950	1900	1900
$MgSO_4.7H_2O$	370	370	370
KH_2PO_4 (monobasic)	150	150	150
K_2HPO_4 (dibasic)	20	20	20
KCl	750	-	-
H_3BO_3	6.20	6.20	6.20
$MnSO_4.H_2O$	16.80	16.80	16.80
$ZnSO_4.7H_2O$	8.60	8.60	8.60
KI (Potassium Iodite)	0.83	0.83	0.83
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25
$CuSO_4.\ 5H_2O$	0.025	0.025	0.025
$CoCl_2.\ 6H_2O$	0.025	0.025	0.025
Na_2EDTA	37.25	37.25	37.25
$FeSO_4.\ 7H_2O$	27.85	27.85	27.85
$CaCl_2\ .2H_2O$	439.95	439.95	439.95
Myo inositol	100	100	100
Thiamine HCL (hydrochloride)	10	10	10
Pyridoxine HCL (hydrochloride)	10	10	10
Nicotinic acid	5	5	5
Glycine	2	2	2
Malt extract	500	500	500
lactose	-	70000	70000
glutamine	1550	-	-
Sucrose	50000	-	-
Agar	-	1200	1600
hygromycin	15	20	25
cefotaxime	250	250	250

Table A-6. Preparation of embryo maturation medium

Components	mg/L
NH_4NO_3	1650
KNO_3	1900
$MgSO_4.7H_2O$	370
KH_2PO_4 (monobasic)	150
K_2HPO_4 (dibasic)	20
H_3BO_3	6.20
$MnSO_4.H_2O$	16.80
$ZnSO_4.7H_2O$	8.60
KI (Potassium Iodite)	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.\ 5H_2O$	0.025
$CoCl_2.\ 6H_2O$	0.025
Na_2EDTA	37.25
$FeSO_4.\ 7H_2O$	27.85
$CaCl_2\ .2H_2O$	439.95
Myo inositol	100
Thiamine HCL (hydrochloride)	10
Pyridoxine HCL (hydrochloride)	10
Nicotinic acid	5
Glycine	2
sorbiol	1822 (0.1M)
galacttose	1802 (0.1M)
Adenine	0.7
Gelrite	2000
pH	5.7-5.8

Table A-7. Preparation of embryo germination medium

Components	mg/L
NH_4NO_3	1650
KNO_3	1900
$MgSO_4.7H_2O$	370
KH_2PO_4 (monobasic)	150
K_2HPO_4 (dibasic)	20
H_3BO_3	6.20
$MnSO_4.H_2O$	16.80
$ZnSO_4.7H_2O$	8.60
KI (Potassium Iodite)	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.\ 5H_2O$	0.025
$CoCl_2.\ 6H_2O$	0.025
Na_2EDTA	37.25
$FeSO_4.\ 7H_2O$	27.85
$CaCl_2\ .2H_2O$	439.95
Myo inositol	100
Thiamine HCL (hydrochloride)	10
Pyridoxine HCL (hydrochloride)	10
Nicotinic acid	5
Glycine	2
Cocconut water	20 ml
Coumarin	0.0146
NAA	0.02
GA_3	1
Sucrose	30000
Gelrite	2000
pH	5.7-5.8

Table A-8. Preparation of MT medium (to make normal plants)

Components	mg/L
NH_4NO_3	1650
KNO_3	1900
$MgSO_4.7H_2O$	370
KH_2PO_4 (monobasic)	150
K_2HPO_4 (dibasic)	20
H_3BO_3	6.20
$MnSO_4.H_2O$	16.80
$ZnSO_4.7H_2O$	8.60
KI (Potassium Iodite)	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.\ 5H_2O$	0.025
$CoCl_2.\ 6H_2O$	0.025
Na_2EDTA	37.25
$FeSO_4.\ 7H_2O$	27.85
$CaCl_2.2H_2O$	439.95
Myo inositol	100
Thiamine HCL (hydrochloride)	10
Pyridoxine HCL (hydrochloride)	10
Nicotinic acid	5
Glycine	2
Sucrose	30000
Agar	8000
pH	5.7-5.8

Molecular analysis solution:

Southern blot analysis solution

5 M NaCl

292.2 g NaCl dissolve into 1000 ml H_2O

10 N NaOH

100 g NaOH dissolve into 250 ml H_2O

10% SDS

10 g SDS dissolve into 100 ml H_2O

Depurination solution or buffer : 250 mM HCl – 500 mL

Denaturation solution or buffer : 1.5 M NaCl and 0.5M NaOH

Deneutralization solution or buffer : 1.5 M NaCl and 0.5 M tris HCl (pH-7.5)

Homogenization solution or buffer : 10x SSC

Hybridization buffer

Buffer 1 (Maleic acid buffer) (1L)

0.15 M NaCl ; 8.77 g

0.1 M Meleic acid : 11.607 g

NaOH pellets (1M) : 7.5 g

pH adjusted 7.5 with NaOH

Autoclaved

10% blocking solution (200 ml)

Blocking reagents : 20g

Buffer 1 : 200 ml

Dissolved it with heating with micro oven and then autoclavesd at 115ºC15 mins.

Buffer 2 (blocking solution)

10% blocking solution	: 10 ml
Buffer 1	: 40 ml

Buffer 3 (Detection buffer) (1L)

0.1 M tris	: 12.114 g
0.1 M NaCl	: 5.884 g

Adjusted pH 9.5 with HCl / NaOH and autoclaved

20X SSC (1L)

0.3M Sodium Citrate	: 88.2 g
3M NaCl	: 175.3 g

Adjust pH to 7.0

Autoclaved

30X SSC (300 mL)

NaCl	: 78.89 g
Trisodium citrate dehydrate	: 39.69 g

Autoclaved

1M sodium phosphate (250 mL)

0.2 M Na2HPO4	: 71.628 g/ 200 mL DW
0.2 M NaH2PO4	: 31.202 g/ 200 mL DW

Above sample mixed together with 3:2 and pH adjusted 7.0 and finally autoclaved.

10% N- lauroylsarcosine (50 mL)

lauryl sarcosine 5 g in 50 ml DW, filtering and autoclaved.

Hybridizaion buffer (high-SDS hybridization buffer 200 mL)

100% formamide	: 100 mL (50%)
30X SSC	: 33.3 mL (5XSSC)
1 M sodium phosphate, pH 7.0	: 10 mL (50 mM)
10% blocking solution	: 40 mL (2%)
10% N-lauroyl sarcosine	: 2 mL (0.1%)

Serially add above material and after that add 14 g SDS (7%) and stire with heating. After dissolving made 200 ml and autoclaved. Before use it was incubated at 65℃.

2X washing buffer (low stringency wash buffer, 1L)

20X SSC	: 100 mL
10% SDS	: 10 mL
Autoclaved DW	: 890 mL

Before use incubate at 37℃

0.1X wash buffer (high stringency wash buffer, 1L)

20X SSC	: 5 mL
10% SDS	: 10 mL
Autoclaved DW	: 985 mL

Before use incubate at 65℃.

Washing buffer (washing buffer for antibody treated membrane)

Buffer 1	: 300 mL
Tween 20	: 900 μL (0.3%)

Autoclaved.

Western Blot Analysis

10X TBS (1Litre)

1M Tris (pH 7.5)	: 200 ml
5M NaCl	: 300 ml
DDW	: 400 ml

pH adjusted 7.5 with 6MHCl

TBST (1L)

10X TBS (pH 7.5)	: 100 ml
Water (DDW)	: 800 ml

Adjust pH 7.4-7.5 by adding 6M HCl

Added 500 µl Tween -20 and finally made 1 litre with DDW

5X Sample buffer (10 ml)

3.1 ml 1M Tris HCl (pH 6.8)	: 312.5 mM
5 ml glycerol	: 50%
SDS (biotechnology grade MW288.38)	: 500 mg
0.5 ml bromophenol blue	: 0.05%

1.4 ml DDW

100 mM DTT were used for miraculin dimerization test.

10X SDS-Running buffer (1L)

glycine	:144 g
tris base	: 30 g
SDS	: 10 g

autoclaved

10X semi-dry transfer buffer (1L)

tris base	: 30.2 g
glycine	: 144.0 g

autoclaved.

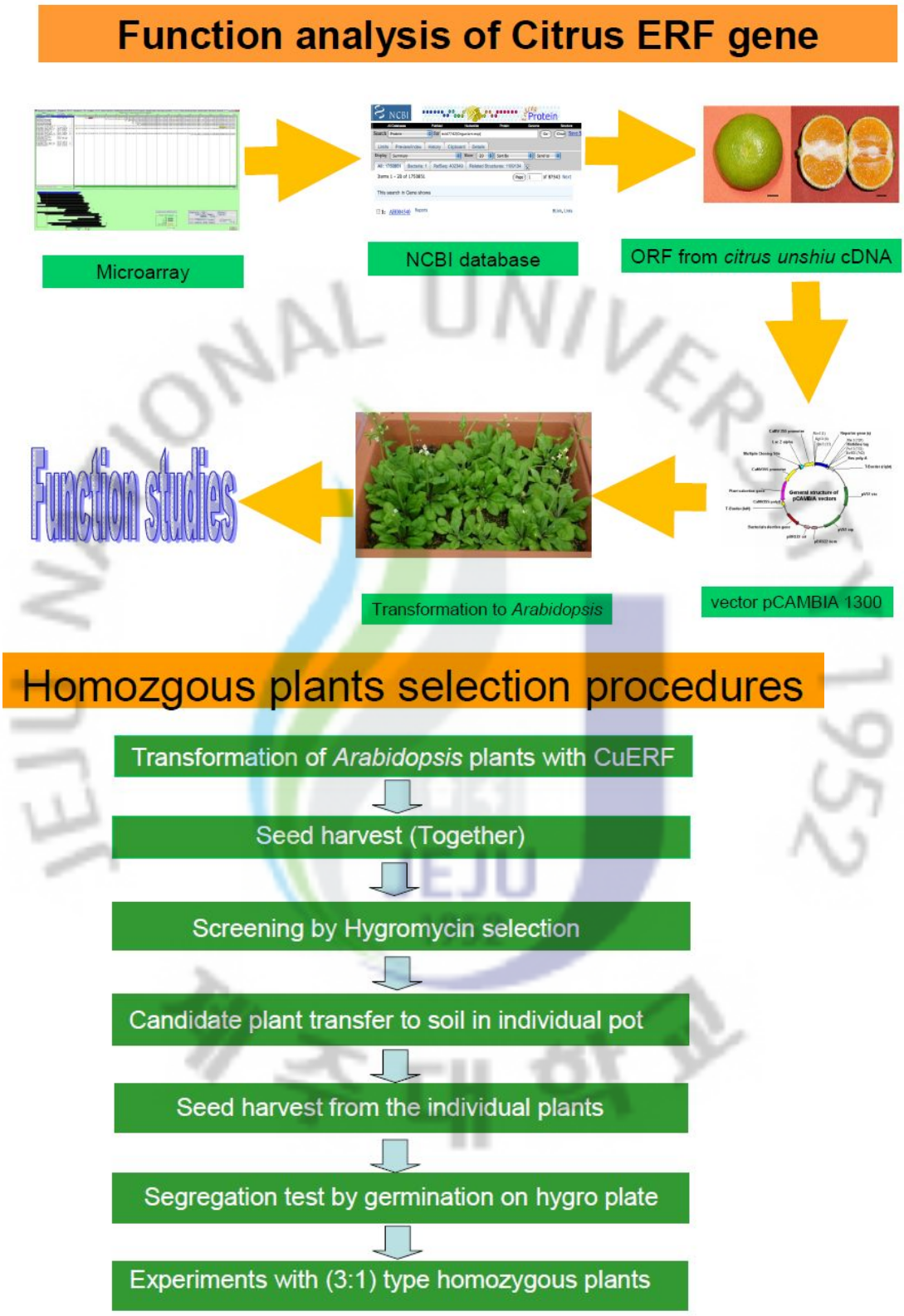

Fig. A-1. Schamatic representation of the functional analysis of CuERF genes (up) and procedure of transgenic Arabidopsis homozygous plants selection (bottom)

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