A THESIS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

OVEREXPRESSION OF *ARABIDOPSIS ABF3* GENE ENHANCED TOLERANCES TO MULTIPLE STRESSES IN TRANSGENIC *LACTUCA SATIVA*. L AND *AGROSTIS MONGOLICA* ROSHEV.

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Department of Agricultural Chemistry
GRADUATE SCHOOL
CHEJU NATIONAL UNIVERSITY

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CONTENTS

요약문

본 연구는저온, 건조 등의 환경스트레스에 대하여 저항성을 갖는 상추 및 잔디를 만들기 위해서 수행되었다. 상추(*Lactuca sativa* L.)는 전 세계적으로 가장 많이 소비되고 있는 엽채소중의 하나이며, 국내에서도 겨울철 온실 재배를 포함하여 연중 생산되고 있는 채소이다. 상추에 환경스트레스 저항성 유전자를 도입하기 위해서는 조직배양계를 확립하는 것이 중요하다. 먼저 상추의 품종 및 배양부위에 따른 식물체 재분화 효율을 검토하였고, 또한 최적의 식물체 재분화 조건을 이용하여 환경스트레스 저항성 유전자인 ABF3 를 상추에 형질전환 시킨 후 그 특성을 조사하였다.

청치마 품종의 cotyledon 절편은 다른 품종의 절편들보다 재분화율이 높았고, 부정아 형성 또한 매우 높았다. 각 절편에서 재분화된 부정아 형성율은 cotyledon 에서 가장 높았으며 다음으로 petiole, hypocotyl 순으로 재분화 효율을 보여 주었다. 따라서 청치마의 cotyledon 절편을 본 연구의 실험재료로 선택하였으며, 최적의 식물체 재분화 조건은 0.5mg/l kinetin 과 0.05mg/l NAA 의 혼합처리구가 이용되었다.

ABA 는 고등식물이 생장하는 동안 여러가지 스트레스 반응에 관여하는 생장조절물질이다. 따라서 ABA 생성에 관여하는 많은 유전자들이 보고되고 있다. 그 중에서 애기장대에서 유래된 ABF3 유전자는 전사인자로서 ABA-반응에 관련된 유전자로 알려졌다. 이러한 ABF3 유전자는 상기의 상추 조직배양계를 이용하여 *Agrobacterium* 법을 통한 형질전환을 유도하였고, 결과적으로 10.8%의 형질전환 식물체를 선발하였다.

ABF3 유전자가 도입된 형질전환 상추는 일반상추에 비해서 저온과 건조에 저항성을 갖는 특성이 관찰되었다. 또한 형질전환 식물체는 대부분 T_1 세대에서 hygromycin 저항성과 비저항성 형질이 3:1 로 분리되었다. Southern blot 분석결과 ABF3 유전자가 T_1 세대 형질전환식물체의 염색체에 안정하게 삽입 된 것을 확인하였다. Northern blot 분석결과 ABF3 유전자가 T_1 세대 식물체에서 높게 발현되는 것으로 나타났다. 또한 형질전환식물체 종자의 형태적 특성을 관찰한 결과 일반상추와 형태적으로 차이가 없었으며 발아율도 정상적이었다.

몽고잔디(*Agrostis mongolica* Roshev.)는 골프 코스의 putting green, 잔디밭, 도로주변, 목장등에 광범위하게 이용되는 몽고지역의 중요한 잔디이다. 또한 이 식물체는 포복경이 있으며, 크리핑 밴트그라스와 매우 유사하다.

몽고잔디의 캘러스 유도 및 식물체 재분화 조건을 검토한 결과 2mg/l 2.4-D 와 0.01mg/l BA 가 포함된 배지에서 1type 의 캘러스가 가장 많이 유도되었고, 0.05mg/l TDZ 가 포함된 배지에서 식물체의 분화율(캘러스당 22.1 개)이 가장 높았다.

1type 의 캘러스에 상기와 동일한 ABF3 유전자를 *Agrobacterium* 감염법을 사용하여 형질전환 시켰다. 형질전환된 캘러스를 선발하기 위해 hygromycin 이 포함된 배지에 배양하여 증식이 좋은 캘러스를 선발하고 식물재분화 배지에 치상하였다. 그 결과 23.4%의 형질전환 식물체를 선발하였다. 이러한 효율은 단자엽 식물의 형질전환에서는 대단히 높은 수치이다. 형질전환 잔디는 일반잔디에 비해서 건조, 저온, 고온에도 저항성형질이 뚜렷하게 나타났고, 또한 형질전환 식물체의 기공은 일반식물체의 기공보다 작았으며, 수분 함량은 가뭄조건에서 일반식물체보다 3-4 배 높았다. 그리고 형질전환식물체는 열과 수분 스트레스를 주었을 때 일반 식물체보다 2-3 배 높은 생존률을 보였다. 형질전환 식물체의 염색체에 삽입된 유전자는 형질전환 식물계통에 따라 다르지만 1-2 copy 가 삽입된 것으로 Southern blot 결과 확인되었다. 또한 캘러스 및 식물체에서도 GFP 유전자가 발현되었다. Northern blot 분석 결과 형질전환 식물체에서는 ABF3 유전자의 발현이 높게 관찰되었다. 형질전환 식물체는 일반식물체와 형태적으로 거의 차이점은 없었다. 그러나 몽고 잔디는 제주도의 기후적 특성이 원인인지 불분명하지만 3 년동안 재배한 결과 일반 식물체와 동일하게 형질전환 식물체에서도 개화가 관찰되지 않았다.

List of Figures

List of Tables

SUMMARY

Lettuce (*Lactuca sativa* L.) is one of the most popular vegetables that is cultivated worldwide. In this study, we identified genotypes and explant sources with high potential for adventitious shoot regeneration, subsequently established the optimal conditions for plant regeneration in each genotype and explant source. Shoot regeneration potential of cotyledon explants of Chongchima lettuce was somewhat higher and also showed better adventitious shoot formation than the other explants of the genotypes. The number of regenerated shoots per explant was dependent on the explant type, cotyledon explants showed the best shoot regeneration and formation ability among the tested three explant types, followed by petiole and hypocotyl in consecutive order. Based on the results, cotyledon explants of Chongchima lettuce was selected as the best plant material and 0.5 mg/L kinetin in combination with 0.05 mg/L NAA was established for the best condition of its shoot regeneration.

Abscisic acid (ABA) plays an important role in environmental stress responses of higher plants during vegetative growth by regulating the expression of numerous stress-responsive genes. The gene *ABF3* encodes a transcription factor regulating the expression of ABA-responsive genes. Here we report the transformation of lettuce (*Lactuca sativa* L. cv. Chongchima, leaf type) with *ABF3* derived from *Arabidopsis* using *Agrobacterium tumefaciens*-mediated system and characteristics of the transgenic plants including responses to drought and cold stresses. Transgenic plants could be

successfully obtained using a two-step selection/regeneration procedure with 10.8% of transformation efficiency. Among the tested nine transgenic lines, eight gave 3:1 segregation ratio in the T_1 progenies of self-pollinated T_0 transgenic plants, suggesting single copy integration of the transgene. One line gave 1:1 ratio, suggesting integration of more than one copy in this line. The result of Southern blot analysis of T_1 progenies showed that the *ABF3* transgene was stably integrated into the genome of transgenic plants. Northern blot analysis confirmed that the *ABF3* was highly expressed in T_1 progenies of transgenic plants. Transgenic plants showed normal growth in terms of morphologies of whole plant and seed. The transgenic plants showed higher tolerances than wild-type plants against drought as well as cold stress.

Agrostis mongolica Roshev. is an important endemic bentgrass of Mongolia widely used for golf course putting greens, lawn and roadside grassing, and livestock pasture. The plant type is stoloniferous and it has the closest genetic relationship to creeping bentgrass. Here, we found a high regenerable callus type, subsequently established the optimal conditions for callus type maintenance and plant regeneration of *A. mongolica* and established an efficient regeneration procedure. The type 1 callus was selected as the best callus type and 2 mg/L 2.4-D in combination with 0.01 mg/L BA and 0.05 mg/L TDZ was established for the best condition of type 1 callus maintenance and its shoot regeneration, respectively. It can be noticed that the selection of regenerable callus type of *A. mongolica* was one of the most critical factors to insure success in this study. The highest number of shoot (22.1 per callus) was regenerated on 0.05 mg/L TDZ

containing medium. TDZ at such a low concentration showed more effective at inducing shoot regeneration from callus of *A. mongolica* than BA either alone or in combinations with NAA.

The loss of plant productivity caused by high temperature is often serious when combined with the other environmental stresses such as water deficit. Plant hormone abscisic acid (ABA) is essential for the adaptive responses of plants to both water and heat stresses. Here we also established an efficient *Agrobacterium*-mediated transformation procedure for *Agrostis mongolica* Roshev. and generated transgenic plants tolerant to heat stress under water deficit condition using a regulatory gene, *ABF3*, which controls the ABA-dependent adaptive responses. The employment of the selected regenerable callus type (type 1) of *A. mongolica* was one of the most critical factors to insure success in this study. The transformation efficiency was 23.4% and GFP was strongly expressed in hygromycin-resistant callus and seedlings. The result of Southern blot analysis showed that the ABF3 transgene was stably integrated into the genome of the transgenic plants. Among the five transgenic lines analyzed, single transgene integration was observed in two, and two copy integration was observed in three transgenic lines. Northern blot analysis confirmed that ABF3 was highly expressed in transgenic plants, and gene silencing was not observed. Transgenic plants showed normal growth and morphology. Interestingly, both transgenic and wild-type plants did not flower during over the 3 years growth in the open field under Jeju climate. The stomata of the transgenic plants opened smaller than did the wild-type plants, and water content of the transgenic leaves remained about

3-4 fold higher than wild-type leaves under water deficit condition. These transgenic plants showed about 2-3 fold higher survival rates under heat and water stress conditions than wild-type plants.

INTRODUCTION

Abiotic stresses, such as drought, salinity, extreme temperatures, chemical toxicity and oxidative stress are serious threats to agriculture and result in the deterioration of environment. Abiotic stresses are the primary causes of crop loss worldwide, reducing average yields for most major crop plants by more the 50% (Boyer, 1982; Bray et al., 2000). Abiotic stresses lead to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity (Wang et al., 2001). Many abiotic environmental stresses, such as salinity, low temperature, heat and drought, cause water deficit in plants (Ingram and Bartels, 1996). Also, drought, salinity, extreme temperatures and oxidative stress are often interconnected, and may induce similar cellular damage (Wang et al., 2003). The complex plant response to abiotic stresses, which involves many genes and biochemical-molecular mechanisms, is schematically represented in Fig. 1. Primary stresses, such as drought, salinity, cold, heat and chemical pollination are often interconnected, and cause cellular damage and secondary stresses, such as osmotic and oxidative stress. The initial stress signals (e.g. osmotic and ionic effects, or temperature, membrane fluidity changes) trigger the downstream signaling process and transcription controls which activate stress-responsive mechanisms to re-establish homeostasis and protect and repair damaged proteins and membranes. Inadequate response at one or several steps in the signaling and

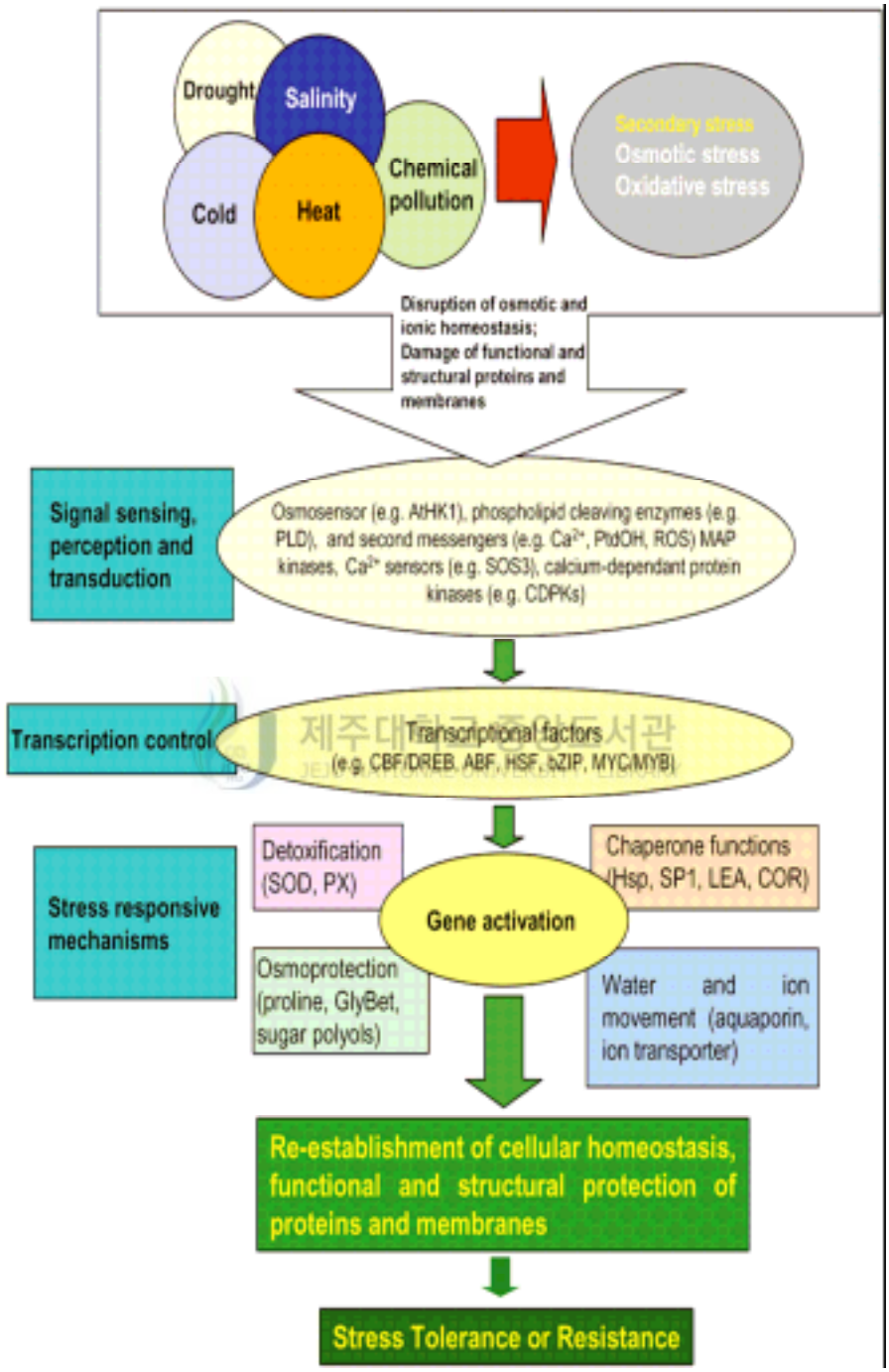


Fig. 1. The complexity of the plant response to abiotic stress.

gene activation may ultimately result in irreversible changes of cellular homeostasis and in the destruction of functional and structural proteins and membranes, leading to cell death.

Breeding for abiotic stress tolerance in crop plants (for food supply) and in forest trees (a central component of the global ecosystem) should be given high research priority in plant biotechnology programs. Molecular control mechanisms for abiotic stress tolerance are based on the activation and regulation of specific stress-related genes. These genes include three major categories (Wang et al., 2003): (1) those that are involved in signaling cascades and in transcriptional control, such as MyC, MAP kinases and SOS kinase (Shinozaki and Yamaguchi-Shinozaki, 1997; Munnik et al., 1999; Zhu, 2001), phospholipases (Chapman, 1998; Frank et al., 2000), and transcriptional factors such as HSF, and the CBF/DREB and ABF/ABAE families (Stochinger et al., 1997; Schoffl et al., 1998; Choi et al., 2000; Shinozaki and Yamaguchi-Shinozaki, 2000); (2) those that function directly in the protection of membranes and proteins, such as heat-shock proteins (Hsps) and chaperones, late embryogenesis abundant (LEA) proteins (Vierling, 1991; Ingram and Bartels, 1996; Thomashow, 1998, 1999; Bray et al., 2000), osmoprotectants, and free-radical scavengers (Bohnert and Sheveleva, 1998); (3) those that are involved in water and ion transporters (Maurel, 1997; Serrano et al., 1999; Tyerman et al., 1999; Zimmermann and Sentenac, 1999; Blumwald, 2000).

Plant modification for enhanced tolerance is mostly based on the manipulation of genes that protect and maintain the function and structure of cellular components. Present engineering strategies rely on the transfer of one several genes that are either involved in signaling and regulatory pathways, or that encode enzymes present in pathways leading to the synthesis of functional and structural protectants, such as osmolytes and antioxidants, or that encode stress-tolerance-conferring proteins (Wang et al., 2003).

Responses of plants to environmental stresses are largely controlled by plant hormone abscisic acid (ABA) (Zeevaart and Creelman, 1988). ABA signaling plays a vital role in plant stress responses as evidenced by the fact that many of the drought-inducible genes studied to date are also induced by ABA. Two transcription factor families bZIP and MYB, are involved in ABA signaling and its gene activation. Many ABA-inducible genes share the (C/T) ACGTGGC consensus, *cis*-acting ABA-responsive element (ABRE) in their promoter regions (Guiltinan et al., 1990; Mundy et al., 1990). Many *cis*-regulatory elements responsible for the ABA-regulated gene expression have been determined (Busk and Pages, 1998; Rock, 2000), and several transcription factors have been reported that interact *in vitro* with the ABRE. Recently, a small family of ABRE-binding bZIP proteins, named as **A**BRE **b**inding **f**actors (**ABF**s)/ABA-responsive element binding factors (AREBs) have been isolated by yeast one-hybrid screens (Choi et al., 2000; Uno et al., 2000). The major function of ABFs is to mediate ABA-dependent stress

signaling during vegetative growth. Constitutive expression of ABF3 or ABF4, members of ABF family, demonstrated enhanced drought tolerance in *Arabidopsis* plants, which altered expression of ABA/stress-responsive genes, e. g. *rd29B*, *rab18*, *ABI1* and *ABI2* (Kang et al., 2002), and ABF3 also demonstrated enhanced low/high temperature tolerances in *Arabidopsis* plants (Kim et al., 2004). Several ABA-associated phenotypes, such as ABA and sugar hypersensitivities were observed in transgenic plants. Moreover, salt hypersensitivity was observed on ABF3- and ABF-4-overexpressing plants at the germination and young seedling stage, indicating the possible participation of ABF3 and ABF4 in the salt response at these particular developmental stages.

Because of overexpression of ABF3 gene leads to tolerances to these stresses in transgenic plants, we tried to construct these stresses tolerant transgenic plants by introducing ABF3 gene into lettuce (*Lactuca sativa* L.) and *Agrostis mongolica* Roshev.

Part I

Plant regeneration from cotyledon, petiole and hypocotyl explants in four genotypes of lettuce (*Lactuca sativa* L.)

1. ABSTRACT

Lettuce is a dicotyledonous leafy vegetable that belongs to the family of Compositae. In this study, we identified genotypes and explant sources with high potential for adventitious shoot regeneration, and subsequently established the optimal conditions for their plant regeneration. Shoot regeneration potential of cotyledon explants of Chongchima lettuce was somewhat higher and also showed better adventitious shoot formation than the other explants of the genotype. The number of regenerated shoots per explant was dependent on the explant type, cotyledon explants is the best in term of its shoot regeneration and formation ability among the tested three explant types and followed by petiole and hypocotyl in consecutive order. Based on the above results, we selected the cotyledon of Chomgchima lettuce as an explant source and 0.5 mg/L kinetin in combination with 0.05 mg/L NAA was established for its optimal culture condition.

2. INTRODUCTION

Lettuce (*Lactuca sativa* L.), one of the most popular vegetables that is cultivated worldwide (Michelmore and Eash, 1988). The leaves of lettuce are edible and important source of vitamins A and C, and lactupicrin (Ryder, 2002), which are known to prevent cancers and insomnia disease (Resh, 2001).

The focus of modern lettuce breeding objectives is to improve horticultural characteristics such as quality, resistance to early bolting and breeding for resistance to pests and diseases (Hunter et al., 2002). Since lettuce is drought and cold sensitive, it would be desirable to develop drought and cold tolerant lettuce varieties. It is possible these qualities may be improved using genetic engineering. The success of gene transformation in plants largely depends on the establishment of effective *in vitro* plant regeneration system (Dandekar, 1992; Yepes and Aldwinkle, 1994). In the previous lettuce studies, lettuce adventitious shoots have been produced from cotyledon (Doerschug and Miller, 1967; Webb et al., 1984; Woo et. al., 1999; Hunter et al., 2002) and hypocotyl explants (Sasaki, 1979; Woo et. al., 1999) using tissue culture. Moreover, the effect of lettuce genotype on adventitious shoot production had been tested (Xinrun et al., 1992; Thorpe, 1994; Ampomah-Dwamena et al., 1997; Hunter et al., 2002).

The objectives of this study were to develop a reproducible method to obtain more shoot production through optimization of regeneration medium

among wide range of combinations of growth regulators, to identify genotypes and explant sources with high potential for shoot regeneration and to establish the optimal conditions for plant regeneration in each genotype and explant source.

3. MATERIALS AND METHODS

3.1. Plant materials

Four genotypes of lettuce, three (Red curled leaf lettuce, Sunny lettuce and Chongchima) from leaf type and one (Sakurament) from crisphead type were used.

3.2. Preparation of explants

Mature seeds of four genotypes of lettuce were surface-sterilized in sodium hypochlorite solution (2% active chloride) containing 0.1% (v/v) of Tween 20 for 15 min, then rinsed three times with sterilized water. The seeds were plated on a medium comprising basal salts and vitamins of MS (Murashige and Skoog, 1962) supplemented with 100 mg/L myo-inositol, 3% (w/v) sucrose, 0.2% (w/v) phytagel (MSBM). The medium was adjusted to pH 5.8, and then autoclaved at 1.2 - 1.3 kg/cm^2 pressure and 121 °C for 20 min. The cultures were maintained under *in vitro* culture conditions adjusted to 25 ± 1 ^{o}C and 18-h photoperiod of 45 μmol m^{-2} s^{-1} illumination provided by cool white fluorescent lamps. Each petridish (90 mm in diameter) contained 30 ml of medium and was sealed with Micropore Surgical Tape (3M Health Care, USA). After 7 days of culture, explants (5 mm in diameter cotyledon, 5 mm in length petiole and hypocotyl) were aseptically excised from lettuce seedlings and used for further experiments. All cultures were maintained under the same *in vitro* culture conditions as described above.

3.3. Plant regeneration from explants

To determine the effect of growth regulators on shoot regeneration from the cotyledon, petiole and hypocotyl explants of each genotypes, the MSBM media containing various combinations of kinetin (0, 0.25, 0.5, 1, or 2 mg/L) with NAA (0, 0.05, 0.1, 0.2, 0.4, or 1.6 mg/L) were used. Each of the treatment was carried out in triplicate (10 explants in a petridish per replication) and the shooting test was repeated three times separately. After 4 weeks of culture, the numbers of regenerated shoots were counted.

For rooting, cotyledon explants with adventitious shoots were incubated in a medium containing 1/2 MS salts and vitamins with 50 mg/L myo-inositol, 1.5% (w/v) sucrose, and 0.2% (w/v) phytagel for 2 weeks. After root formation, the plants were transferred to 20 x 20 cm plastic pots with peat moss and perlite (1:1) and grown in an environmentally controlled glasshouse adjusted to 20 ± 2 °C, 60 ± 10% relative humidity, and 18-h photoperiod of 30 μmol m^{-2} s^{-1} illumination provided by cool white fluorescent lamps.

4. RESULTS

4.1. Shoot regeneration from cotyledon explants

A great number of shoots were regenerated from cotyledon within a range of combinations of kinetin 0.25-1 mg/L with 0.05 or 0.1 mg/L NAA in all four genotypes, while shoot formation was greatly stimulated by ratios of kinetin and NAA, 5:1 or 10:1 (Fig. 2-5). The highest number of shoots per cotyledon was obtained at following conditions; Sunny lettuce (7.35) on 0.25 mg/L kinetin and 0.05 mg/L NAA, Red curled leaf lettuce (8.6), Chongchima (10.9) and Sakurament (8.97) on 0.5 mg/L kinetin and 0.05 mg/L NAA, respectively. In contrast, no shoot was regenerated when NAA used alone without kinetin, and also kinetin alone was not effective for optimal shoot regeneration from cotyledons of all four genotypes. Shoot regeneration was dramatically decreased with increasing NAA and kinetin concentrations, respectively (Fig. 2-5). Even though in Red curled leaf lettuce, the high concentration of kinetin (2 mg/L) in combinations with NAA and high concentration of NAA (1.6 mg/L) in combinations with kinetin completely inhibited shoot regeneration (Fig. 2).

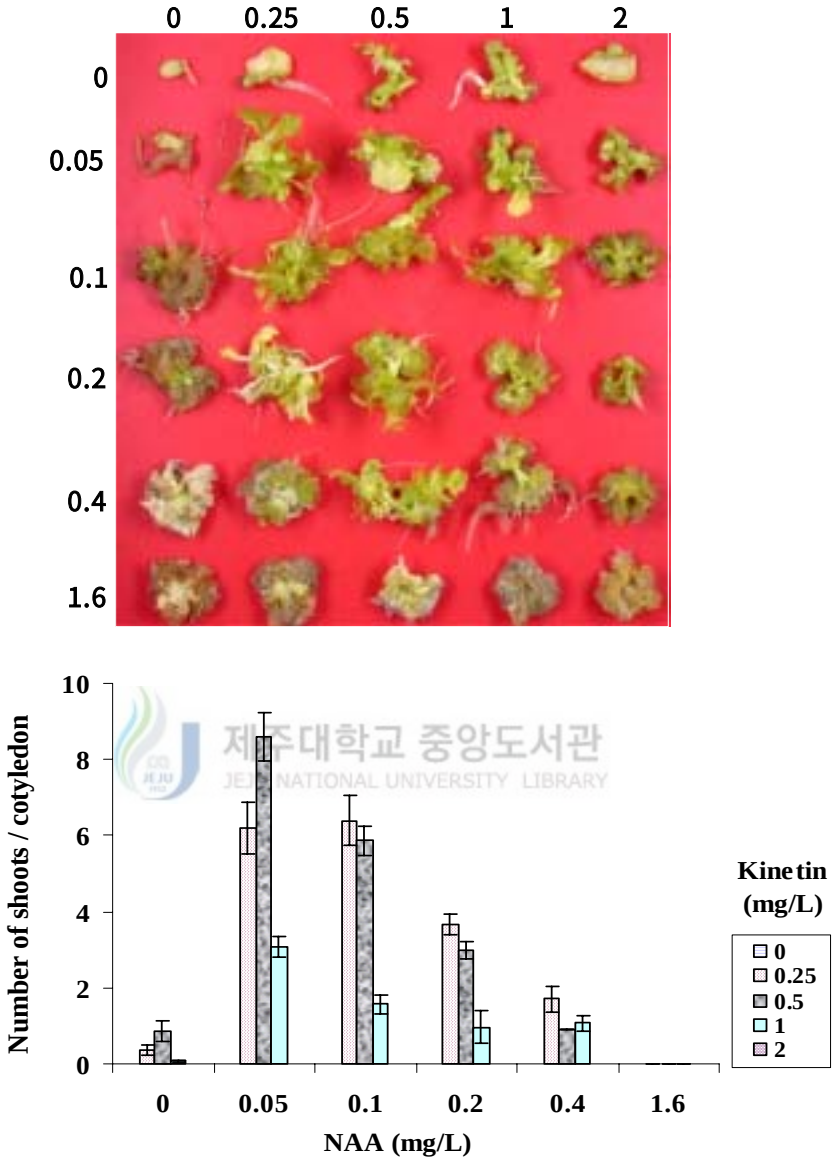


Fig. 2. Shoot regeneration from cotyledon explants of *Lactuca sativa* L. cv. Red curled leaf lettuce on MSBM medium containing various combinations of kinetin with NAA. Columns indicate the mean number of regenerated shoots per explant of three replicates. Bars refer to standard error.

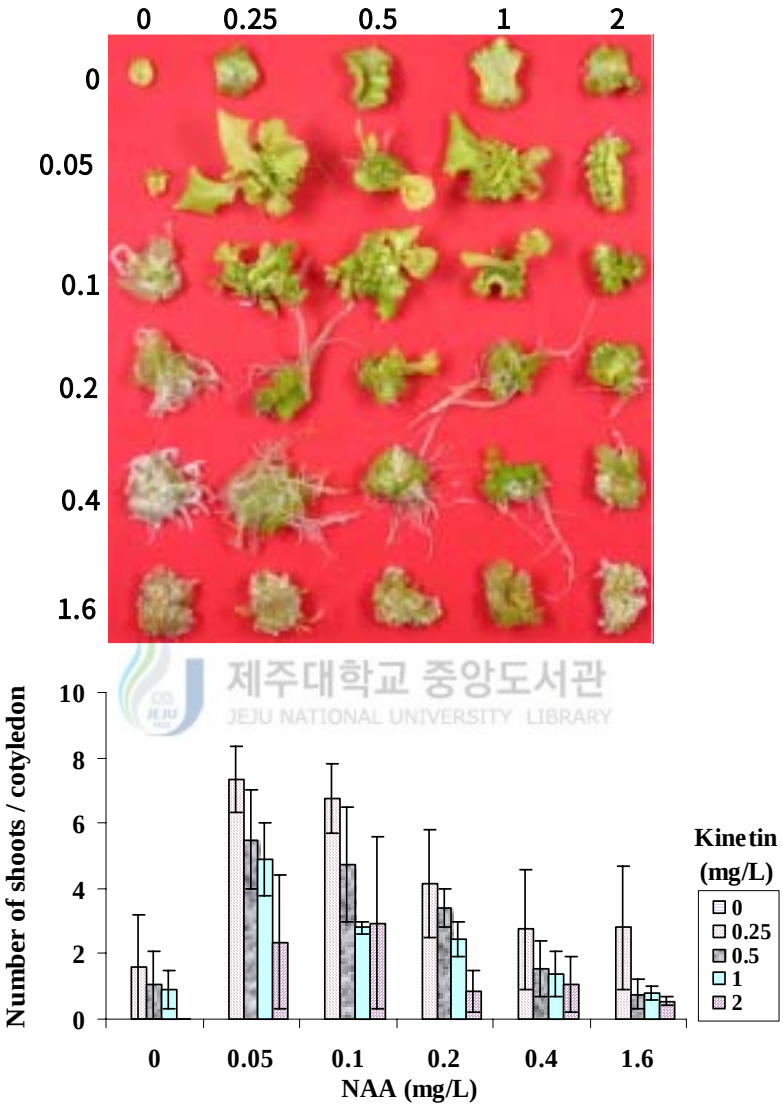


Fig. 3. Shoot regeneration from cotyledon explants of *Lactuca sativa* L. cv. Sunny lettuce on MSBM medium containing various combinations of kinetin with NAA. Columns indicate the mean number of regenerated shoots per explant of three replicates. Bars refer to standard error.

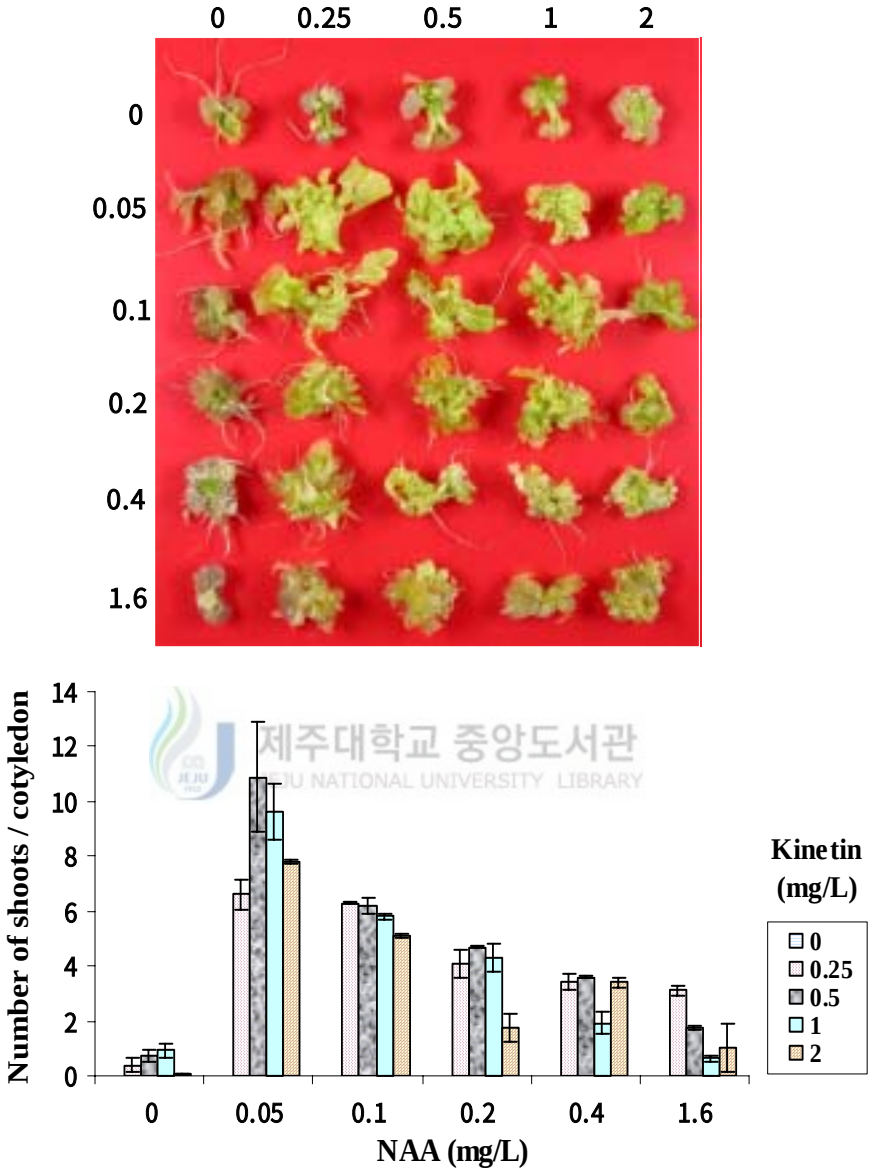


Fig. 4. Shoot regeneration from cotyledon explants of *Lactuca sativa* L. cv. Chongchima on MSBM medium containing various combinations of kinetin with NAA. Columns indicate the mean number of regenerated shoots per explant of three replicates. Bars refer to standard error.

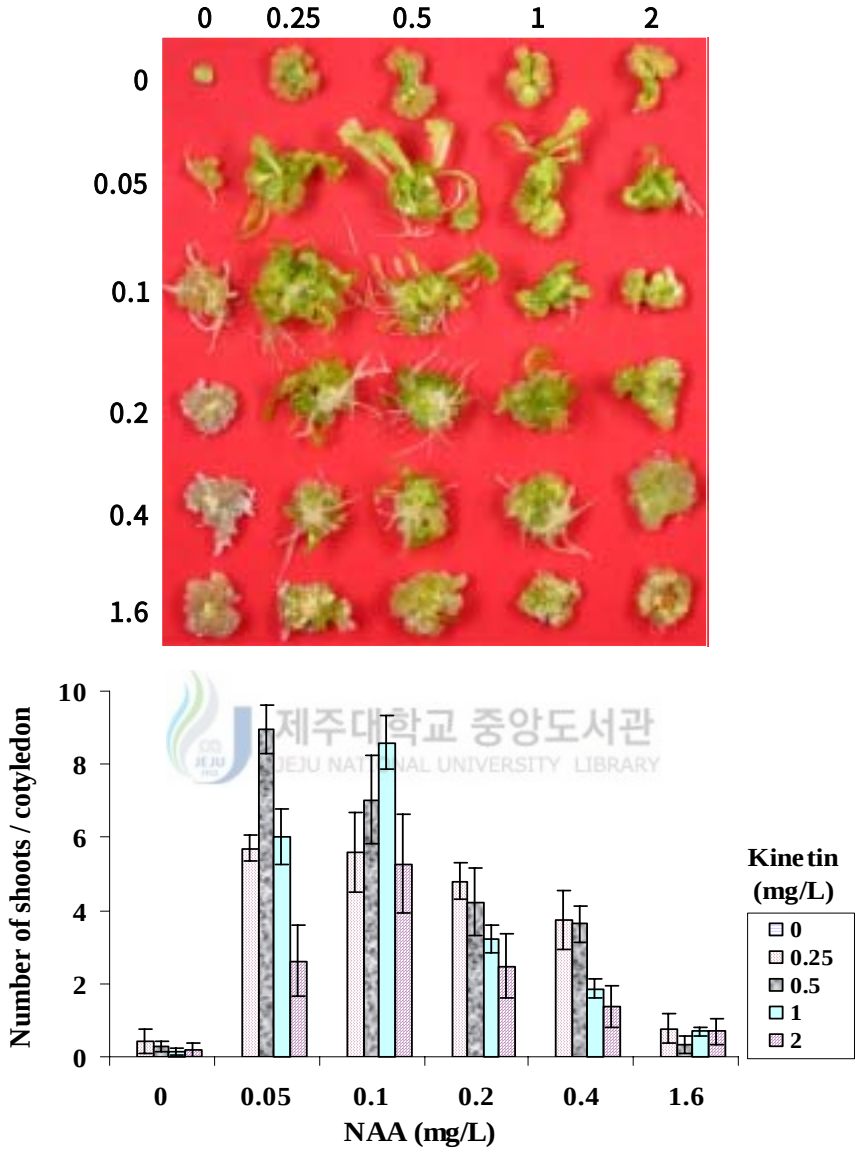


Fig. 5. Shoot regeneration from cotyledon explants of *Lactuca sativa* L. cv. Sakurament on MSBM medium containing various combinations of kinetin with NAA. Columns indicate the mean number of regenerated shoots per explant of three replicates. Bars refer to standard error.

4.2. Shoot regeneration from petiole explants

A great number of shoots were regenerated from petiole within a range of combinations of kinetin 0.25 or 0.5 mg/L with 0.05-0.2 mg/L NAA in all four genotypes, while shoot formation was greatly stimulated by ratios of kinetin and NAA, 2.5:1 or 5:1 (Fig. 6-9). The highest number of shoots per petiole was obtained at following conditions; Red curled leaf lettuce (7.4) and Sunny lettuce (9.5) on 0.25 mg/L kinetin and 0.1 mg/L NAA, Chongchima (8.13) on 0.5 mg/L kinetin and 0.2 mg/L NAA and Sakurament (8.97) on 0.5 mg/L kinetin and 0.1 mg/L NAA, respectively. Shoot regeneration was dramatically decreased with increasing kinetin and NAA concentrations. Higher concentrations of NAA in combinations with kinetin only produced callus formations from petiole explants of all the genotypes. Moreover, no shoot was regenerated when NAA used alone without kinetin, and also kinetin alone was produced only a few number of shoots in all genotypes of leaf type lettuce, but in Sakurament, it was completely inhibited the shoot regeneration.

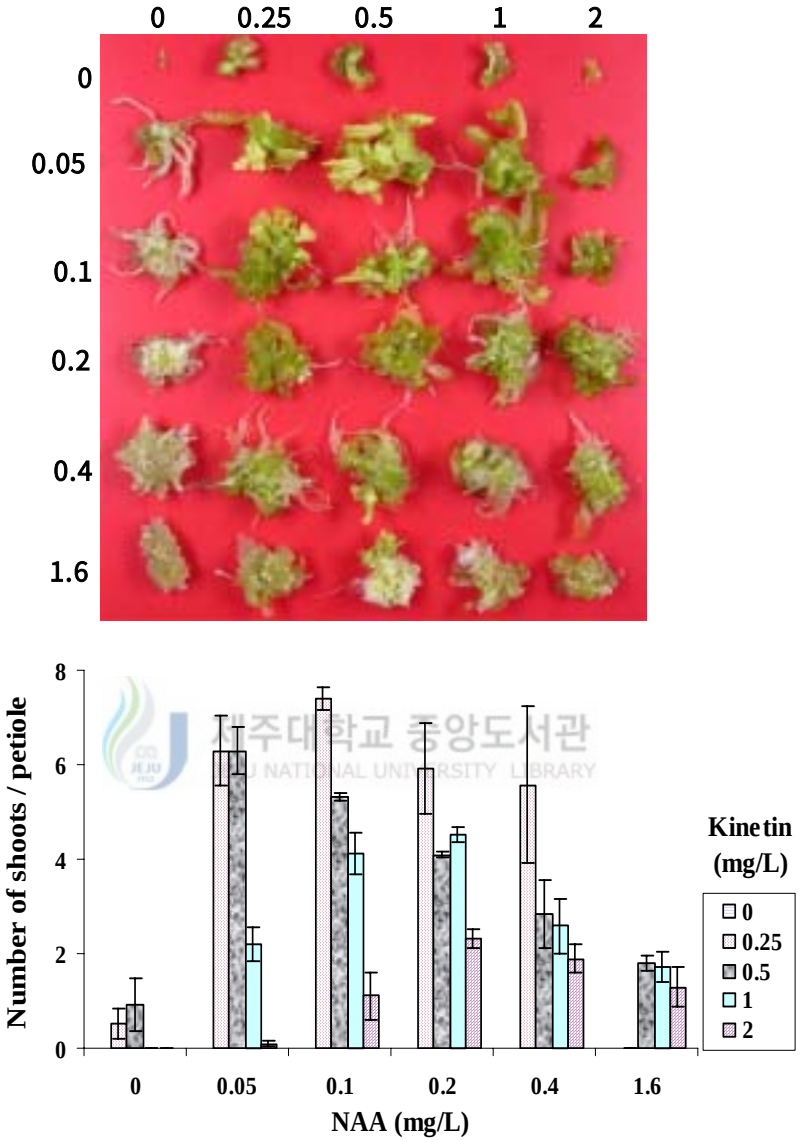


Fig. 6. Shoot regeneration from petiole explants of *Lactuca sativa* L. cv. Red curled leaf lettuce on MSBM medium containing various combinations of kinetin with NAA. Columns indicate the mean number of regenerated shoots per explant of three replicates. Bars refer to standard error.

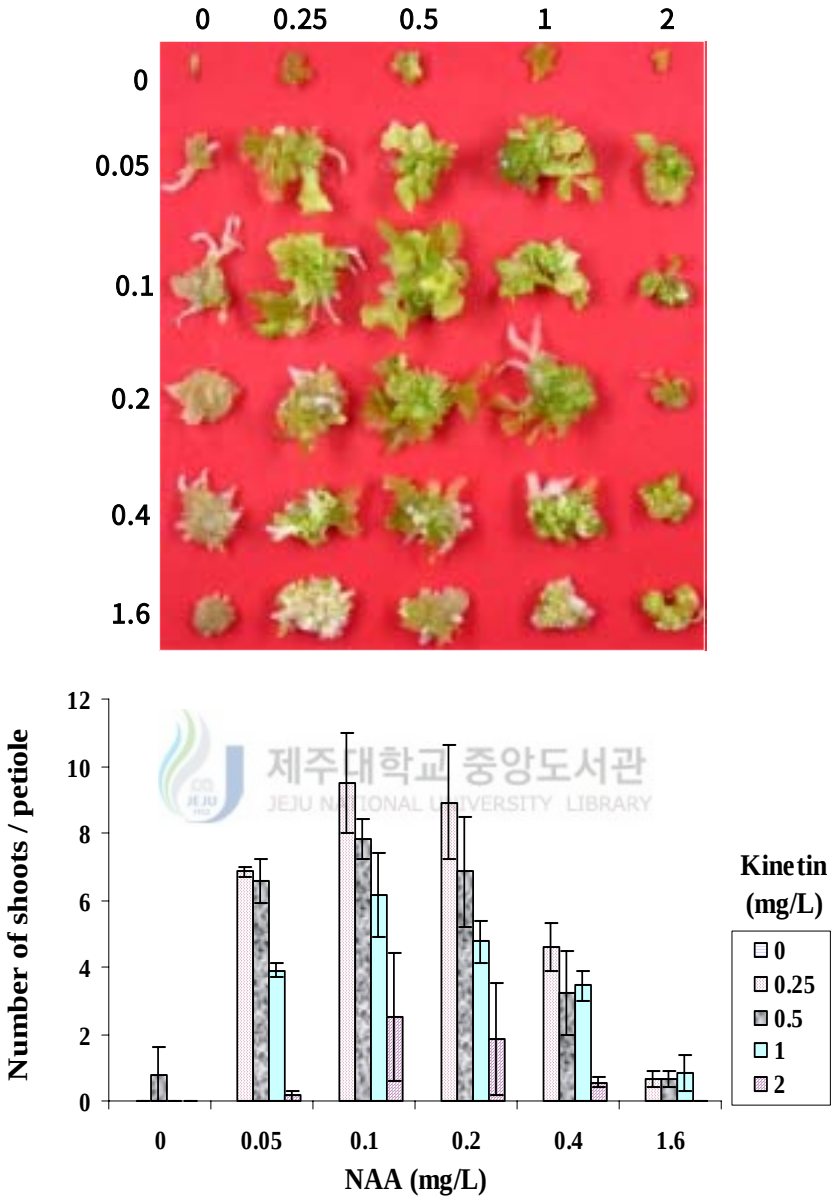


Fig. 7. Shoot regeneration from petiole explants of *Lactuca sativa* L. cv. Sunny lettuce on MSBM medium containing various combinations of kinetin with NAA. Columns indicate the mean number of regenerated shoots per explant of three replicates. Bars refer to standard error.

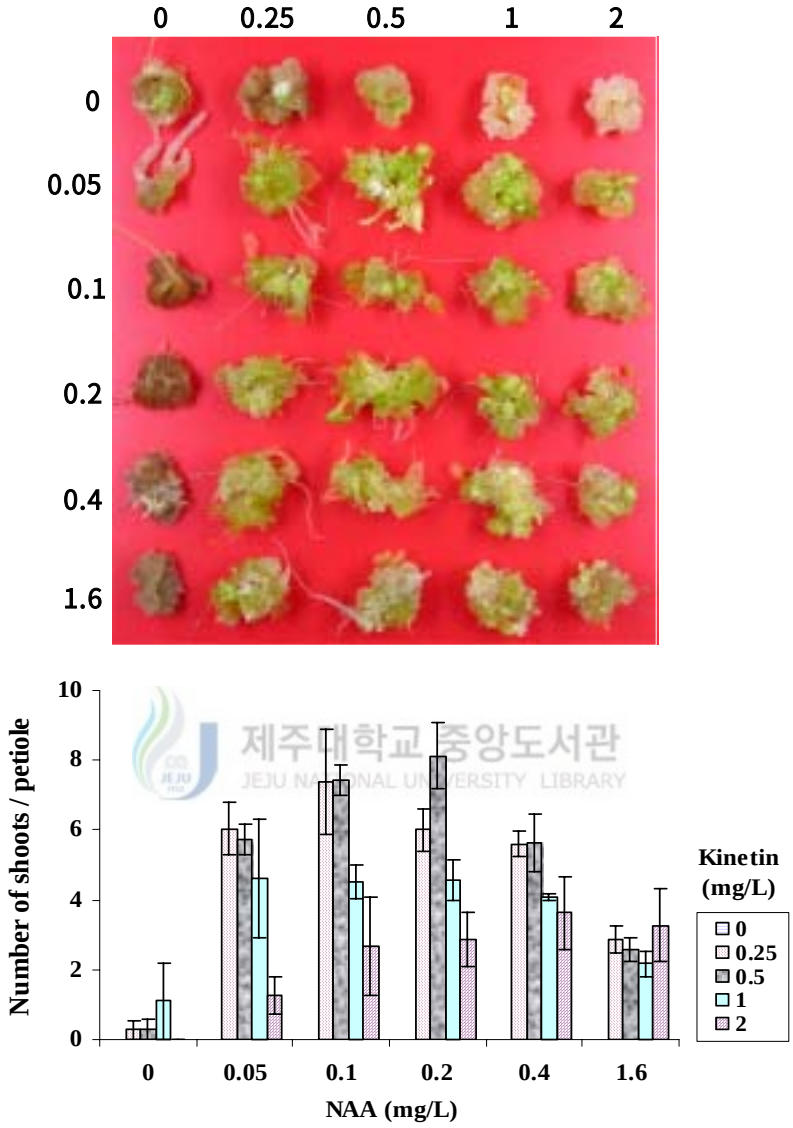


Fig. 8. Shoot regeneration from petiole explants of *Lactuca sativa* L. cv. Chongchima on MSBM medium containing various combinations of kinetin with NAA. Columns indicate the mean number of regenerated shoots per explant of three replicates. Bars refer to standard error.

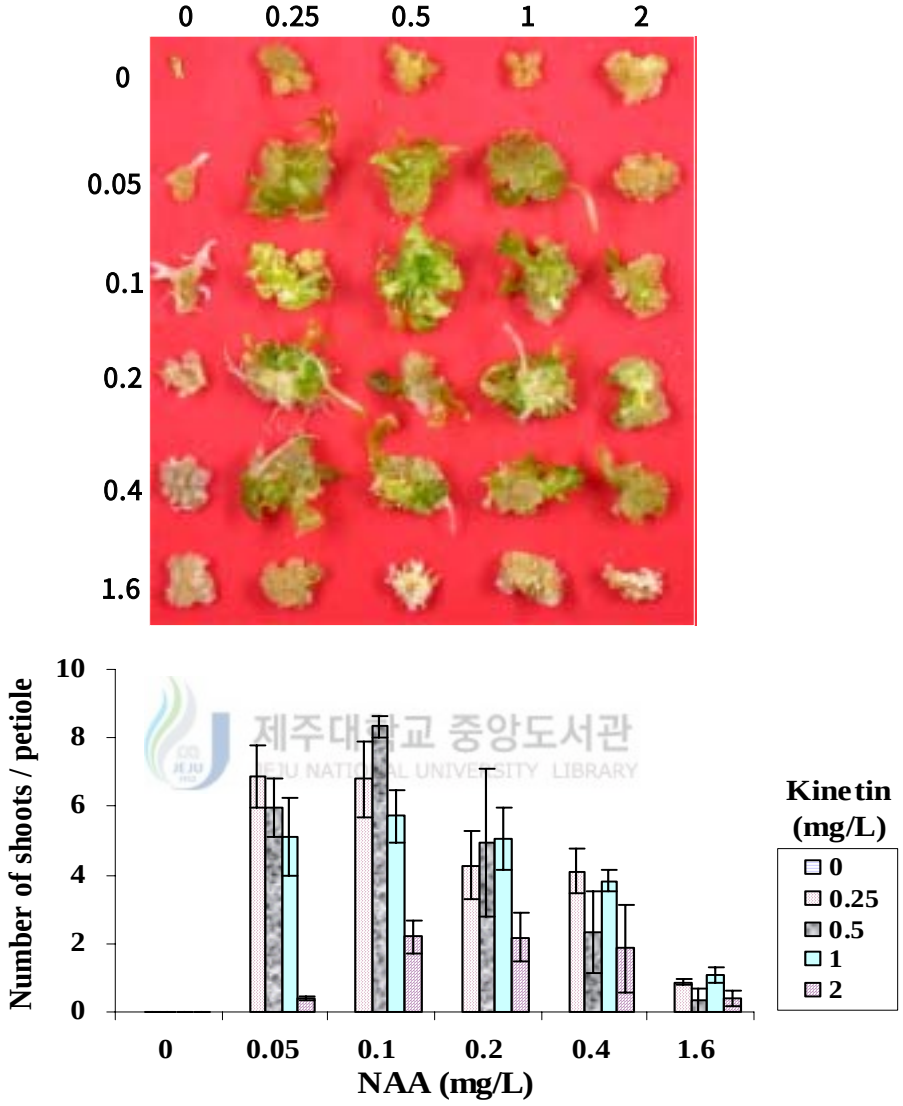


Fig. 9. Shoot regeneration from petiole explants of *Lactuca sativa* L. cv. Sakurament on MSBM medium containing various combinations of kinetin with NAA. Columns indicate the mean number of regenerated shoots per explant of three replicates. Bars refer to standard error.

4.3. Shoot regeneration from hypocotyl explants

A great number of shoots were regenerated from hypocotyl within a range of combinations of kinetin 0.25 or 0.5 mg/L with 0.1 or 0.2 mg/L NAA in all four genotypes, while shoot formation was not observed on all combinations of mediums (Fig. 10-13). The highest number of shoots per hypocotyl was obtained at following conditions; Red curled leaf lettuce (6.5) and Sunny lettuce (4.0) on 0.25 mg/L kinetin and 0.1 mg/L NAA, Chongchima (5.8) and Sakurament (5.6) on 0.5 mg/L kinetin and 0.1 mg/L NAA, respectively. As a result, NAA alone was completely inhibited the shoot regeneration and also kinetin alone was produced only a few number of shoots in three genotypes but in Sunny lettuce it was inhibited shoot regeneration completely. Shoot regeneration was dramatically decreased with increasing kinetin and NAA concentrations. The higher concentrations of kinetin in combination with NAA as well as the higher concentrations of NAA in combinations with kinetin also produced only callus formations from hypocotyl explants of all the genotypes.

The regenerated shoots from cotyledon and petiole explants were rooted all and more than 90% of these regenerants survived under the environmentally controlled glasshouse conditions and developed into mature, fully formed plants which were morphologically similar to seed-grown plants. Since adventitious shoots formation was not observed in hypocotyl, we failed to develop fully formed plants from hypocotyl.

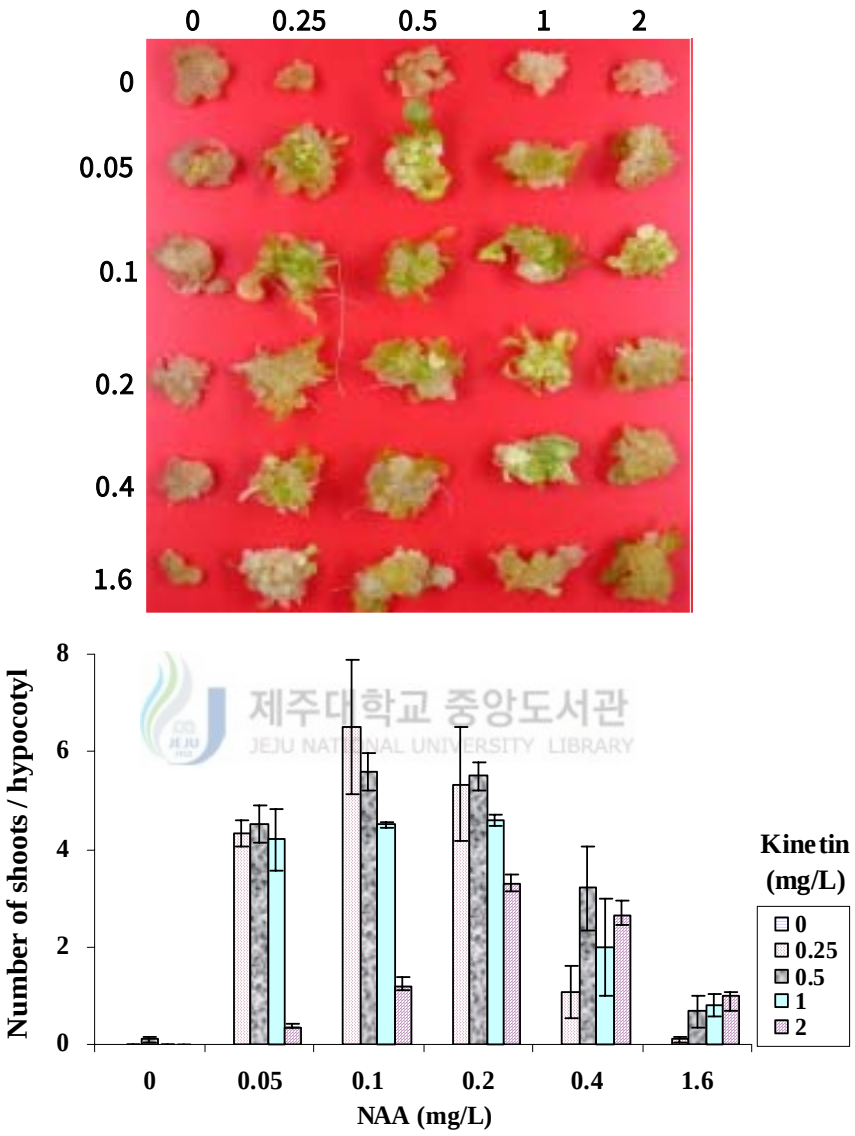


Fig. 10. Shoot regeneration from hypocotyl explants of *Lactuca sativa* L. cv. Red curled leaf lettuce on MSBM medium containing various combinations of kinetin with NAA. Columns indicate the mean number of regenerated shoots per explant of three replicates. Bars refer to standard error.

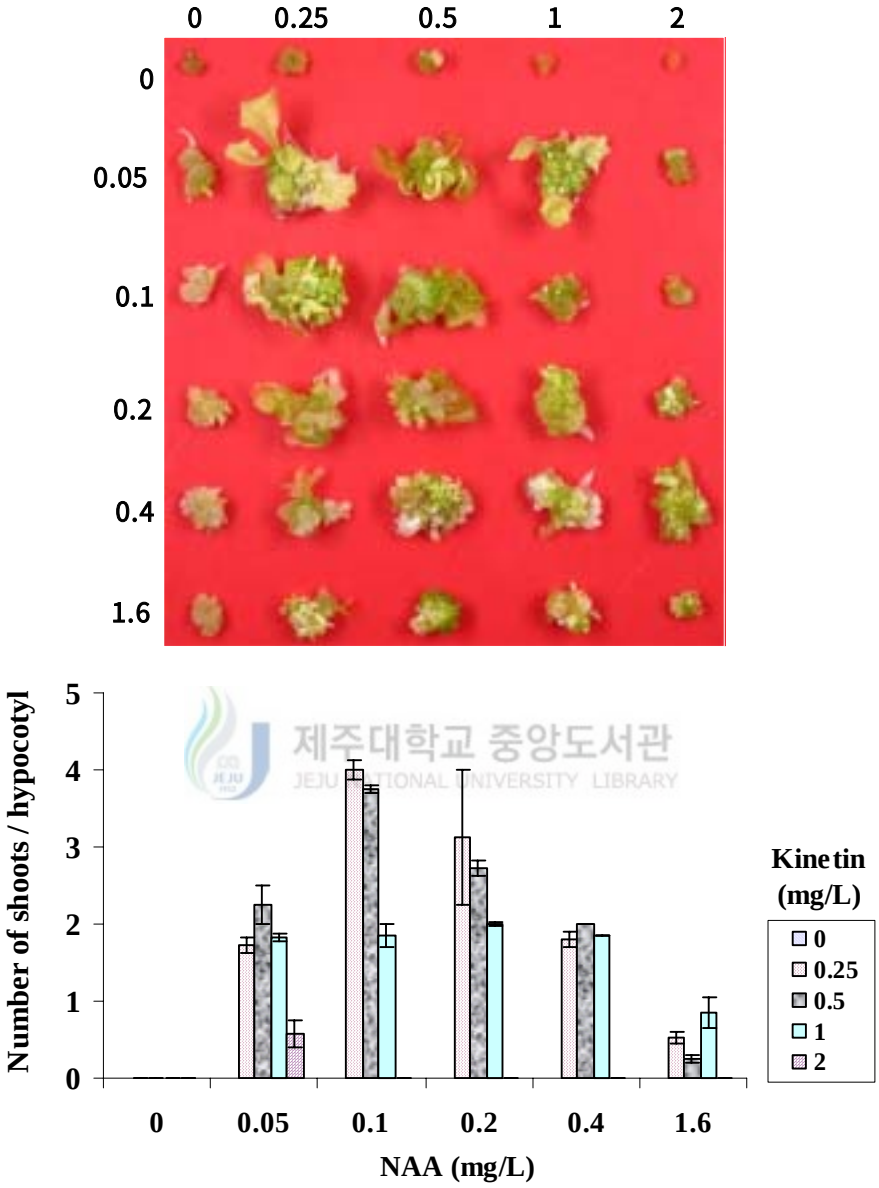


Fig. 11. Shoot regeneration from hypocotyl explants of *Lactuca sativa* L. cv. Sunny lettuce on MSBM medium containing various combinations of kinetin with NAA. Columns indicate the mean number of regenerated shoots per explant of three replicates. Bars refer to standard error.

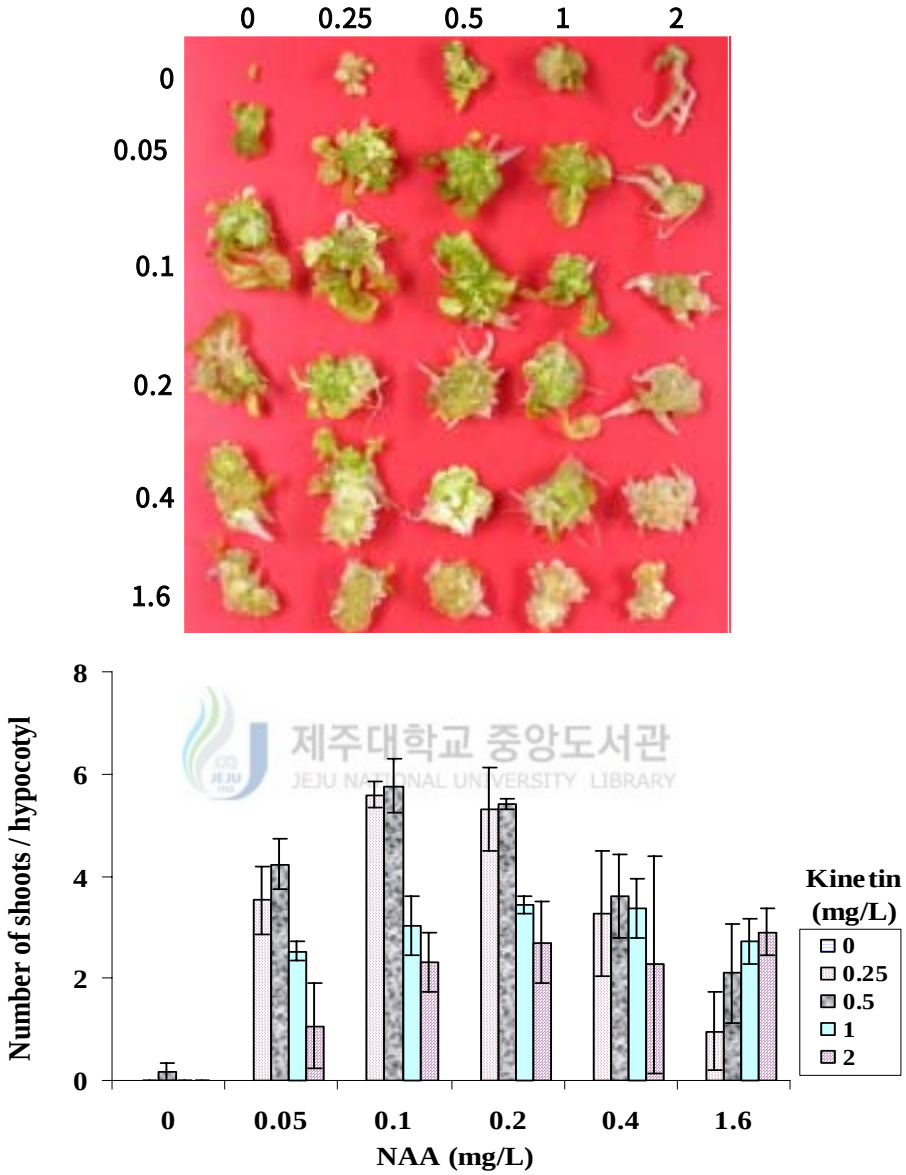


Fig. 12. Shoot regeneration from hypocotyl explants of *Lactuca sativa* L. cv. Chongchima on MSBM medium containing various combinations of kinetin with NAA. Columns indicate the mean number of regenerated shoots per explant of three replicates. Bars refer to standard error.

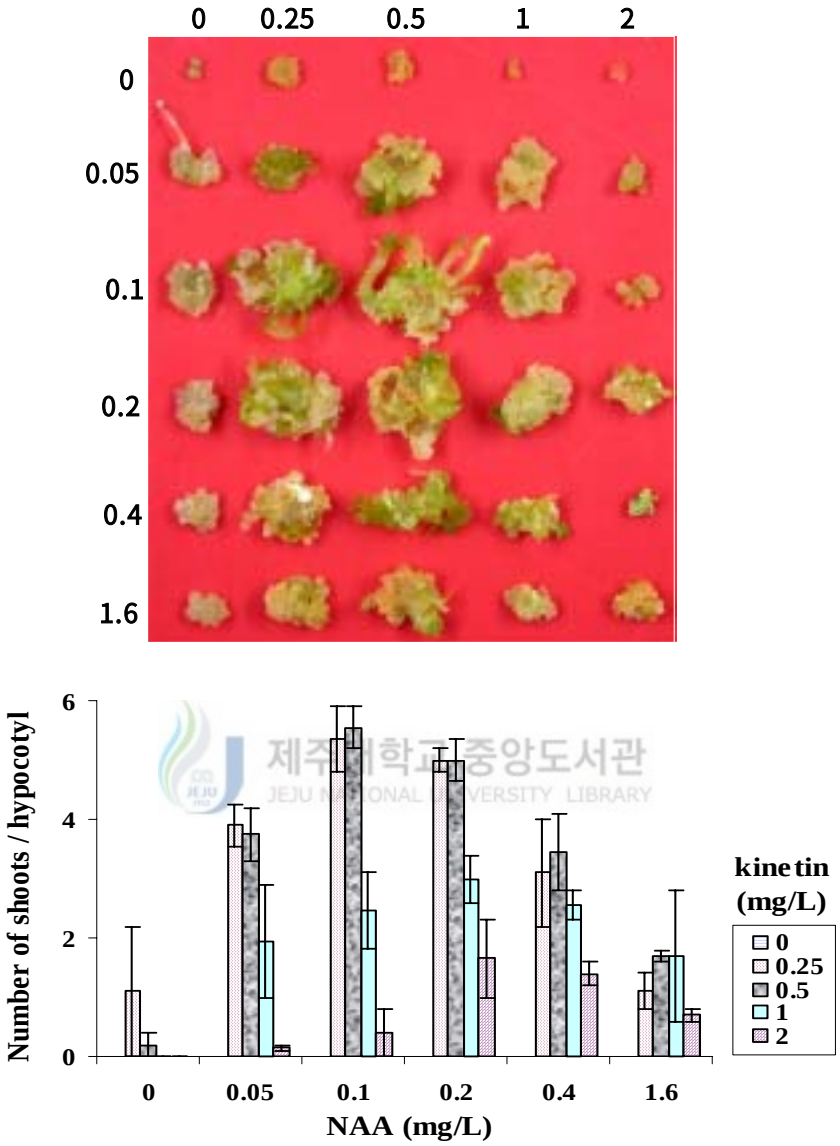


Fig. 13. Shoot regeneration from hypocotyl explants of *Lactuca sativa* L. cv. Sakurament on MSBM medium containing various combinations of kinetin with NAA. Columns indicate the mean number of regenerated shoots per explant of three replicates. Bars refer to standard error.

5. DISCUSSION

Shoot regeneration potential of cotyledon explants of Chongchima genotype was somewhat higher and also showed better adventitious shoot formation than the other explants of the genotypes. Other genotype with comparable regeneration capacity was Sakurament and also its cotyledon explants. It is well known that genotype can significantly influence the regenerative capacity in lettuce (Xinrun and Conner, 1992; Ampomah-Dwamena et al., 1997). And also the genotype showed an important factor in many other important crops, such as chickpeas (Polisetty et al., 1997), potatoes (Carputo et al., 1995), oats (Chen et al., 1995), peanuts (McKently, 1995) and pea (van Doorne et al., 1995). Our results also support the hypotheses of these previous studies.

The number of regenerated shoots per explant was dependent on the explant type. From the results, it can be concluded that the regeneration efficiency and adventitious shoot formation from the cotyledon explants is the best among the three explant types tested, followed by petiole and hypocotyl in consecutive order. This result was consistent with the results in lettuce described by Woo et al. 1999.

Both kinetin and NAA alone in the culture medium was not effective to generate shoots from all explants of all the genotypes, indicating incorporation of both exogenous kinetin and NAA is essential for optimal shoot regeneration from lettuce. Moreover, shoot regeneration was

influenced by the ratio of kinetin to NAA. The optimal ratio of growth regulators was dependent on genotypes and their explants.

In this study, we identified genotypes and explant sources with high potential for adventitious shoot regeneration and established the optimal conditions for plant regeneration in each genotype and explant source. Based on the results, we selected the cotyledon explant of Chongchima lettuce and 0.5 mg/L kinetin in combination with 0.05 mg/L NAA for its optimal culture condition for the further transformation experiment, since the success of genetic transformation largely depends on the efficiency of the in vitro plant regeneration.

Part II

Overexpression of *Arabidopsis ABF3* Gene Enhances Tolerance to Drought and Cold in Transgenic Lettuce (*Lactuca sativa* L. cv. Chongchima)

1. ABSTRACT

Abscisic acid (ABA) plays an important role in environmental stress responses of higher plants during vegetative growth via regulating the expression of numerous stress-responsive genes. The gene *ABF3* encodes a transcription factor for the expression of ABA-responsive genes. Here we report the transformation of lettuce (*Lactuca sativa* L. cv. Chongchima, leaf type) with *ABF3* derived from *Arabidopsis* using *Agrobacterium tumefaciens*-mediated system and the characteristics of the transgenic plants including responses to drought and cold stresses. Transgenic plants could be successfully obtained using a two-step selection/regeneration procedure with 10.8% of transformation efficiency. Among the tested nine transgenic lines, eight gave 3:1 segregation ratio in the T_1 progenies of self-pollinated T_0 transgenic plants, suggesting single copy integration of the transgene. One line gave 1:1 ratio, suggesting integration of more than one copy in this line. The result of Southern blot analysis of T_1 progenies showed that the *ABF3* transgene was stably integrated into the genome of transgenic plants. Northern blot analysis confirmed that the *ABF3* was highly expressed in T_1

progenies of transgenic plants. Transgenic plants showed normal growth in terms of morphologies of whole plant and seed. The transgenic plants showed higher tolerances than wild-type plants against drought as well as cold stress.

2. INTRODUCTION

Lettuce productivity is usually limited by drought and cold stresses, even though plants have their intrinsic capability to respond and adapt to harsh environmental conditions. Responses of plants to environmental stresses are largely controlled by the plant hormone abscisic acid (ABA) (Zeevaart and Creelman, 1988). Numerous studies have shown that ABA is essential for the normal adaptive response to the water stress imposed by drought (Koorneef et al., 1984; Leon-Kloosterziel et al., 1996; Xiong et al., 2002). The importance of ABA in low-temperature adaptation is somewhat controversial (Thomashow, 1999). However, a number of studies have demonstrated that ABA is essential for cold acclimatization and full development of freezing tolerance (Lang et al., 1994; Mantyla et al., 1995). Recently, a small family of abscisic acid- responsive element (ABRE)-binding bZIP proteins, named as ABRE binding factors (ABFs)/ABA-responsive element binding factors (AREBs) have been isolated by yeast one-hybrid screens (Choi et al., 2000; Uno et al., 2000). The major function of ABFs is to mediate ABA-dependent stress signaling during vegetative growth. Indeed, over-expression of ABF3, one of the ABF family members, in *Arabidopsis* enhanced tolerances to drought (Kang et al., 2002) and low/high temperature (Kim et al., 2004). Based on these results, we tried to construct drought and cold tolerant transgenic plants by introducing *ABF3* gene into lettuce. *Agrobacterium*-mediated transformation has been

successfully applied to lettuce mainly for establishing transformation system or improving disease-resistance capacity. Because lettuce productivity is usually limited by drought and cold stresses, it would be desirable to develop lettuce plants resistant to these stresses. Therefore the objectives of this study were to establish an efficient transformation procedure in lettuce, to transform *ABF3* gene into lettuce genome, to determine its inheritance to their progenies, and to evaluate the enhancement of drought and cold tolerance in these transgenic lettuce plants.

3. MATERIALS AND METHODS

3.1. Preparation of cotyledon explants

Mature seeds of lettuce (*Lactuca sativa* L. cv. Chongchima, leaf type lettuce) were surface-sterilized in sodium hypochlorite solution (2% active chloride) containing 0.1% (v/v) of Tween 20 for 15 min and then rinsed three times with sterilized water. The seeds were plated on medium comprising basal salts and vitamins of MS (Murashige and Skoog, 1962) supplemented with 100 mg/L myo-inositol, 3% (w/v) sucrose, 0.2% (w/v) phytagel (MSBM). The medium was adjusted to pH 5.8, and then autoclaved at 1.2 - 1.3 kg/cm^2 pressure and 121 °C for 20 min. The cultures were maintained under *in vitro* culture conditions adjusted to 25 ± 1 ^{o}C and 18-h photoperiod of 45 μmol m^{-2} s^{-1} illumination provided by cool white fluorescent lamps. Each petridish (90 mm in diameter) contained 30 ml of medium and was sealed with Micropore Surgical Tape (3M Health Care, USA). After 7 days of culture, explants (5 mm in diameter) were aseptically excised from cotyledons of lettuce seedlings and were used for further experiments. All cultures except those for co-cultivation were maintained under the same *in vitro* culture conditions as described above. The summary of media used in the lettuce tissue culture and transformation system is showed in Table 1.

3.2. Bacterial strain and plasmid

Agrobacterium tumifaciens strain EHA 105 harboring a binary vector pCUMB was used for transformation experiments. The binary vector carries a target gene ABA-responsive element binding factor3 (*ABF3*) under ubi promoter, reporter genes green fluorescence protein - β-glucuronidase (*gfp-gus*) under 35S promoter, and a selectable marker gene hygromycin phosphotransferase (*hph*) under 35S promoter (Fig. 14).

3.3. *Agrobacterium*-mediated transformation protocol

(1) The cotyledon explants (5 mm in diameter) were pre-cultured on MSSR medium for 3 days.

(2) *A. tumefaciens* EHA 105 containing pCUMB was grown in 50 ml Luria-Bertani (LB) medium supplemented with 100 mg/L kanamycin, 100 mg/L rifampicin with agitation at 160 rpm in a shaking incubator at 28ºC until an $OD_{600\ nm}$ value of 0.6-0.8.

(3) The overnight culture of *A. tumefaciens* was centrifuged at 2000 rpm for 15 min, and pellet was resuspended in a 50 ml resuspension medium (MSBM without $CaCl_2$, 100 mg/L acetosyringone, 20 mg/L pluronic F68, pH 5.2) with vortex.

(4) Pre-cultured lettuce cotyledon explants were briefly surface-dried on sterilized filter paper, and immersed in suspended bacterium culture for 10 min at 28 ºC with shaking at 160 rpm. The explants were then briefly dried

on filter paper, and cultured on co-culture medium (MSSR without $CaCl_2$, 100 mg/L acetosyringone, pH 5.2) at 25 ^{o}C in the dark for 3 days.

(5) Prior to bacteria elimination, a range of carbenicillin concentrations (MSSR medium with 0, 50, 125, 250, 500, or 1000 mg/L carbenicillin) was investigated to determine the maximum suitable concentration at shoot regeneration from cotyledon explants of wild-type lettuce plants and it was used to eliminate bacteria.

(6) For bacteria elimination, co-cultured explants were washed in sterile water containing 500 mg/L carbenicillin, then cultured on bacteria elimination medium (MSSR with 500 mg/L carbenicillin) for 9 days.

(7) Prior to selection steps, a range of hygromycin concentrations (MSSR medium with 0, 5, 10, 20, 30, 40, or 50 mg/L hygromycin) was investigated to determine the minimum fatal concentration at shoot regeneration from cotyledon explants of wild-type lettuce plants, and it was used to select the putative transformed plants.

(8) For the first step selection, the explants were cultured on MSSR medium supplemented with 20 mg/L hygromycin, and 250 mg/L carbenicillin for 2 weeks.

(9) For the second step selection, hygromycin-resistant calli derived from the explants were cultured on MSSR medium supplemented with 25 mg/L hygromycin, and 250 mg/L carbenicillin for 3 weeks.

(10) The hygromycin-resistant shoots (5-10 mm in height) derived from those calli were cultured on MSBM medium containing 25 mg/L hygromycin, and 250 mg/L carbenicillin for 2 weeks.

(11) After root formation, hygromycin-resistant plants were transferred to 20 x 20 cm plastic pots with peat moss and perlite (1:1), and grown in an environmentally controlled glasshouse adjusted to 20 ± 2 °C, 60 ± 10% relative humidity, and 18-h photoperiod of 30 μmol m^{-2} s^{-1} illumination provided by cool white fluorescent lamps.

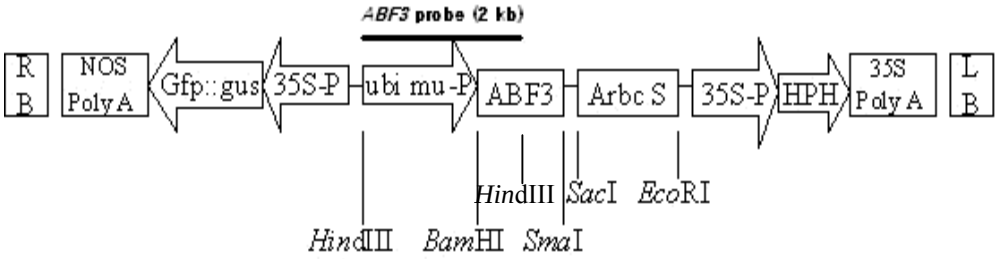


Fig. 14. Schematic diagram of T-DNA region of vector pCUMB. 35S-P: CaMV 35S promoter of cauliflower mosaic virus, ubi mu-P: maize ubiquitin promoter, gfp-gus: gene for green fluorescent protein and β-glucuronidase, ABF3: gene for ABA-responsive element factor3, hph: gene for hygromycin phosphotransferase, NOS Poly A, Arbc S and 35S-Poly A: terminators.

3.4. Transgene segregation test in T_1 progenies of transgenic lettuce plants

Self-pollinated seeds (T_1) of transgenic plants were placed on segregation medium comprising MSBM with 30 mg/L hygromycin. The treatment was carried out in triplicate (10 seeds per dish) and was repeated three times in each transgenic line. After 4 weeks, hygromycin-resistant plants developing true leaves were counted, and data were analyzed by the X^2 test at P = 0.05. To determine the minimum fatal concentration at seed germination of wild-type lettuce plants, a range of hygromycin concentrations (MSBM with 0, 5, 10, 20, 30, 40, or 50 mg/L hygromycin) was tested. Each of the treatment was carried out in triplicate (15 seeds per dish) and was repeated three times. After 4 weeks, hygromycin-resistant plants producing true leaves were also counted.

3.5. Histochemical GUS staining

Histochemical GUS staining was performed as described by Jefferson et al. (1987). Whole plants and mature seeds were incubated overnight at 37 °C in X-gluc reaction buffer contains 34 mM phosphate buffered saline, 10 mM EDTA (pH 8.0), 0.5 mM $K_3[Fe(CN_6)] \cdot 3H_2O$, 0.5 mM $K_4[Fe(CN_6)] \cdot 3H_2O$, 2 mM X-gluc, and 0.1% triton X-100. Chlorophyll was removed from the plant tissues by soaking in 70% ethanol.

Table 1. Summary of media used in lettuce tissue culture and transformation system

Medium	Composition
Basal media (MSBM)	MS salts and vitamins, 30 g/L sucrose, 2 g/L phytagel, pH 5.8
Seed germination	MSBM
Shoot regeneration(SR)	MSBM with 0.5 mg/L kinetin, 0.05 mg/L NAA
Root regeneration	1/2 MSBM
Agrobacterium culture	LB (10 g/L tryptone peptone, 5 g/L bacto-yeast extract, 5 g/L NaCl, 1 g/L D-glucose) with 100 mg/L kanamycin, 100 mg/L rifampicin, pH 7.0
Agrobacterium suspension	SR (without $CaCl_2$) with 100 mg/L acetosyringone, 20 mg/L pluronic F68, pH 5.2
Pre-cultivation	SR medium
Co-cultivation	SR (without $CaCl_2$) with 100 mg/L acetosyringone, pH 5.2
Bacteria elimination	SR with 500 mg/L carbenicillin
Shoot regeneration and selection 1	SR with 20 mg/L hygromycin, 250 mg/L carbenicillin
Shoot regeneration and selection 2	SR with 25 mg/L hygromycin, 250 mg/L carbenicillin
Root regeneration and selection	MSBM with 25 mg/L hygromycin, 250 mg/L carbenicillin
Seed germination and selection	MSBM with 30 mg/L hygromycin

3.6. Comparison of growth characters of transgenic and wild-type plants

To compare the seed germination speed of wild-type and transgenic plant seeds, mature seeds were surface-sterilized in sodium hypochlorite solution (2% active chloride) containing 0.1% (v/v) of Tween 20 for 15 min and then rinsed three times with sterilized water. The seeds were plated on MSBM medium, and incubated under the same *in vitro* culture conditions as described above. Each of the treatment was carried out in triplicate (20 seeds in a petridish per replication) and the germination test was repeated three times. The germination was scored at 24, 36, 48, 60, 72, and 96 h after culturing.

Mature seeds of wild-type and T_1 transgenic lettuce lines T104, T107, and T108 were sown on a 1:1 mixture of peatmoss and perlite, and placed at the environmentally controlled glasshouse as described in above and they were irrigated once a day. During the growth period, number of plant height, leaf number per plant, leaf size (leaf length x leaf width) of 1- and 3-month-old plants, also leaf number per plant, plant height, flower number per plant, seed number per flower, and weight of 10 seeds of 5-month-old plants were evaluated.

3.7. Southern and Northern blot analysis in T_1 progeny of transgenic plants

Genomic DNA was isolated from young leaf tissue of T_1 transgenic and wild-type plants according to CTAB method (Saghai-Maroof, 1984).

Polymerase chain reactions (PCR) were carried out on the hygromycin-resistant plants by amplifying the coding regions of transgenes using the following sets of oligonucleotide primers: *ABF3* 5'-AGAACCTCAACCGGTGGAGAGTG-3' (forward) and 5'-GGAGTCAGATCAGGTGACATCTGG-3' (reverse); *hph* 5'-GATGTAGGAGGGCGTGGATATGTC-3' (forward) and 5'-CTTCTACACAGCCATCGGTCCAGA-3'(reverse); *gus* 5'-CAACGAACTGAACTGGCAGA-3' (forward) and 5'-GGCACAGCACATCAAAGAGA-3' (reverse). The expected PCR products were 480 bp for *ABF3*, 1080 bp for *hph* and 966 bp for *gus*. The total volume of reaction mixture was 13 μl, including 25 ng genomic DNA or 5 ng plasmid DNA (as serving control), 1 μl of each primer (10 pmols) and 7 μl Premix Ex Taq (Takara, Japan). Cycling parameters began with an initial hot start at 95 °C for 5 min, then 35 cycles of denaturation 95 °C for 30 s, annealing at 57 °C for 1 min and extension at 72 °C for 45 s, followed by a final extension of 5 min at 72 °C. PCR amplification products were analyzed by electrophoresis in 0.8% agarose gel and stained with ethidium bromide (EtBr) and visualized with UV. For southern blot analysis, about 25 μg of *Hin*dIII-digested genomic DNA samples were electrophoresed on 0.8% (w/v) agarose gel, followed by depurination in 0.25 N HCl for 10 min, denaturation in 0.5 N NaOH and 1.5 N NaCl for 1 h, neutralization in 1 M Tris (pH 7.5) and 1.5 M NaCl for 1 h, and homogenization in 10 x SSC for 1 h.

Total RNA was isolated from young leaf tissue of T_1 transgenic and wild-type plants using Tri-Reagent (MRC, Inc., Cincinnati, OH) by the method of Choi et al. (2000). For northern blot analysis, the reaction mixture was contained 2 μl of 10 x MOPS, 4 μl of formaldehyde, 10 μl of formamide and 25 μg of total RNA for each sample, followed incubation in the water bath at 65 °C for 15 min and chilled on the ice for 3 min. The samples were then electrophoresed on 1.2% (w/v) agarose gel containing 2.2 M formaldehyde at 50 volt for 3 h. The gel was stained with EtBr and visualized on UV, and then it was washed by deionozed sterile water for 45 min and by 20 x SSC for 1 h.

The gels were blotted onto an uncharged nylon membrane (Amersham Pharmacia Biotech, UK) using 20 x SSC by download capillary transfer method (Koetsier et al., 1993). Hybridization probe, a PCR-amplified product of *ABF3*, was [^{32}P] dCTP-labelled using the Rediprime II Random Prime Labelling System (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instruction, and the unincorporated nucleotides were removed by passing through the MicroSpin^{TM} G-25 Columns (Amersham Biosciences, Piscataway, NJ). Prehybridization with 2 x SSC and 0.1% SDS was carried out at 65 °C for 30 min. Hybridization was performed overnight at 65 °C in Rapid-hyb buffer (Amersham Pharmacia Biotech, UK). Then the membranes were initially washed in 2 x SSC and 0.1% SDS at 65 °C for 30 min, and repeated in the same buffer at room temperature for 15 min, thereafter in 0.2 × SSC and 0.1% SDS with the same agitation at room

temperature for 30 min. The blot was exposed to Kodak Biomax (Kodak, Stuttgart) film for 24 h at 80 °C.

3.8. Analysis of drought and cold tolerances in T_1 progeny of transgenic plants

The 5-week-old T_1 transgenic and wild-type plants derived from the seeds were transferred to 6 x 6 cm plastic pots with peatmoss and perlite (1:1) and grown in the environmentally controlled glasshouse until emerging four to five true leaves. The plants were irrigated once a day with water (approx. 100 ml), and used for subsequent experiments. For drought stress test, T_1 transgenic and wild-type plants were kept without any water supply in the environmentally controlled glasshouse. After 1 month, the plants were rewatered, and plant survival rate was scored 10 days after rewatering. For cold stress tolerance assay, T_1 transgenic and wild-type plants were kept at -4 ± 1 °C, 50% relative humidity with 18-h photoperiod of 45 μmol m^{-2} s^{-1} illumination provided by cool white fluorescent lamps in a low temperature incubator (LS-103 MP-L, LS Tech, Korea). After 2 days, the plants were transferred to the environmentally controlled glasshouse, and the plant survival rate was evaluated after 7 days since transferring. The survival rates in both tests were defined as healthy and recovered plant number / total plant number x 100%. The 15 pots from each T_1 transgenic and wild-type plant were used for drought and cold stress tests, and each test was repeated three times.

4. RESULTS

4.1. Selection and regeneration of transgenic plants

To conduct the transgenic lettuce plants with ABF3 gene, cotyledon explants were inoculated with A. tumefaciens harboring a binary vector pCUMB carrying *ubi::ABF3*, *35S::gfp-gus*, and *35S::hph* genes. To eliminate excess bacteria, the maximum suitable concentration of carbenicillin at shoot regeneration from cotyledon explants of wild-type lettuce was determined. Lower concentrations of carbenicillin were not influenced on shoot regeneration, whereas higher concentrations such as 500 and 1000 mg/L reduced shoot regeneration but not influenced on cotyledon expansion (Fig. 15). According to this result, 250 mg/L carbenicillin was contained in shoot or root regeneration and selection medium and 500 mg/L carbenicillin was added in bacteria elimination medium.

Hygromycin-resistant plants were obtained from a two-step selection/regeneration procedure. Prior to the selections, the minimum fatal concentration of hygromycin at cotyledon explants of wild-type lettuce plants was determined. Hygromycin at 20 mg/L or higher concentrations, explants growth was completely inhibited and eventually died within 4 weeks of culture (Fig. 16). Based on this result, 20 mg/L hygromycin was chosen for the first step selection and 25 mg/L hygromycin was chosen for the second step selection. After 2 weeks of the first step selection, a total

number of 53 hygromycin-resistant calli was generated and all the calli were formed only on the edges of the explants (Table 2, Fig. 17A). After 3 weeks of second step selection, 9 independent lines of hygromycin-resistant shoots were regenerated from these calli (Table 2, Fig. 17B). All the resistant shoots were rooted on the medium comprising MSBM with 25 mg/L hygromycin within 2 weeks of culture (Fig. 17C) and GUS activity was detected in all putative transgenic plants, and the transformation efficiency was 10.8% (Table 2). These plants were transplanted in the environmentally controlled glasshouse and flowered after 3 months (Fig. 17D) and self-pollinated seeds were produced after 3 weeks since flowering (Fig. 17E). The transgenic plants exhibited normal growth in terms of morphology and seed yield.

4.2. Transgene segregation in T_1 progeny of transgenic plants

Among the progenies of self-pollinated T_1 transgenic plant lines, 8 lines showed 3:1 segregation ratio (X_2=0.0002-0.221, P=0.64-0.99), suggesting single copy integration of the transgene, and 1 line (Line No.5) showed a 1:1 ratio (X_2=0.131, P=0.72), suggesting semidominant (Table 3). These segregation ratios were determined at 30 mg/L hygromycin-containing MSBM medium. The cotyledons and radicles derived from seeds of wild-type lettuce plants were completely abolished and eventually died at 30 mg/L or higher concentrations of hygromycin-containing medium within 4 weeks of culture (Fig. 18). In contrast, the hygromycin-resistant seedlings grew normally and their true leaves were fully expanded (Fig. 17F). Furthermore,

histochemical GUS staining of these hygromycin-resistant T_0 and T_1 plants was examined. GUS was expressed strongly in leaves of hygromycin-resistant plants, whereas it was not expressed in wild type plants (Fig. 19). In case of results in T_1 plants, cotyledons, leaves, stems, roots of adult plants, and flower parts except for ovaries showed strong GUS expression (Fig. 20B-G), whereas mature seeds (Fig. 20A), newly emerging leaves (Fig. 20B), young roots (Fig. 20B-D), and ovaries (Fig. 20G) did not show GUS expression clearly. Also, GUS expression was not detected in the semidominant line plants, although the *gus* transgene in their genome was confirmed by PCR.

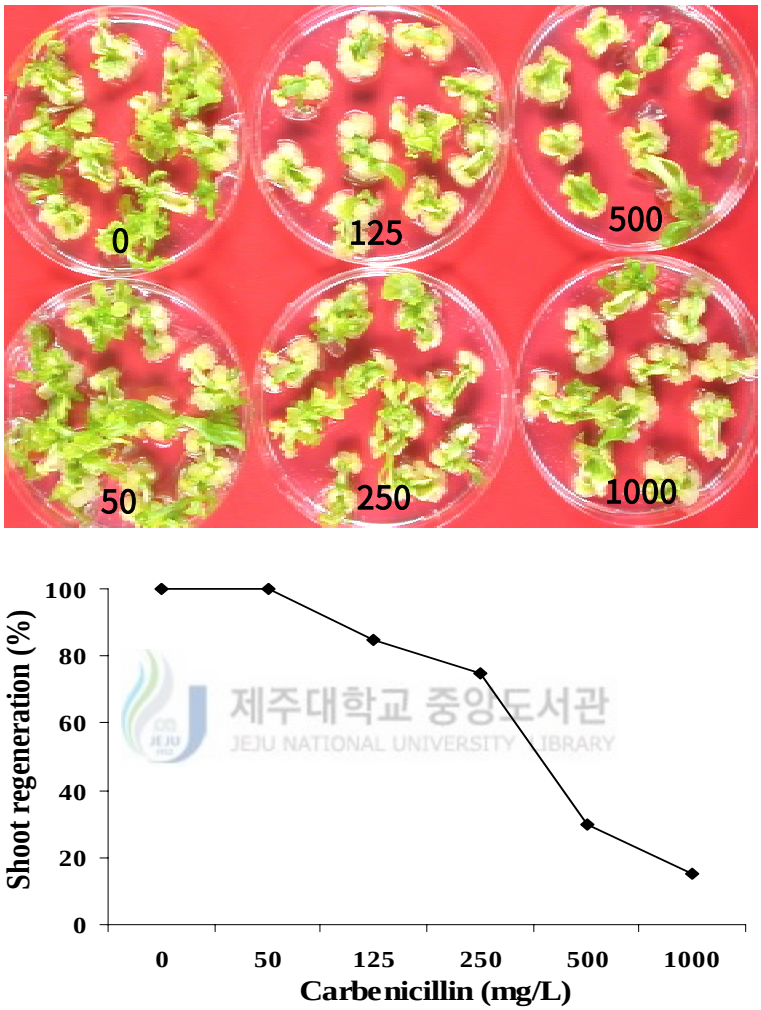


Fig. 15. Effect of carbenicillin concentrations on shoot regeneration from cotyledon explants of wild-type *Lactuca sativa* L. cv. Chongchima. Data was collected after 4 weeks of cotyledon culture on medium comprising MS supplemented with 0.5 mg/L kinetin, 0.05 mg/L NAA and various concentrations of carbenicillin.

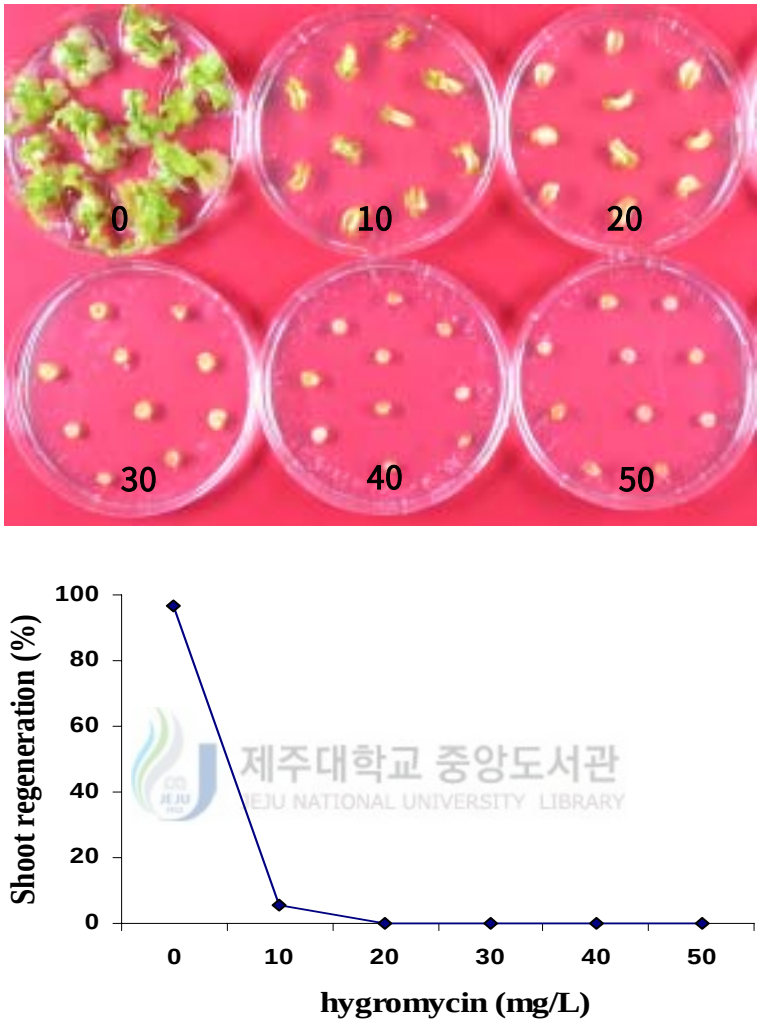


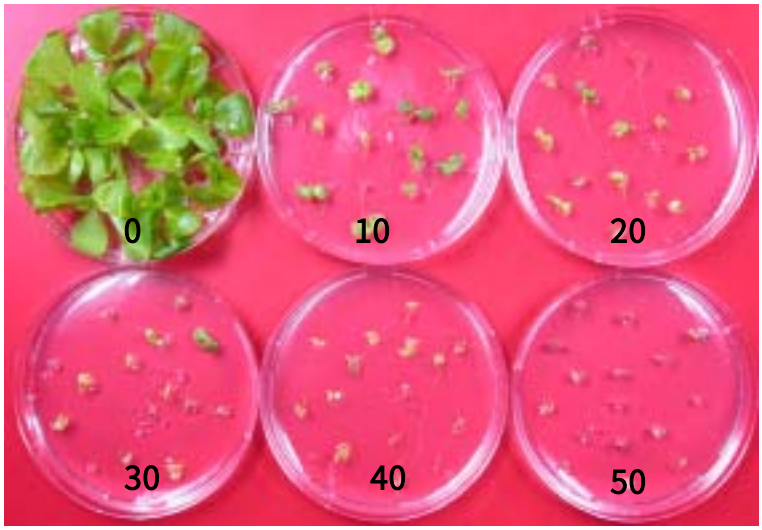
Fig. 16. Effect of hygromycin concentrations on shoot regeneration from cotyledon explants of wild-type *Lactuca sativa* L. cv. Chongchima. Data was collected after 4 weeks of cotyledon culture on medium comprising MS supplemented with 0.5 mg/L kinetin, 0.05 mg/L NAA and various concentrations of hygromycin.

Fig. 17. Transformation stages of *Lactuca sativa* L. cv. Chongchima mediated by *Agrobacterium tumefaciens*. A: Hygromycin-resistant calli were generated on the edges of cotyledon explants after the first step selection. B: Hygromycin-resistant shoots regenerated from the hygromycin-resistant calli after the second step selection. C: Roots induced from hygromycin-resistant shoots. D: Transgenic plant at flowering stage. E: Seed ripen of transgenic plant. F. Selfed seeds of wild-type (*left*) and T_1 transgenic plants (*right*) were germinated on 30 mg/L hygromycin-containing MSBM medium.

Table 2. Transformation efficiency (T.E.) of *Lactuca sativa* L. cv. Chongchima mediated by *Agrobacterium tumefaciens*

Co-cultivated explants[1] (A)	Produced Hyg^R calli	Produced Hyg^R plants	Produced Hyg^R plants with GUS activity (B)	T.E. (%) (B/A)
83	53	9	9	10.8

[1]7-day-old cotyledon explants were used.



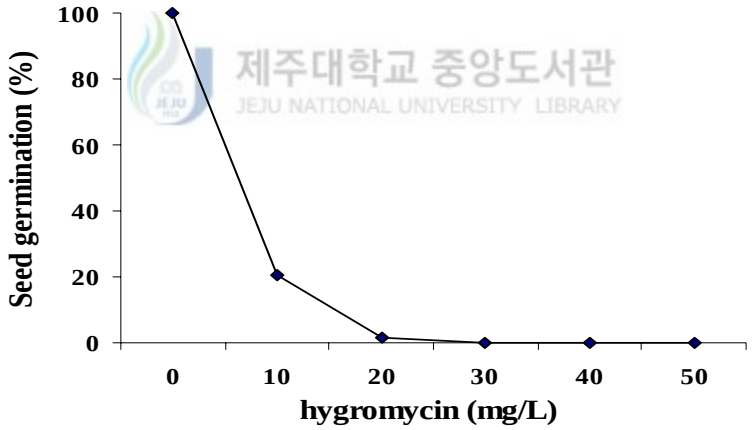


Fig. 18. Effect of hygromycin concentrations on seed germination from mature seeds of wild-type *Lactuca sativa* L. cv. Chongchima. Data was collected after 4 weeks of cotyledon culture on medium comprising MS with various concentrations of hygromycin.

Table 3. Segregation of hygromycin resistance in T_1 progeny of transgenic plants

Plant code	Resistant (R) progeny	Susceptible (S) progeny	Ratio (R:S)	Expected ratio	X^2-value	*P*-value
101	7.0 ± 1.0	2.3 ± 0.9	3.0:1	3:1	0.0002	0.99
102	7.3 ± 0.7	1.7 ± 0.3	4.3:1	3:1	0.179	0.67
103	7.0 ± 0.4	3.2 ± 0.5	2.2:1	3:1	0.221	0.64
104	6.7 ± 0.3	2.0 ± 0.6	3.3:1	3:1	0.018	0.89
105	4.3 ± 0.7	3.3 ± 1.5	1.3:1	1:1	0.131	0.72
106	7.7 ± 0.7	2.3 ± 0.7	3.3:1	3:1	0.021	0.89
107	6.7 ± 0.7	2.3 ± 0.3	2.9:1	3:1	0.001	0.97
108	5.3 ± 0.9	2.0 ± 0.6	2.7:1	3:1	0.022	0.88
109	6.7 ± 1.2	3.0 ± 1.0	2.2:1	3:1	0.182	0.67

Each value represents mean ± S.E. of three replicates. Selfed seeds were germinated on MSBM medium containing 30 mg/L hygromycin, and cultured for 4 weeks at 25 ± 1 ^{o}C and an 18-h photoperiod of 45 μmol m^{-2} s^{-1} illumination provided by cool white fluorescent lamps. X^2-values indicate significant fit to the expected ratio.

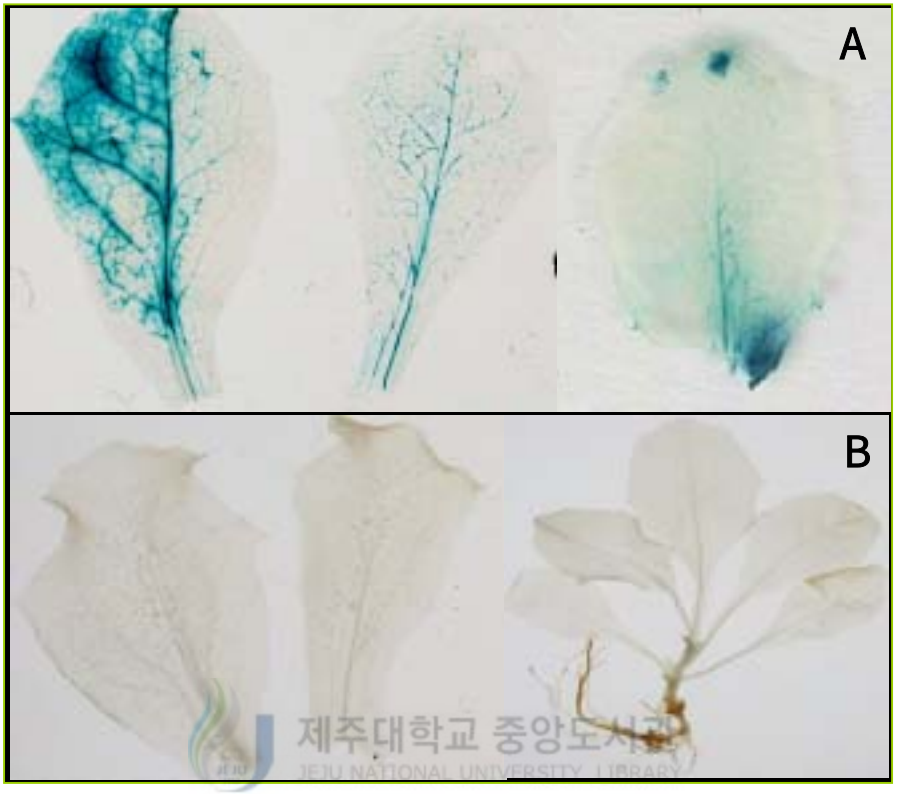


Fig. 19. Histochemical GUS expression in leaves of T_0 transgenic (A) and wild-type (B) *Lactuca sativa* L. cv. Chongchima. Blue staining indicates GUS expression.

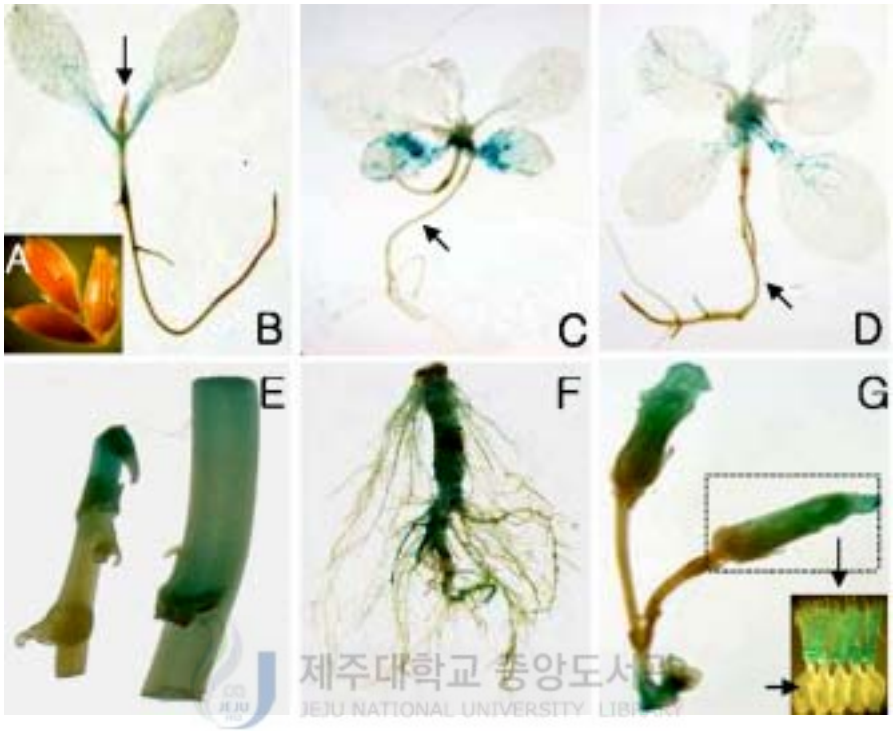


Fig. 20. Histochemical GUS staining of T_1 transgenic plants. Blue staining indicates GUS expression. A: Mature seeds. B: 7-day-old cotyledon. C: 2-week-old seedling. D: 4-week-old seedling. E: Segments of stem. F: Root of adult plant. G: Flower. Arrows in (B) indicate the newly emerging leaf and young root, (C, D) show young roots, and (G) show ovary and pappus of flower.

4.3. Growth phenotypes of *ubi::ABF3* overexpressing T_1 transgenic plants

The coding region of *ABF3* was fused to maize ubiquitin promoter was used to transform lettuce plants. T_1 transgenic lines with higher *ABF3* expression levels were selected for more detailed phenotype comparison analysis.

The seed germination of *ubi::ABF3* transgenic lettuce plants were delayed several hours compared with wild-type plants (Fig. 21).

Compared with 1-month-old wild-type plants, *ubi::ABF3* transgenic plants exhibited mild growth retardation in plant height and leaf size, however number of leaf per plant was the same to each other (Fig. 22). At 3 month, transgenic plant height and leaf size did reach the same level as that of the wild-type plants. Furthermore, no significant differences in number of leaf per plant, plant height, flower number per plant, seed number per flower, and seed weight were found at 5-month-old transgenic and wild-type plants (Fig. 22).

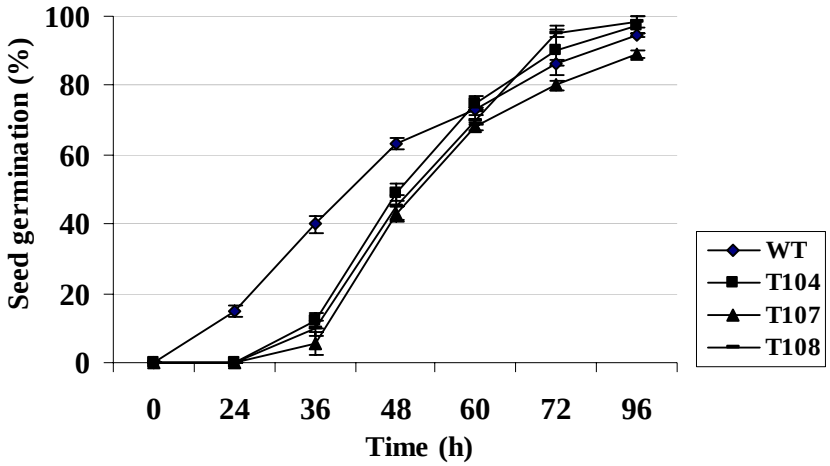


Fig. 21. Seed germination of wild-type (WT) and T_1 transgenic lettuce lines T104, T107, and T108. Seeds were plated on MS hormone-free medium, and seed germination was scored at various times. Each value represents the mean of three replicates. Bars indicate to standard error.

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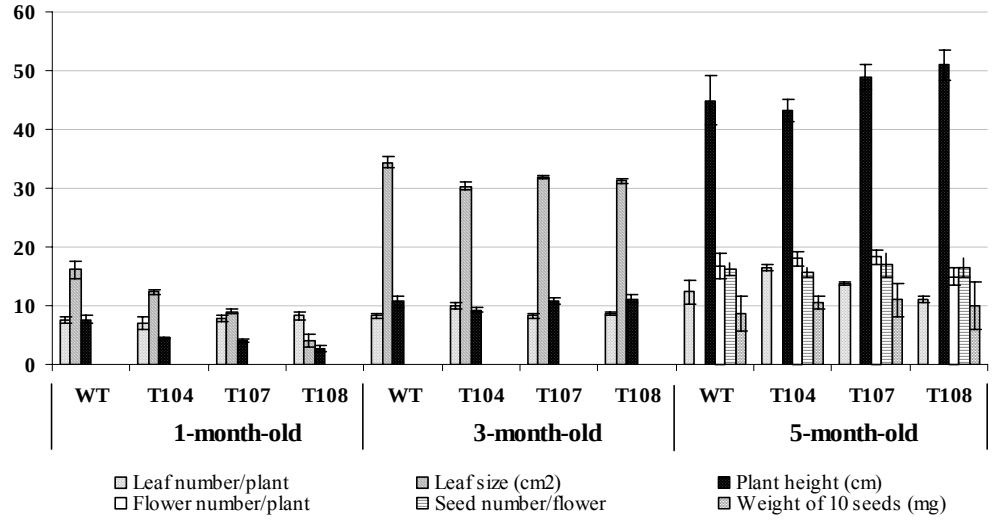

Fig. 22. Comparison of growth phenotypes of T_1 transgenic and wild-type plants at 1, 3, and 5 months of growth period. The columns indicate the mean of five replicates. Bars refer to standard error.

4.4. Confirmation of *ABF3* gene integration and expression in T_1 transgenic plants

The hygromycin-resistant T_1 plants were analyzed by PCR and Southern blot analysis to confirm the transgenes integration. Gel electrophoresis of the PCR amplification products revealed in all cases the presence of a 480-bp *ABF3*, a 1080-bp *hph* and a 966-bp *gus* band of the expected size in the hygromycin-resistant plants and positive control, and its absence in the wild-type plants (Fig. 23), indicating that the selection was stringent. These plants were further confirmed by Southern blot analysis. A genomic DNA from the resistant T_1 plants and wild-type plants was digested with *Hin*dIII and hybridized with the ABF3 probe. The wild-type plant was shown no band (Fig. 24, WT lane), whereas the resistant plants showed the expected bands at 2 kb (Fig. 24, lane 101, 104, 107, and 108) indicating that the gene was successfully integrated in the genomes of transgenic plants, and inherited to their progenies. In order to confirm the stable expression of *ubi::ABF3*, we performed Northern blot analysis with the hygromycin-resistant T_1 plants. *ABF3* gene expression was not detected in wild-type plant (Fig. 25D, WT lane), while it was detected homogenous high expression levels in four independent lines of T_1 transgenic plants (Fig. 25D, lane 101, 104, 107, and 108), suggesting that stabilized expression of introduced genes.

4.5. Tolerances to drought and cold in T_1 transgenic plants

The T_1 transgenic and wild-type plants planted in soil were subjected to drought and cold stress tests. After the drought stress test, 52.1% of the transgenic, whereas only 10% of the wild-type plants were recovered (Fig. 25A, C). After the cold stress test, 68.5% of the transgenic, while 31.7% of the wild-type plants were recovered (Fig. 25B, C). The *ABF3* gene was strongly expressed in the transgenic plants (Fig. 25D, lane 2-5). These results indicated that the transgenic plants are more tolerant to drought and cold stresses than those of wild-type plants.

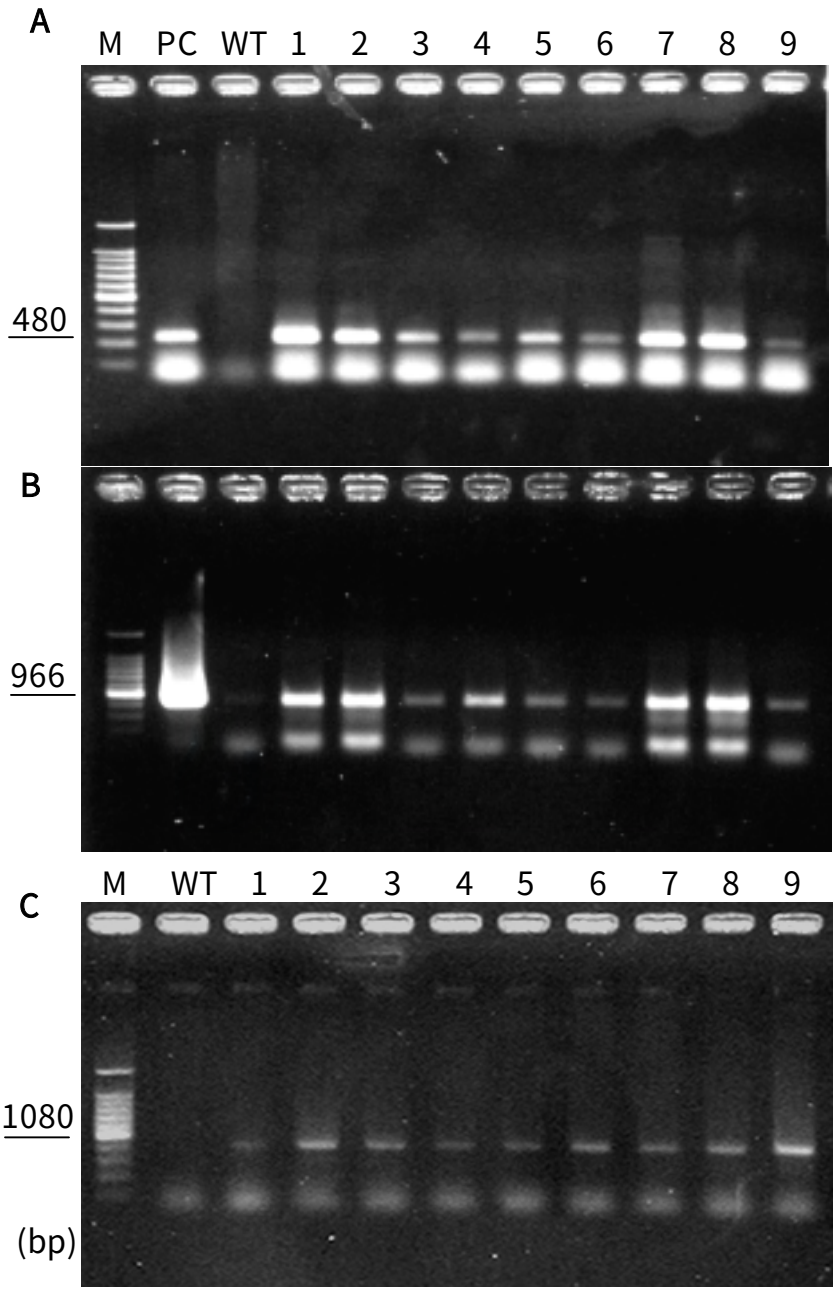

Fig. 23. PCR products amplified with ABF3 (A), GUS (B) and hygromycin (C) gene primers. M: DNA marker, PC: positive control, WT: wild-type lettuce, Lines (1-9): putative transgenic lettuce lines T101-109.

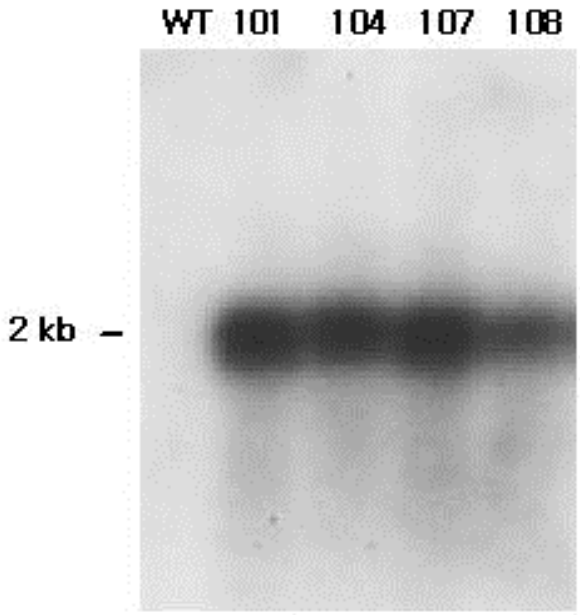

Fig. 24. Southern blot analysis of wild-type and T_1 transgenic lettuce lines 101, 104, 107, and 108. About 25 μg of *Hin*dIII-digested genomic DNA samples were probed with ^{32}P labeled ABF3 probe.

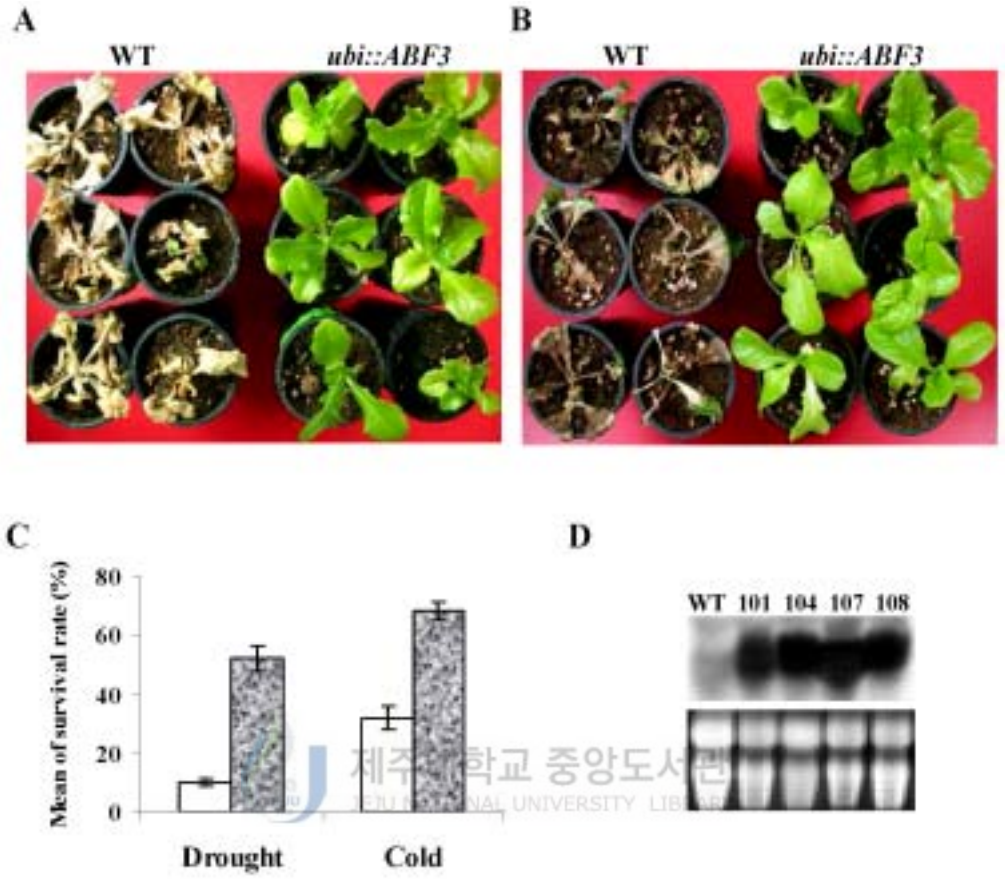

Fig. 25. Drought (A) and cold (B) stress tests in T_1 transgenic and wild-type lettuce plants. The drought test was carried out for 1 month at 20 ± 2 °C, 60 ± 10% relative humidity with 18-h photoperiod of 30 μmol m^{-2} s^{-1} illumination provided by cool white fluorescent lamps. The cold test was carried out for 2 days at -4 ± 1 °C, 50% relative humidity with 18-h photoperiod of 45 μmol m^{-2} s^{-1} illumination provided by cool white fluorescent lamps. The photographs were taken after 10 days from rewatering, and after 7 days from transferring to the glasshouse conditions, respectively. C: Mean of survival rate of 15 pots of each wild-type (white

column) and T_1 transgenic plants (gray column) after drought and cold stress tests. Each test was replicated three times. Bars indicate the standard error. D: Northern blot analysis on wild-type and T_1 transgenic lines 101, 104, 107, and 108. About 25 μg of total RNA samples were probed with ^{32}P labeled ABF3 probe. The bottom panel shown ethidium bromide staining of the RNA gel.

5. DISCUSSION

According to previous reports, overexpression of ABF3 enhances the tolerances to drought (Kang et al., 2002) and low/high temperature in *Arabidopsis* (Kim et al., 2004). Therefore, we expected that ABF3 could be used for the production of tolerant transgenic lettuce against drought and cold stresses. In this study, we established the optimum procedure for *Agrobacterium*-mediated transformation in lettuce and screening of transformants. And then we generated transgenic plants, analyzed the introduced genes and phenotypes of plants focusing on drought and cold tolerances. In several previous studies on *Agrobacterium*-mediated transformation of lettuce, nurse cultures using *Nicotiana plumbaginifolia* (Michelmore et al., 1987) or *Petunia hybrida* (Curtis et al., 1994) cells were employed for increasing transformation efficiency. However, this procedure is somewhat complicated and not efficient enough. Here we established more convenient and simple procedure using only acetosyringone in co-culture medium. The transgenic plants could be obtained by employing this procedure with 10.8% of transformation efficiency. Although no nurse cultures were used in the present study, the final transformation efficiency was comparable to those of the previous studies.

The analysis of hygromycin resistance of T_1 progenies showed a typical Mendelian segregation pattern in 8 out of 9 transgenic plant lines. This indicated that the 8 transgenic lines have single copy of the gene. The

result of Southern blot analysis of T_1 progenies showed that the *ABF3* transgene was stably integrated into the genome of transgenic plants. Moreover, Northern blot analysis confirmed that the ABF3 was highly expressed in T_1 progenies of transgenic plants.

The reporter gene *gus* was expressed in young seedlings as well as in adult transgenic plants. However, hygromycin-resistant plants lacking of GUS expression also were observed in the plants of the semidominant line. This lack of GUS expression is not caused by gene escape, because the gus transgene in their genome was already determined by PCR. Therefore, this phenomenon might be resulted from genomic position effects (Finnegan and McElroy, 1994; Loo and Rine, 1994), deletion of promoter or GUS coding region, or gene silencing due to DNA methylation (Renckens et al., 1992; Finnegan and McElroy, 1994).

Overexpression of ABF3 exerted an inhibitory effect on both germination and seedling growth. Except for slightly growth retardation at the early developmental stage, *ubi::ABF3* plants did not show any abnormality in the general developmental processes. Thus, its overexpression affected growth rate but not developmental processes. This result was consistent with the result reported in *Arabidopsis* (Kang et al., 2002). The transgenic lettuce plants at middle and post developmental stages were normal in terms of morphologies of whole plant and seed yield, indicating transformation with ABF3 did not cause such severe growth retardation as ABF2 or ABF4 (Kim et al., 2004).

The transgenic plants with *ABF3* showed higher tolerances than wild-type plants against drought as well as cold stress. Major cause of freezing damage is the freeze-induced dehydration because once ice crystals form in the extracellular spaces of plant cells, water moves out of the cell resulting in water deficit (Steponkus and Webb, 1992; Thomashow, 1998). Therefore the basic mechanisms of plant response to drought and freezing may be similar to each other at the molecular level (Shinozaki and Yamaguchi-Shinozaki, 2000; Thomashow, 2001). Our results were consistent with this hypothesis. The ABF3 gene derived from *Arabidopsis* was functional in lettuce and its over-expression appeared to enhance drought and cold tolerances in transgenic plants. Therefore, we concluded that ABF3 can be used to improve stress tolerance to adverse environmental conditions, especially to drought and cold in many other agriculturally important crops.

Part III

Callus selection and plant regeneration of *Agrostis mongolica* Roshev.

1. ABSTRACT

We established an efficient regeneration procedure for *Agrostis mongolica* Roshev. First, we found a high regenerable callus type and subsequently established the optimal conditions for callus type maintenance and plant regeneration of *A. mongolica*. The type 1 callus was selected as the best callus type and 2 mg/L 2.4-D in combination with 0.01 mg/L BA and 0.05 mg/L TDZ were established for the best conditions of type 1 callus maintenance and its shoot regeneration, respectively. The selection of regenerable callus type of *A. mongolica* was one of the most critical factors to ensure success in this study. The highest number of shoot (22.1 per callus) was regenerated on 0.05 mg/L TDZ containing medium. TDZ at such a low concentration was more effective at inducing shoot regeneration from callus of *A. mongolica* than BA either alone or in combinations with NAA.

2. INTRODUCTION

Agrostis mongolica Roshev. (*A. mongolica*) is a monocotyledonous plant belongs to family of Poaceae. The plant type is stoloniferous, and it has the closest genetic relationship to creeping bentgrass (Vergara and Bughrara, 2003). *A. mongolica*, like other bentgrass species, is susceptible to various diseases like dollar spot, brown patch and pythium blight, and it affects by harsh environmental stresses and becomes dormant, injured or even died during hot and dry weather. Breeding for improved varieties relied mainly on conventional approaches, which had the limitations on the accessibility and use of diverse genetic materials.

Bentgrass, like other monocotyledonous plants, is recalcitrant to manipulation *in vitro*. To perform efficient genetic transformation of bentgrass, it is necessary to optimize tissue culture conditions. Embryogenic callus induction and plant regeneration systems have been established for creeping bentgrass (*Agrostis palustris* Huds.) (Krans et al., 1982; Blanche et al., 1986; Zhong et al., 1991, Chai et al., 2000), velvet bentgrass (*Agrostis canina* L.) and colonial bentgrass (*Agrostis capillaries* L.) (Wang et al., 2002). But the establishment of those systems has not reported on *Agrostis mongolica* Roshev.

Here we have reported for the first time the result of successful establishment of the efficient and simple system of regenerable callus induction and plant regeneration for *A. mongolica.*

3. MATERIALS AND METHODS

3.1. Callus induction

Mature seeds of *A. mongolica* were dehusked with sand paper and surface-sterilized in sodium hypochlorite solution (2% active chloride) containing 0.1% (v/v) of Tween 20 for 15 min, and then rinsed three times with sterilized water. The seeds were plated on wet filter paper in petri dish (90 mm in diameter) sealed with Micropore Surgical Tape (3M Health Care, USA) and germinated under *in vitro* culture conditions adjusted to 25 ± 1 ^{o}C and 18-h photoperiod of 45 μmol m^{-2} s^{-1} illumination provided by cool white fluorescent lamps. To determine the effect of growth regulators on callus induction, the germinated seeds were cultured on callus induction medium comprising MS (Murashige and Skoog, 1962) salts and modified vitamins (4 mg/L thiamine HCl), 100 mg/L myo-inositol, 3% (w/v) sucrose, various combinations of 2.4-D (0.5, 1, or 2 mg/L) with BA (0, 0.5, or 1 mg/L), and 0.2% (w/v) phytagel. The mediums were adjusted to pH 5.8 before autoclaving at 1.2 - 1.3 kg/cm^2 pressure and 121 °C for 20 min. All cultures were incubated at 25 °C in the dark in a low temperature incubator (LS-103 MP-L, LS Tech, Korea). Each treatment was carried out in triplicates (10 seeds in a petri dish per replication) and tests were repeated three times. After 2 months of culture, the number of seeds with induced callus was counted, and then callus types were characterized by their morphology.

The characterized five types of calli were divided half, respectively, and ½ was used to test their regeneration and transformation ability for selecting the best callus type, and the other ½ was kept culturing continuously.

3.2. Callus growth and maintenance

To examine growth and maintenance of selected type of callus, type 1 calli were transferred to medium comprising MSBM containing various combinations of 2.4-D (0, 0.5, 1, 2, or 4 mg/L) with BA (0. 0.01, 0.05, 0.1, or 0.2 mg/L) and incubated at 25 °C in the dark. Initially about 200 mg in fresh weight of the calli was used in each treatment. After five weeks, the five callus types once again formed from the type 1 calli were weighed, and their ratios (fresh weight of each type callus / total fresh weight of callus x 100%) were calculated.

3.3. Shoot regeneration from callus

To determine the effect of growth regulators on shoot regeneration, various combinations of TDZ or BA (0, 0.05, 0.5, 1, or 2 mg/L) with NAA (0, 0.05, 0.1, or 0.2 mg/L) were tested. TDZ was dissolved in dimethyl sulfoxide (DMSO) (Sigma Chemical Co., USA) and sterilized by hydrophobic PTFE membrane with 0.5 ㎛ pore size (MFS, Inc., USA), and it was then added into autoclaved medium comprising basal salts and vitamins of MS supplemented with 100 mg/L myo-inositol, 3% (w/v) sucrose, 0.2% (w/v) phytagel (MSBM). Each treatment was carried out in triplicates (10

calli in a petri dish per replication) and shooting tests were repeated three times. All cultures were incubated under *in vitro* culture conditions as described in above. After 5 weeks of culture, the numbers of regenerated shoots were counted.

3.4. Root regeneration and plant acclimatization

These regenerated shoots, without root, were transferred into MSBM medium supplemented with various combination of NAA (0, 0.5, 1, and 2 mg/L) with BA (0, 0.05, 0.1, and 0.5 mg/L) to test the effect of growth regulators on rooting. After 3 weeks of culture under *in vitro* culture conditions as described in above, the numbers of regenerated roots were counted. On the other hand, the shoots with small roots were simply bolted by wet kimwipes (Yuhan-Kimberly Co., Ltd), they were then put into the water for a week to elongate roots. Then, the plants were transferred to 20 x 20 cm plastic pots with peat moss and perlite (1:1), and grown in an environmentally controlled glasshouse adjusted to 20 ± 2 °C, 60 ± 10% relative humidity, and 18-h photoperiod of 30 μmol m^{-2} s^{-1} illumination provided by cool white fluorescent lamps.

4. RESULTS

4.1. Induction condition and characteristics of callus

The combinations of 2.4-D (0.5, 1, and 2 mg/L) with BA (0, 0.5, and 1 mg/L) were studied to determine the callus induction frequency from mature seeds of *A. mongolica*. To avoid the influence of seed germination on callus induction efficiency, the germinated seeds were cultured on callus induction mediums. Within 2 months, approximately from 56 to 92.5% of calli were induced (Table 4). Calli were classified into five types according to their morphology: type 1, whitish color, compact, non friable and non watery (Fig. 26A); type 2, whitish yellow color, with green spot, compact and non watery (Fig. 26B); type 3, yellow color, compact, friable and non watery (Fig. 26C); type 4, whitish color, watery and sticky (Fig. 26D); type 5, brown color, compact, friable and non watery (Fig. 26E). The highest frequency of each type was obtained at following conditions; type 1 (82.6%) on 1 mg/L 2.4-D and 0.5 mg/L BA, type 2 (70.8%) on 0.5 mg/L 2.4-D and 0.5 mg/L BA, type 3 (58.1%) on 1 mg/L only 2.4-D, type 4 (14.1%) on 0.5 mg/L 2.4-D and 1 mg/L BA and type 5 (5.4%) on 2 mg/L 2.4-D and 1 mg/L BA, respectively (Table 4). Among these callus types, type 1 and 2 were highly regenerable, whereas type 3 and 5 did not show any regenerated shoot (Table 5) but type 3 showed only root formation on MSSR medium (data not shown). Type 2 callus showed approximately 1.2 fold higher shoot regeneration ability than type 1 callus, however, type 1 callus produced 2

fold higher hygromycin-resistant calli than type 2 (Table 5). Therefore, the type 1 callus was selected for the further studies.

4.2. Effects of growth regulators on callus growth and maintenance

Type 1 calli were cultured on various combinations of 2.4-D (0, 0.5, 1, 2, or 4 mg/L) with BA (0. 0.01, 0.05, 0.1, or 0.2 mg/L) containing medium and their produced various mixtures of calli of types 1, 2, 3, 4 and 5. The best proliferation yields for type 1 and type 2 calli (550 mg, 79%; 240 mg, 48%) were observed on 2 and 0.5 mg/L 2.4-D in combination with 0.01 mg/L BA containing mediums, respectively (Table 6). Whereas, higher proliferation yields for non-regenerable type 3 calli were observed on 2.4-D alone or high concentrations of 2.4-D combined with BA mediums.

The proliferation yield of non-regenerable type 3 calli was reduced by decreasing 2.4-D concentrations and adding BA at the same time. Based on this result, 2 mg/L 2.4-D in combination with 0.01 mg/L BA was chosen for callus growth and maintenance condition. Moreover, about 90% of the type 1 callus is still able to regenerate into green plantlets after 4 years of subculture.

Table 4. Callus induction frequency from mature seeds of *A. mongolica*[a]

GR (mg/L)		Callus	Callus types (%)				
2.4-D	BA	induction (%)[b]	Type 1	Type 2	Type 3	Type 4	Type 5
0.5	0	56.0 ± 7.1	3.2 ± 0.5	0	49.5 ± 1.8	0	3.3 ± 2.3
0.5	0.5	89.8 ± 5.0	19.0 ± 1.4	70.8 ± 6.3	0	0	0
0.5	1	82.5 ± 9.1	51.1 ± 7.3	14.8 ± 4.5	0	14.1 ± 1.9	2.5 ± 1.6
1	0	60.3 ± 3.2	0	0	58.1 ± 3.3	0	2.2 ± 1.4
1	0.5	92.5 ± 8.7	82.6 ± 3.2	5.5 ± 2.5	0	2.1 ± 0.7	2.3 ± 2.2
1	1	85.0 ± 3.5	50.0 ± 3.6	20.5 ± 3.2	0	14.5 ± 1.5	0
2	0	58.5 ± 4.4	0	0	55.0 ± 2.8	0	3.5 ± 4.5
2	0.5	78.1 ± 7.8	57.3 ± 5.4	0	20.8 ± 5.6	0	0
2	1	62.2 ± 4.5	28.2 ± 3.4	0	28.6 ± 6.2	0	5.4 ± 2.3

[a]Germinated seeds were cultured on MS medium (4 mg/L thiamine-HCl, 30 mg/L sucrose, 2 mg/L phytagel) containing various combinations of 2.4-D with BA and incubated in the dark at 25°C for 2 months. [b]Number of seeds with induced callus / number of cultured seeds x 100%. Each value indicates the mean ± S.E. of three replicates.

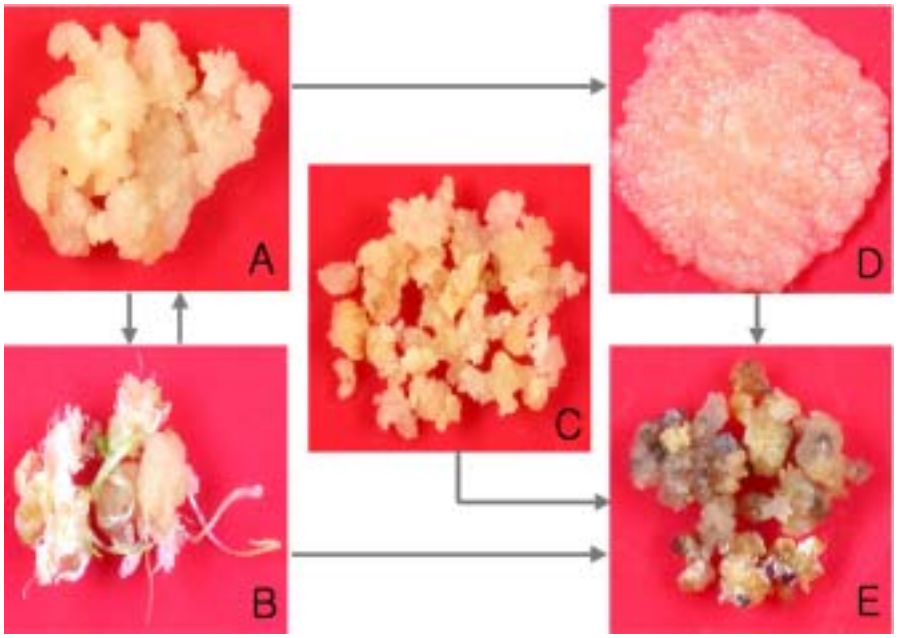

Fig. 26. Callus types derived from mature seeds of *A. mongolica*. A: Type 1 callus is whitish color, compact, non friable and non watery. B: Type 2 callus is whitish yellow color, with green spot, compact and non watery. C: Type 3 callus is yellow color, compact, friable and non watery. D: Type 4 callus is whitish color, watery and sticky. E: Type 5 callus is brown color, compact, friable and non watery.

Table 5. The callus types influenced on efficiency of shoot regeneration and hygromycin-resistant callus production

Callus type	No. of calli with regenerated shoot/No. of cultured calli	Shoot regeneration (%)[a]	No. of Hyg^R calli/ No. of calli infected	Hyg^R calli production (%)[b]
1	76/92	82.6	43/95	45.3
2	95/100	95.0	31/140	22.1
3	0/120	0	0/100	0
4	11/84	13.1	1/54	1.85
5	0/70	0	0/43	0

[a]Number of callus with regenerated shoots / Number of cultured calli x 100%.

[b]Number of Hyg^R callus / Number of callus infected x 100%. Shoots were regenerated on MSSR medium. Hygromycin-resistant callus was produced on 50 mg/L hygromycin-containing MSSR medium. Each data was collected in 5 weeks of culture.

Table 6. Differentiation characteristics of callus types affected by various combinations of 2.4-D with BA

2.4-D (mg/L)	BA (mg/L)	Callus fresh weight (mg)						Mixture ratio (Type 1:2:3:4:5)
		Total	1	2	3	4	5	
0	0	350	250	0	0	100	0	71: 0 : 0 :29 : 0
	0.01	430	230	100	0	50	50	53:23: 0 :12:12
	0.05	400	200	100	0	30	70	50:25 :0 : 8 :17
	0.1	400	150	100	0	100	50	37:25 :0 :25:13
	0.2	400	130	100	0	100	70	33:25: 0 :25:17
0.5	0	460	210	0	200	0	50	46: 0 :43: 0 :11
	0.01	500	100	240	0	80	80	20:48: 0 :16:16
	0.05	500	300	100	0	50	50	60:20: 0: 10:10
	0.1	460	230	100	0	100	30	50:22: 0 :22: 6
	0.2	570	200	190	0	100	80	35:33: 0:18: 14
1	0	650	100	0	550	0	0	15: 0 :85: 0 : 0
	0.01	500	280	100	0	0	120	56:20: 0 : 0 :24
	0.05	420	150	50	0	120	100	36:12: 0 :28:24
	0.1	400	190	20	0	100	90	48: 5 : 0 :25:22
	0.2	500	250	100	0	100	50	50:20: 0 :20:10
2	0	520	200	0	320	0	0	38: 0 :62: 0 : 0
	0.01	700	550	50	20	30	50	79: 7 : 3 : 4 : 7
	0.05	740	350	150	100	0	140	47:20:13 :0 :20
	0.1	630	300	10	200	0	120	48: 2 :31: 0 :19
	0.2	860	168	0	482	0	210	20: 0 :56: 0: 24
4	0	610	110	0	500	0	0	18: 0 :82: 0 : 0
	0.01	530	190	0	340	0	0	36: 0 :64: 0 : 0
	0.05	450	150	0	300	0	0	33: 0 :67: 0 : 0
	0.1	830	180	0	650	0	0	22: 0 :78: 0 : 0
	0.2	900	200	0	700	0	0	22: 0 :78: 0 : 0

Initially, about 200 mg in fresh weight of type 1 calli was used in each treatment, and incubated them at 25^{o}C in the dark for 5 weeks.

4.3. Effects of growth regulators on shoot regeneration from callus

4.3.1. Effects of combinations of TDZ with NAA on shoot regeneration from callus

The combinations of TDZ (0, 0.05, 0.5, 1, and 2 mg/L) with NAA (0, 0.05, 0.1, and 0.2 mg/L) were tested to optimize the concentration of growth regulators for shoot regeneration from callus. The calli grown on hormone-free MS medium did not produce any shoot and the number of regenerated shoots per explant was dramatically decreased with increasing NAA and as well as increasing TDZ concentrations (Fig. 27). In contrast, a great number of shoots per callus were observed on the medium with lower concentrations of TDZ. Based on the number of regenerated shoots (22.1 shoots per callus) and their morphology, 0.05 mg/L TDZ was chosen as the most suitable condition for shoot regeneration from callus (Fig. 27).

4.3.2. Effects of combinations of BA with NAA on shoot regeneration from callus

The combinations of BA (0, 0.05, 0.5, 1, and 2 mg/L) with NAA (0, 0.05, 0.1, and 0.2 mg/L) were tested to optimize the concentration of growth regulators for shoot regeneration from callus. A great numbers of regenerated shoots per explant were observed within a range of combinations of BA 0.5-2 mg/L with 0.05 or 0.1 mg/L NAA (Fig. 28). Based on the number of regenerated shoots (8 shoots per explant) and their morphology, 0.5 mg/L

kinetin in combination with 0.1 mg/L NAA was chosen as the most suitable for shoot regeneration from callus of *A. mongolica*. In contrast, the number of regenerated shoots per explant was decreased with increasing NAA concentrations, moreover BA alone was produced only a few number of shoots (Fig. 28), suggesting that the appropriate combination ratios of BA and NAA is essential for shoot regeneration from callus.

4.4. Effects of combinations of NAA with BA on rooting

The combinations of NAA (0, 0.5, 1, and 2 mg/L) with BA (0, 0.05, 0.1, and 0.5 mg/L) were tested to optimize the concentration of growth regulators for rooting. A great number of regenerated roots per plant were observed on MS hormone free or 0.05 mg/L BA or lower concentrations of NAA containing mediums. Root regeneration was dramatically decreased with increasing NAA and as well as increasing BA concentrations (Fig. 29). Based on the number of regenerated roots (32.8 roots per plant) and their morphology, MS hormone free medium was chosen as the most suitable condition for root regeneration from shoots of *A. mongolica*.

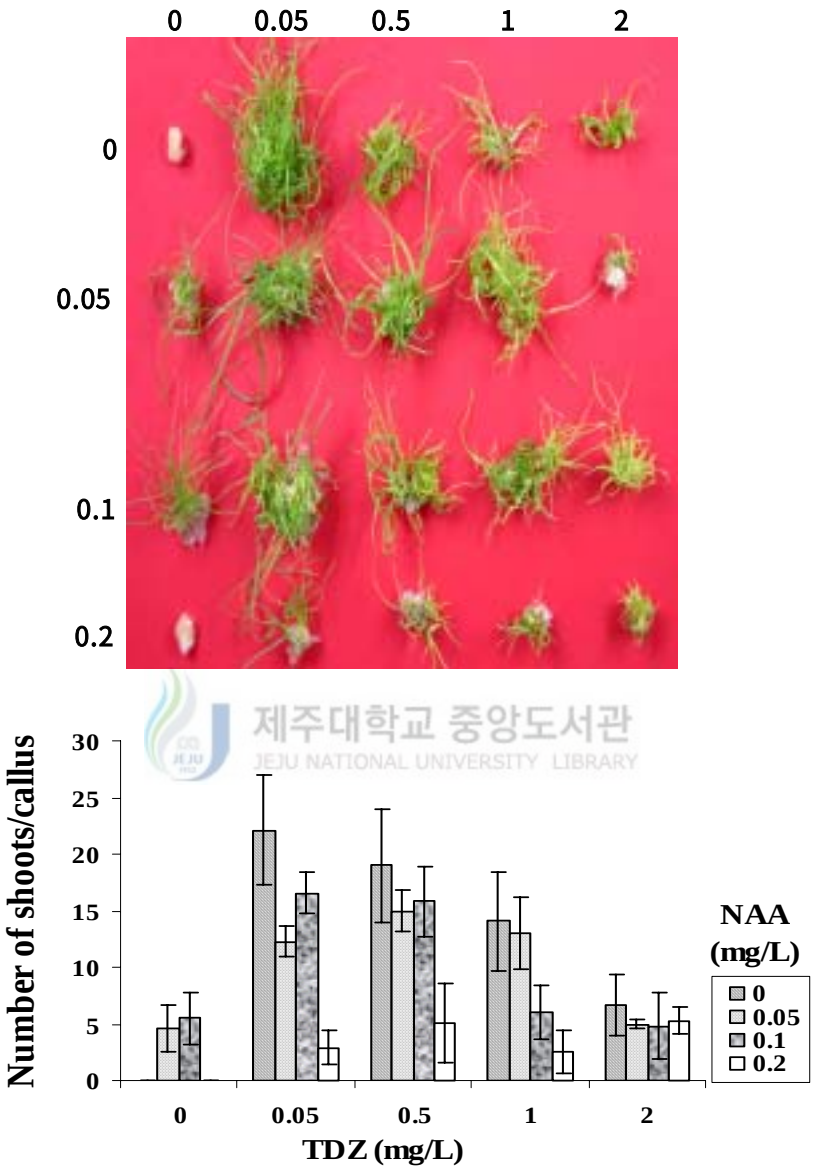

Fig. 27. Shoot regeneration from type 1 callus on MSBM medium containing various combinations of TDZ with NAA. Totally 30 callus were tested in each treatment. Columns indicate the mean number of regenerated shoots per callus of three replicates. Bars refer to standard error.

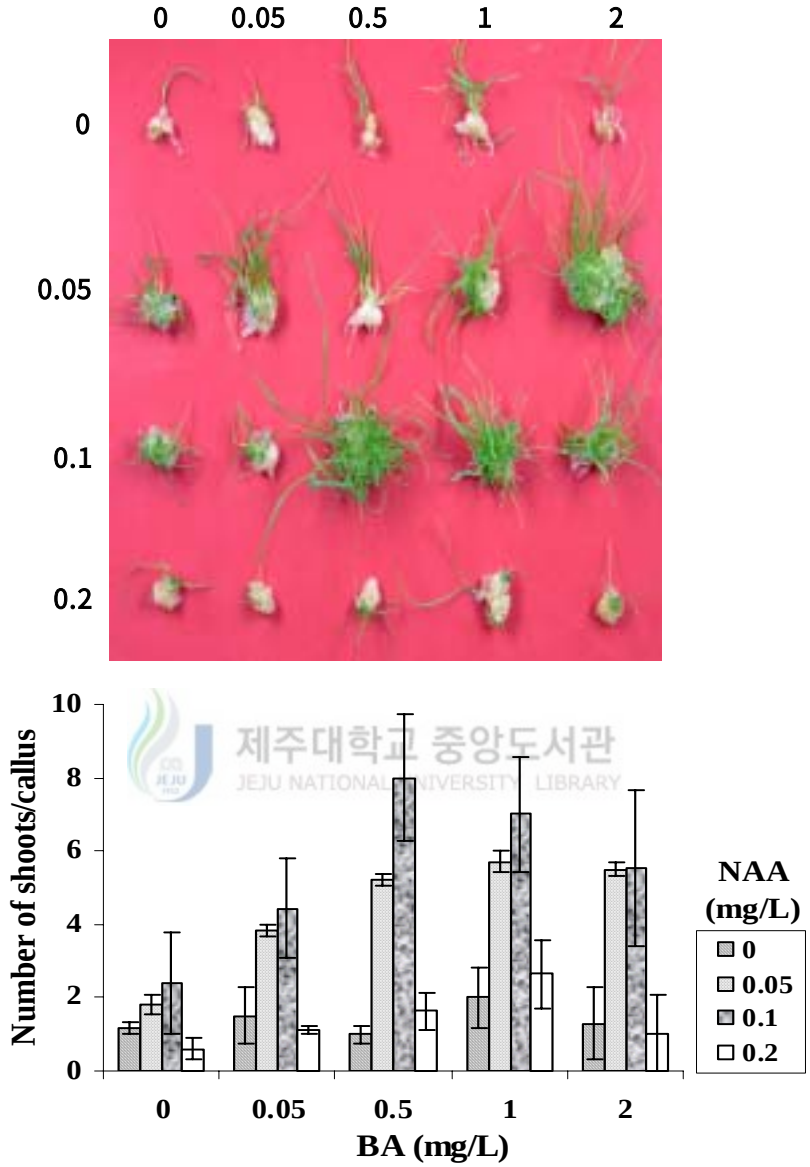

Fig. 28. Shoot regeneration from type 1 callus on MSBM medium containing various combinations of BA with NAA. Totally 30 callus was tested in each treatment. Columns indicate the mean number of regenerated shoots per callus of three replicates. Bars refer to standard error.

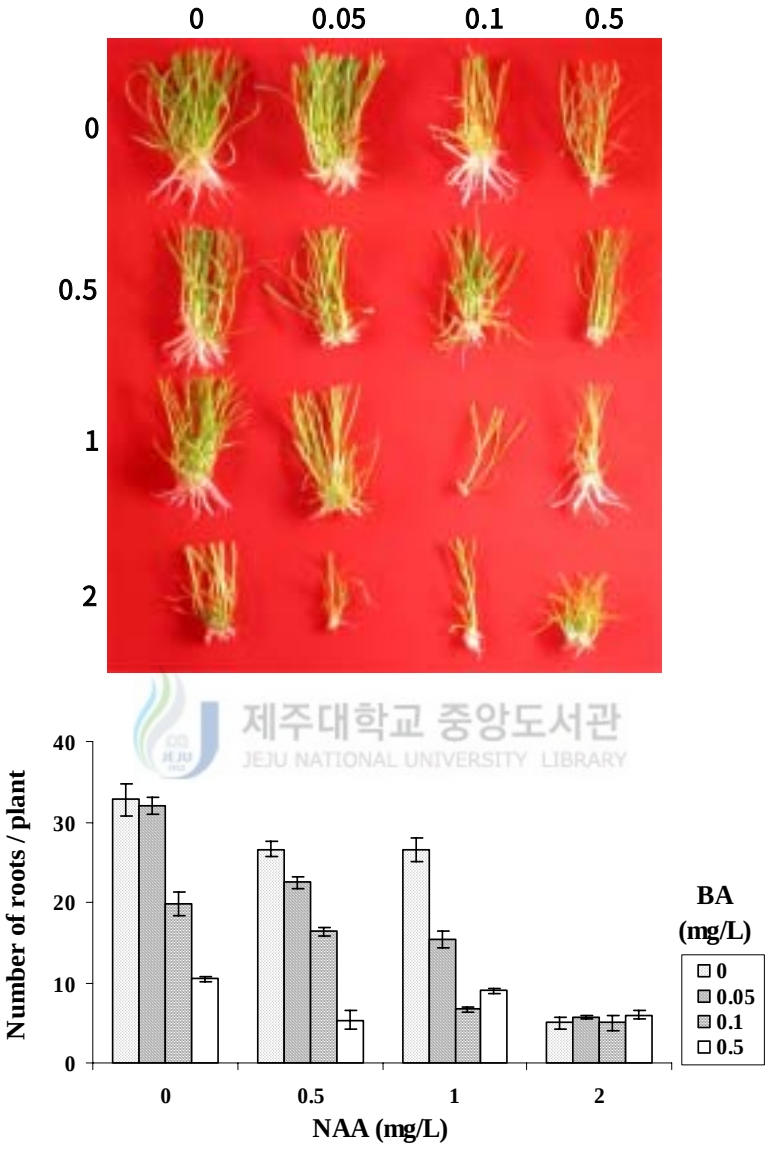

Fig. 29. Root regeneration of *A. mongolica* on MSBM medium containing various combinations of NAA with BA. Columns indicate the mean number of regenerated roots per plant of three replicates. Bars refer to standard error.

5. DISCUSSION

The selection of regenerable callus type of *A. mongolica* was one of the most critical factors to insure success in this study. The correlation between morphological characteristics of calli and their regenerability has been reported for Kentucky blue grass (Kusano et al., 2003), zoysiagrass (Toyama et al., 2003), bermudagrass (Li and Qu, 2004), and bentgrasses (Luo et al., 2004; Chai et al., 2004). The highest number of shoot (22.1 per callus) was regenerated on 0.05 mg/L TDZ containing medium. TDZ either alone at low concentration or in combination with lower concentrations of NAA induced a high number of shoots from type 1 callus. NAA in general was not essential for shoot regeneration of *A. mongolica*. In pigeonpea (*Cajanus cajan* L.) also, TDZ alone at low concentration induced multiple shoots while at higher concentration it caused complete shift in regeneration from induction of adventitious shoots to somatic embryogenesis (Singh et al., 2003). It is interesting to note that increasing the concentration of TDZ in the medium did not significantly affect the frequency of regeneration. Hutchinson and Saxena (1996) and Murch et al. (2000) described a similar phenomenon in *Pelaggonium* and *Hypericum perforatum* hypocotyl explants exposed to TDZ. In both of the plants, longer exposures to higher level of TDZ resulted in residual effects, including a decrease in the number of regenerants, hyperhydricity of the shoots, and poor rooting of regenerants (Lu, 1993; Murthy et al., 1998).

In this study, TDZ was more effective at inducing shoot regeneration from callus of *A. mongolica* than BA either alone or in combinations with NAA. TDZ– a substituted phenyl urea showing cytokinin-like activity similar to that of the N_6 substituted adenine derivatives (Mok et al., 1982). TDZ has been shown to induce the accumulation of both endogenous auxins and cytokinins in legumes and herbaceous species (Murthy et al., 1995; Hutchinson and Saxena, 1996). Also, treatment of soybean callus with TDZ stimulated cytokinin accumulation (Thomas and Ketterman, 1986). This is probably because cytokinins are inactivated irreversibly by two mechanisms; one of the mechanisms is the oxidative cleavage of N_6 side chain of the cytokinin substrate by cytokinin oxidase (Kende and Zeevaart, 1997). TDZ is known to non-competitively inhibit cytokinin oxidase activity (Chatfield and Armstrong, 1986), thereby enhancing the availability of endogenous cytokinins. In this study also, TDZ displayed more effective shoot regeneration ability than BA, supporting these previous hypotheses.

Here, we found a high regenerable callus type and established the optimal conditions for callus type maintenance and plant regeneration of *A. mongolica*. The type 1 callus was selected as the best callus type, and 2 mg/L 2.4-D in combination with 0.01 mg/L BA and 0.05 mg/L TDZ were established for the best conditions of type 1 callus maintenance and its shoot regeneration, respectively, for the further transformation experiment.

Part IV

Enhances tolerance to heat stress under water deficit condition in transgenic *Agrostis mongolica* Roshev. overexpressing *ABF3* gene

1. ABSTRACT

The loss of plant productivity caused by high temperature is often serious when combined with the other environmental stresses such as water deficit. Plant hormone abscisic acid (ABA) is essential for the adaptive responses of plants to both water and heat stresses. We established an efficient *Agrobacterium*-mediated transformation procedure for *Agrostis mongolica* Roshev. and generated transgenic plants tolerant to heat stress under water deficit condition using a regulatory gene, *ABF3*, which controls the ABA-dependent adaptive responses. The employment of the selected regenerable callus type (type 1) of *A. mongolica* was one of the most critical factors to ensure success in this study. The transformation efficiency was 49.2% and GFP was strongly expressed in hygromycin-resistant callus and seedlings. The result of Southern blot analysis showed that the ABF3 transgene was stably integrated into the genome of the transgenic plants. Of the five transgenic lines analyzed, single transgene integration was observed in two, and two copy integration was observed in three transgenic lines. Northern blot analysis confirmed that ABF3 was highly expressed in

transgenic plants and gene silencing was not observed. Transgenic plants showed normal growth and morphology. Interestingly, both transgenic and wild-type plants did not flower during over the 3 years growth in the open field under Jeju climate. The stomatal opening of the transgenic plants was smaller than did the wild-type plants and water content of the transgenic leaves remained about 3-4 fold higher than wild-type leaves under water deficit condition. The transgenic plants showed about 2-3 fold higher survival rates under heat and water stress conditions than wild-type plants.

2. INTRODUCTION

Agrostis mongolica Roshev. (*A. mongolica*) is an important endemic bentgrass of Mongolia widely used for golf course putting greens, lawn and roadside grassing, and livestock pasture. Similar to many other bentgrass species, the growth of *A. mongolica* is favored during cooler period (15-25 °C) of the growing season, and it becomes dormant, injured or even died during hot and dry weather. In nature, a combination of drought and heat stress can represent the conditions encountered by many plants and crops growing within arid and semiarid environments (Mittler et al., 2001), and it is possible to enhance stress tolerances of plants via genetic manipulations. Numerous studies have shown that ABA is essential for the normal adaptive response to the water stress imposed by drought (Koorneef et al., 1984; Leon-Kloosterziel et al., 1996; Xiong et al., 2002). Moreover, ABA is necessary for the protection against high temperature (Gong et al. 1998; Larkindale and Knight 2002).

Over-expression of ABF3 enhanced tolerances to drought, low/high temperature in *Arabidopsis* (Kang et al. 2002; Kim et al. 2004) and also enhanced both drought and cold stress tolerances in lettuce (Enkhchimeg et al. 2005). Based on these results, we tried to produce transgenic *A. mongolica* plants which are tolerant to heat stress under water deficit condition by introducing the *ABF3* gene. Successful productions of transgenic plants using *Agrobacterium*-mediated transformation of creeping

bentgrass (*A. stolonifera*) (Yu et al. 2000; Chai et al. 2003; Luo et al. 2004; Han et al. 2005; Fu et al. 2005) and colonial bentgrass (*A. tenuis*) (Chai et al. 2004) have been reported, although there is no study available for gene transformation of *A. mongolica*. The goals of previous studies were to develop efficient transformation methods and to generate herbicide- or disease-resistant bentgrasses. Since bentgrass is heat-sensitive, it would be desirable to develop heat-tolerant bentgrass varieties. Toward the goal of this study was to establish an efficient *Agrobacterium*-mediated transformation procedure for *A. mongolica* and to develop transgenic *A. mongolica* plants tolerant to heat stress under water deficit condition employing the procedure.

3. MATERIALS AND METHODS

3.1. Plant material, bacterial strain and plasmid

The type 1 calli propagated on the medium containing 2 mg/L 2.4-D and 0.01 mg/L BA were used as initial plant material.

The summary of media used in the *A. mongolica* tissue culture and transformation system is showed in Table 7.

*Agrobacterium tumifacien*s strain EHA 105 harboring a binary vector pCUMB was used for transformation. The binary vector carries a target gene ABA-responsive element binding factor3 (*ABF3*) under ubi promoter, the reporter genes green fluorescence protein - β-glucuronidase (*gfp-gus*) under 35S promoter, and a selectable marker gene hygromycin phosphotransferase (*hph*) under 35S promoter (Fig. 14).

3.2. *Agrobacterium*-mediated transformation protocol

The *Agrobacterium* was grown in 20 ml Luria-Bertani (LB) medium supplemented with 100 mg/L kanamycin and 100 mg/L rifampicin, and shaken with 160 rpm at 28 ºC until $OD_{600\ nm}$ value of 0.6-0.8. The bacterial suspension was centrifuged at 2000 rpm for 20 min in a 50-ml polypropylene tube (Becton Dickinson Labware, USA) and resuspended in 40 ml resuspension medium (MSBM without $CaCl_2$, 100 mg/L acetosyringone, 20 mg/L pluronic F68, pH 5.8) by vortexing. Calli growing on callus growth medium comprising MSBM with 2 mg/L 2.4-D and 0.01 mg/L BA (MSCG)

were separated into small pieces using pincers and pre-cultured on fresh MSCG medium without $CaCl_2$ for 3 days in the dark at 25 °C. The pre-cultured calli were briefly surface-dried on sterilized filter paper, and immersed in the bacterial suspension for 10 min at 28 ºC with shaking at 160 rpm. The calli were briefly dried on the filter paper again, and cultured on co-cultivation medium comprising MSCG without $CaCl_2$, 100 mg/L acetosyringone, pH 5.8 (MSCC) in the dark at 25 ^{o}C for 10 days. Prior to bacteria elimination, a range of carbenicillin concentrations (0, 50, 125, 250, 500, or 1000 mg/L carbenicillin) was investigated to determine the maximum suitable concentration at shoot regeneration from callus of wild-type *A. mongolica*, and it was used to eliminate excess bacteria.

For bacteria elimination, co-cultured calli were washed in sterile water containing 500 mg/L carbenicillin, then cultured on bacteria elimination medium (MSCG with 500 mg/L carbenicillin) in the dark at 25 ^{o}C for 14 days.

Prior to selection, a range of hygromycin concentrations (0, 10, 20, 30, 40, 50, 70, or 100 mg/L hygromycin) was tested to determine the minimum fatal concentration at shoot regeneration from callus of wild-type *A. mongolica*, and it was used to select the putative transformed plants. For shoot regeneration and selection, the calli were cultured on MSBM containing 0.05 mg/L TDZ (MSSR) supplemented with 50 mg/L hygromycin and 250 mg/L carbenicillin medium at 25 ^{o}C in the light for 5 weeks. Then hygromycin-resistant shoots (5-10 mm in height) derived from these calli were transferred

into MSBM medium containing 50 mg/L hygromycin and 250 mg/L carbenicillin for 2 weeks for root induction and selection. The hygromycin-resistant plants were transferred to 20 x 20 cm plastic pots with peat moss and perlite (1:1), and grown in an environmentally controlled glasshouse adjusted to 20 ± 2 °C, 60 ± 10% relative humidity, and 18-h photoperiod of 30 μmol m^{-2} s^{-1} illumination provided by cool white fluorescent lamps. GFP expression of wild-type and hygromycin-resistant calli, also wild-type and putative transgenic plants was examined with a Zeiss LSM510 confocal laser scanning microscope.

Table 7. Summary of media used in the *A. mongolica* tissue culture and transformation system

Medium	Composition
Basal media (MSBM)	MS salts and vitamins, 30 g/L sucrose, 2 g/L phytagel, pH 5.8
Callus induction	MSBM with 4 mg/L thiamine-HCl, 1 mg/L 2.4-D, 0.5 mg/L BA
Callus growth* (CG)	MSBM with 2 mg/L 2.4-D, 0.01 mg/L BA
Shoot regeneration(SR)	MSBM with 0.05 mg/L TDZ
Root regeneration	MSBM
Agrobacterium culture	LB (10 g/L tryptone peptone, 5 g/L bacto-yeast extract, 5 g/L NaCl, 1 g/L D-glucose) with 100 mg/L kanamycin, 100 mg/L rifampicin, pH 7.0
Agrobacterium suspension	SR (without $CaCl_2$) with 100 mg/L acetosyringone, 20 mg/L pluronic F68, pH 5.2
Pre-cultivation*	CG medium without $CaCl_2$
Co-cultivation*	CG (without $CaCl_2$) with 100 mg/L acetosyringone, pH 5.8
Bacteria elimination*	CG with 500 mg/L carbenicillin
Shoot regeneration and selection	SR with 50 mg/L hygromycin, 250 mg/L carbenicillin
Root induction and selection	MSBM with 50 mg/L hygromycin, 250 mg/L carbenicillin

*Filter papers were impregnated on the medium.

3.3. Molecular characterization of transgenic plants

Genomic DNA was isolated from young leaf tissue of transgenic and wild-type plants according to CTAB method (Saghai-Maroof, 1984). Polymerase chain reactions (PCR) were carried out on the hygromycin-resistant plants by amplifying the coding regions of transgenes using the following sets of oligonucleotide primers: *ABF3* 5'-AGAACCTCAACCGGTGGAGAGTG-3' (forward) and 5'-GGAGTCAGATCAGGTGACATCTGG-3' (reverse); *hph* 5'-GATGTAGGAGGGCGTGGATATGTC-3' (forward) and 5'-CTTCTACACAGCCATCGGTCCAGA-3'(reverse); *gus* 5'-CAACGAACTGAACTGGCAGA-3' (forward) and 5'-GGCACAGCACATCAAAGAGA-3' (reverse). The expected PCR products were 480 bp for *ABF3*, 1080 bp for *hph* and 966 bp for *gus*. The total volume of reaction mixture was 13 μl, including 25 ng genomic DNA, 1 μl of each primer (10 pmols) and 7 μl Premix Ex Taq (Takara, Japan). Cycling parameters began with an initial hot start at 95 °C for 5 min, then 35 cycles of denaturation 95 °C for 30 s, annealing at 57 °C for 1 min and extension at 72 °C for 45 s, followed by a final extension of 5 min at 72 °C. PCR amplification products were analyzed by electrophoresis in 0.8% agarose gel and stained with ethidium bromide (EtBr) and visualized with UV. For southern blot analysis, each of 25 μg of *Hin*dIII or *Bam*HI-digested genomic DNA samples were electrophoresed through 0.8% (w/v) agarose gel, followed by depurination in 0.25 N HCl for 10 min, denaturation in 0.5 N

NaOH and 1.5 N NaCl for 1 h, neutralization in 1 M Tris (pH 7.5) and 1.5 M NaCl for 1 h, and homogenization in 10 x SSC for 1 h.

Total RNA was isolated from young leaf tissue of transgenic and wild-type plants using Tri-Reagent made as the total volume of 400 ml containing 47.3 g of guanidine thiocynate, 16.4 g of ammonium thiocynate, 150 ml of phenol A, 12.4 ml of 3M sodium acetate (pH 5.0) and 25 ml of glycerol. RNA isolation was followed by Tri-reagent (MRC, Inc., Cincinnati, OH) manufacturer's protocol. For northern blot analysis, the reaction mixtures containing 2 μl of 10 x MOPS, 4 μl of formaldehyde, 10 μl of formamide and 30 μg of total RNA were prepared, the mixtures were incubated in the water bath at 65 °C for 15 min and chilled on the ice for 3 min. The samples were then electrophoresed on 1.2% (w/v) agarose gel containing 2.2 M formaldehyde at 50 volt for 3 h. The gel was stained with EtBr and visualized on UV, and then it was washed by deionozed sterile water for 45 min and by 20 x SSC for 1 h.

The gels of both southern and northern blot analysis were blotted onto an uncharged nylon membrane (Amersham Pharmacia Biotech, UK) using 20 x SSC by download capillary transfer method (Koetsier et al., 1993), respectively. Hybridization probe, a PCR-amplified product of *ABF3*, was [^{32}P] dCTP-labelled using the Rediprime II Random Prime Labelling System (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instruction, and the unincorporated nucleotides were removed by passing through the MicroSpin^{TM} G-25 Columns (Amersham Biosciences,

Piscataway, NJ). Prehybridization with 2 x SSC and 0.1% SDS was carried out at 65 °C for 30 min. Hybridization was performed overnight at 65 °C in Rapid-hyb buffer (Amersham Pharmacia Biotech, UK). Then the membranes were initially washed in 2 x SSC and 0.1% SDS at 65 °C for 30 min, and repeated in the same buffer at room temperature for 15 min, thereafter in 0.2 × SSC and 0.1% SDS with the same agitation at room temperature for 30 min. The blot was exposed to Kodak Biomax (Kodak, Stuttgart) for 36 h at 80 °C. RT-PCR was performed employing the Access RT-PCR System (Promega). The total volume of reaction mixtures was 25 μl, including 1 μg of total RNA, 5 μl AMV/*TfI* 5XReaction buffer, 0.5 μl 10mM dNTP mixture, 1 μl $MgSO_4$, 2.5 μl of each primer, 0.5 μl AMV reverse transcriptase, 0.5 μl *TfI* DNA polymerase. The set of oligonucleotide primer as described in above were employed as primer for the RT reactions of ABF3. As a standard control, a 303 bp 18S rRNA fragment was amplified using the primers 5'-ATGATAACTCGACGGATCGC-3' and 5'-CCTCCAATGGATCCTCGTTA-3'. PCR reactions comprised 10 pmols of each primer. The following RT-PCR conditions were used: 45 °C for 50 min for cDNA synthesis, cycling parameters began with an initial hot start at 94 °C for 2 min, then 35 cycles of denaturation at 94 °C for 10 s, annealing at 50 °C for 15 s and extension at 68 °C for 1 min 30 s, followed by a final extension of 7 min at 68 °C. Amplified PCR products were separated on 0.8% agarose gel, stained with EtBr and visualized with UV.

3.4. Heat stress test under water deficit

The 3-year-old transgenic and wild-type plants were grown in the environmentally controlled glasshouse and irrigated twice a day. The stress test was carried out in two steps. First step, the plants were withheld from water for 20 days at 25 ± 3 °C and at 60 ± 10% of relative humidity with 18-h photoperiod. Second step, these already stressed plants were directly treated again for 48h under heat stress conditions adjusted to 40 ± 1 °C, 60 ± 10% relative humidity with 18-h photoperiod of 45 μmol m^{-2} s^{-1} illumination provided by cool white fluorescent lamps in a low temperature incubator (LS-103 MP-L, LS Tech, Korea), and then the plants were transferred to the environmentally controlled glasshouse and irrigated again. The survival rate of heat stressed plants was evaluated after 14 days since transferring. 12 pots from transgenic and wild-type plant were used for the stress test and it was repeated three times. The water content of leaves was measured at 0, 5, 10, 15, and 20 days of water deficit treatment and calculated based on the equation as described by Hsieh et al. 2002. The dry weight (DW) of leaves was measured after drying them for 36h at 65 °C. Stomata aperture was also observed at 10 days of water deficit treatment under Axiostar plus, Carl Zeiss microscope (x400). The upper and lower surfaces of detached leaf blades were varnished by a colorless manicure and leaved them to dry then pressed scotch tape on the leaf surface and peel off the nail varnish impression and stick it to a microscope slide to observe the stomatal aperture.

4. RESULTS

4.1. Factors affecting hygromycin-resistant callus production

To evaluate the effect of $CaCl_2$ and acetosyringone in pre-cultivation medium on hygromycin resistant calli production, the type 1 callus were precultured for 3 days on MSCG medium with or without $CaCl_2$ in combinations with acetosyringone (0 or 150 mg/L). The production of hygromycin resistant calli was high in the absence of both $CaCl_2$ and acetosyringone (Fig. 30A). It is probably due to calcium induced defense response of the plant against *Agrobacterium* infection and it was known that the expression of GUS in zoysiagrass was transiently inhibited under the presence of calcium (Toyama et al., 2003). In the previous study it has been hypothesized that the calli pretreated with acetosyringone enhanced transformation efficiency in bentgrass (Luo et al., 2004). However, in this study our results were not consistent with this hypothesis. The effects of pH and acetosyringone concentrations in co-cultivation medium on hygromycin resistant calli production were also determined. The highest frequency (50.9%) of hygromycin resistant calli were produced at pH 5.8, and it was decreased about 1.3 fold at pH 5.2 and 1.5 fold at pH 7.0 (Fig. 30B). Moreover, among the tested acetosyringone concentrations (0, 50, 100, 150, 200, and 300 mg/L), acetosyringone at 100 or 150 mg/L produced the highest frequency of hygromycin resistant calli (Fig. 31A). Therefore, 100 mg/L of acetosyringone was used for the further experiments. To determine

the effect of co-cultivation period on hygromycin resistant calli production, the inoculated calli were co-cultivated on MSCC medium for 5, 7, 10, and 14 days. Only 3.6% of hygromycin-resistant calli were produced from 5 day co-cultivated calli, whereas it was increased about 4 fold (12.6%) from 7 day co-cultivated calli and 11 fold (38.6%) from 10 day co-cultivated calli, respectively (Fig. 31B). As further co-cultivation to 14 days resulted in decrease in the hygromycin-resistant callus production, and therefore 10 day period was used in subsequent experiments.

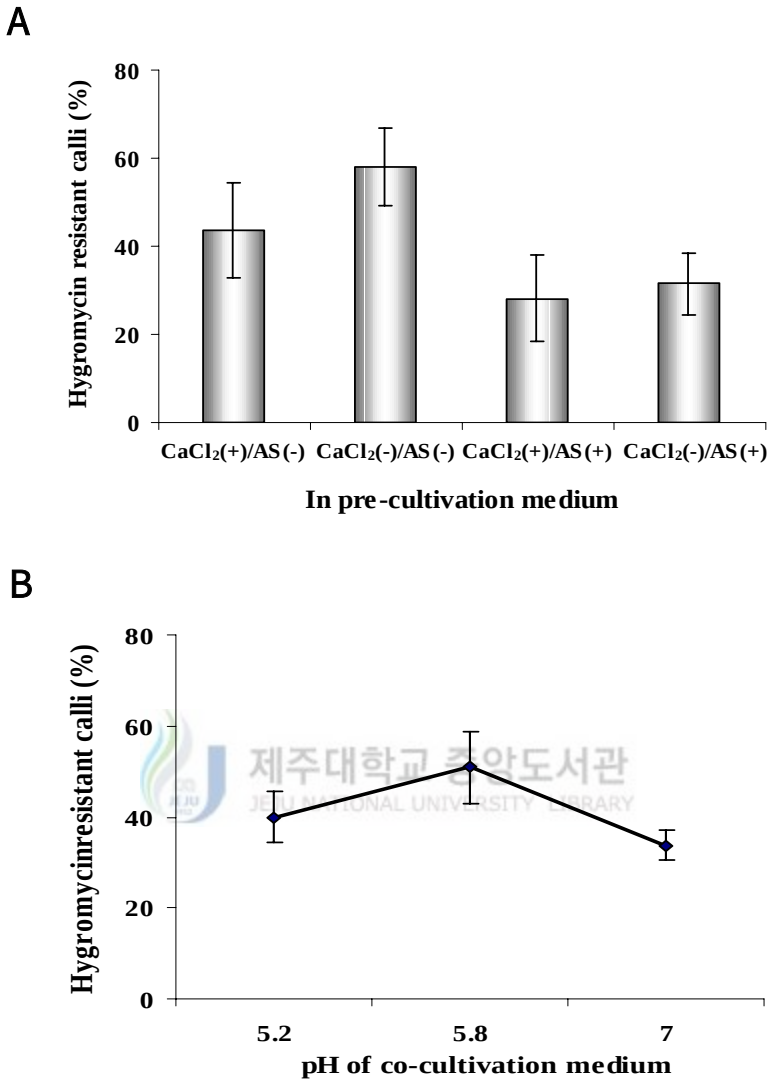

Fig. 30. Effect of with/without $CaCl_2$ and 150 mg/L acetosyringone (AS) in precultivation medium (A) and pH of co-cultivation medium (B) on hygromycin-resistant calli production. Hygromycin resistant calli = number of survived calli / total number of infected calli x 100%. Data were collected in 5 weeks of selection on 50 mg/L hygromycin, respectively. Each value represents the mean of four replicates. Bars refer to standard error.

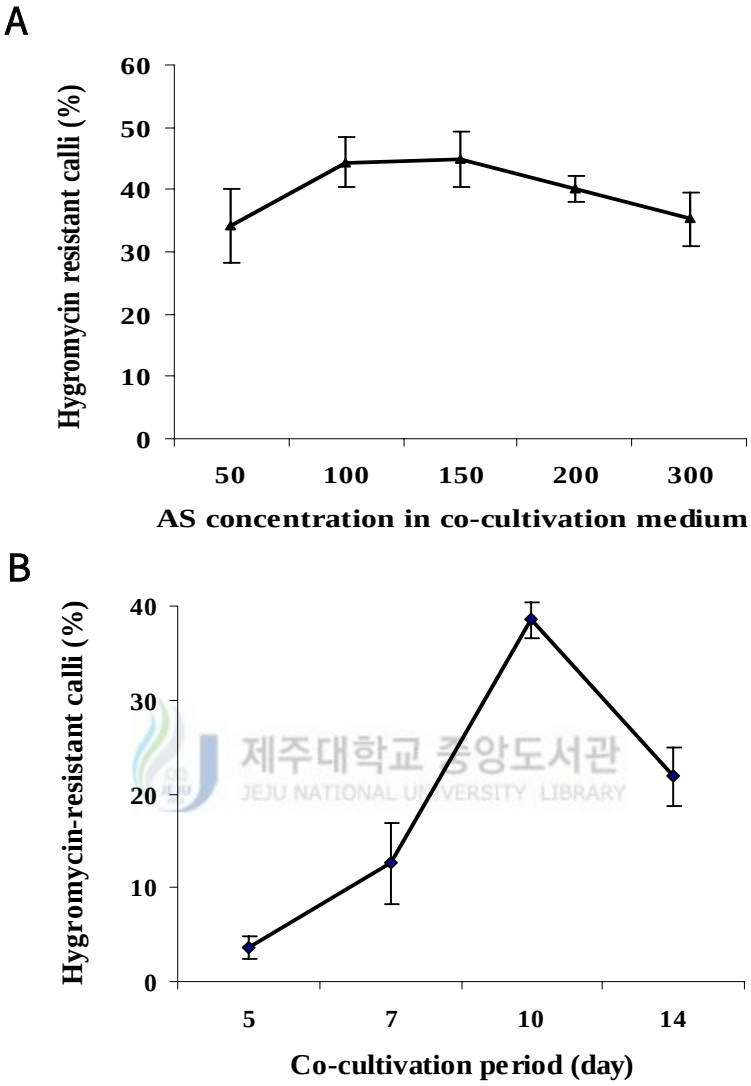

Fig. 31. Effect of acetosyringone (AS) concentration in co-cultivation medium (A) and co-cultivation period (B) on hygromycin-resistant calli production. Hygromycin resistant calli = number of survived calli / total number of infected calli x 100%. Data were collected in 5 weeks of selection on 50 mg/L hygromycin, respectively. Each value represents the mean of four replicates. Bars refer to standard error.

4.2. Selection and regeneration of transgenic plants

To construct the transgenic *A. mongolica* plants with *ABF3* gene, type 1 calli were inoculated with *A. tumefaciens* harboring a binary vector pCUMB carrying *ubi::ABF3*, *35S::gfp-gus*, and *35S::hph* genes. To eliminate excess bacteria, the maximum suitable concentration of carbenicillin at shoot regeneration from callus of wild-type *A. mongolica* was determined. All the tested concentrations of carbenicillin did not influence on shoot regeneration efficiency of calli (Fig. 32). Therefore, 250 mg/L carbenicillin was contained in shoot or root regeneration and selection medium, and 500 mg/L carbenicillin was added in bacteria elimination medium. Also prior to selections, the minimum fatal concentration of hygromycin at shoot regeneration from calli of wild-type plants was determined. From 30 mg/L hygromycin concentration, shoot regeneration was completely inhibited and at 50 mg/L or higher hygromycin concentrations, callus growth was completely inhibited and eventually died within 5 weeks of culture (Fig. 33). Based on this result, 50 mg/L hygromycin was chosen in both shoot regeneration/selection and root induction/selection steps. At the end of the second week of shoot regeneration/selection, the growth of the calli was decreased and gradually changed into brown color and died, while some resistant calli kept growing with result in white to yellowish color (Fig. 34A). At the end of the fifth week, 49.2% (128/260) calli were survived, but only 23.4% (30/128) of them

generated green shoots (Fig. 34B). These hygromycin-resistant shoots were rooted on 50 mg/L hygromycin-containing 1/2 MSBM medium, whereas susceptible-shoots were died within 2 weeks during root induction/selection (Fig. 34C). The hygromycin-resistant plants were transplanted to the environmentally controlled glasshouse and all of them exhibited normal growth and morphology (Fig. 34D), and the stolons were arisen from 3-year-old transgenic plants (Fig. 34E). Interestingly, these transgenic as well as wild-type plants did not flower during over the 3 years growth in the open field.

Although GUS expression was not observed in the other species of *Agrostis* genus, wild-type of *A. mongolica* showed strong GUS expression in callus and seedling (Fig. 35). Therefore, we could not observe the GUS expression from transgenic plants, only GFP expression was evaluated. On the confocal imaging of callus of wild-type plant (Fig. 36) and hygromycin-resistant callus (Fig. 38) was shown in red (Fig. 36A, 38A), green (Fig. 36B, 38B) and transmission (Fig. 36C, 38C). Images of Fig. 36D and 38D are the superimposed imaging of the combination of red and green images, respectively. Co-localization areas are displayed as false color yellow in Fig. 36D and 38D. On the confocal imaging of stem of wild-type plant (Fig. 37) and stem of hygromycin-resistant plant (Fig. 39) was shown in red (Fig. 37A, 39A), green (Fig. 37B, 39B) and transmission (Fig. 37C, 39C). Images of Fig. 37D and 39D are the superimposed imaging of the combination of red and green images, respectively. Co-localization areas are displayed as false color

yellow in Fig. 37D and 39D. High level of GFP expression was detected in the hygromycin-resistant callus (Fig. 38D) and lower part of stem of hygromycin-resistant plants (Fig. 39D). In contrast, GFP expression was not detected in both callus (Fig. 36D) and stem of wild-type plants (Fig. 37D) and also in leaves of putative transgenic plants (data not shown).

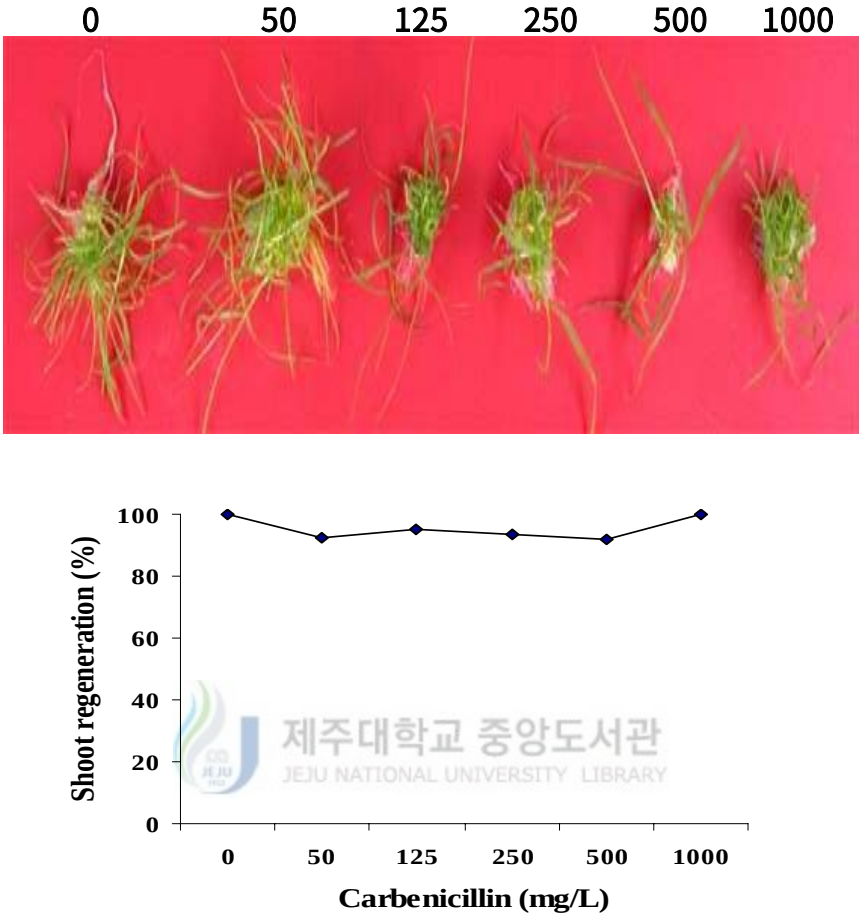

Fig. 32. Effect of carbenicillin concentrations on shoot regeneration from callus of wild-type *A. mongolica*. Data was collected after 5 weeks of callus culture on medium comprising MS supplemented with 0.05 mg/L TDZ and various concentrations of carbenicillin.

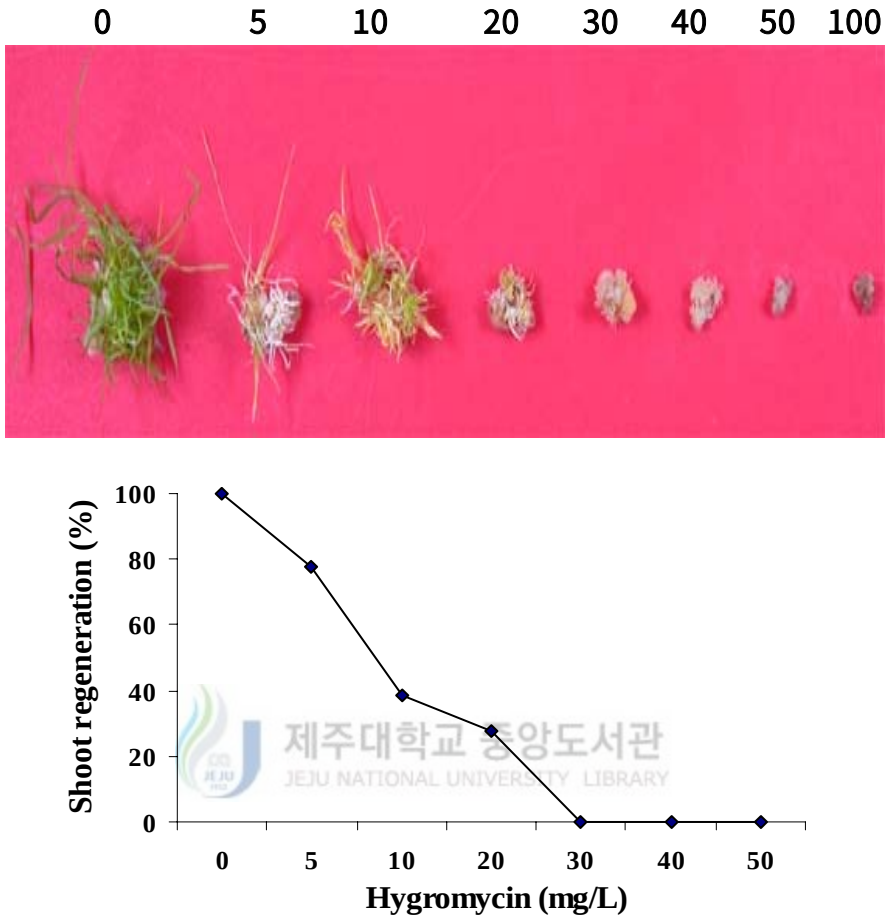

Fig. 33. Effect of hygromycin concentrations on shoot regeneration from callus of wild-type *A. mongolica*. Data was collected after 5 weeks of callus culture on medium comprising MS supplemented with 0.05 mg/L TDZ and various concentrations of hygromycin.

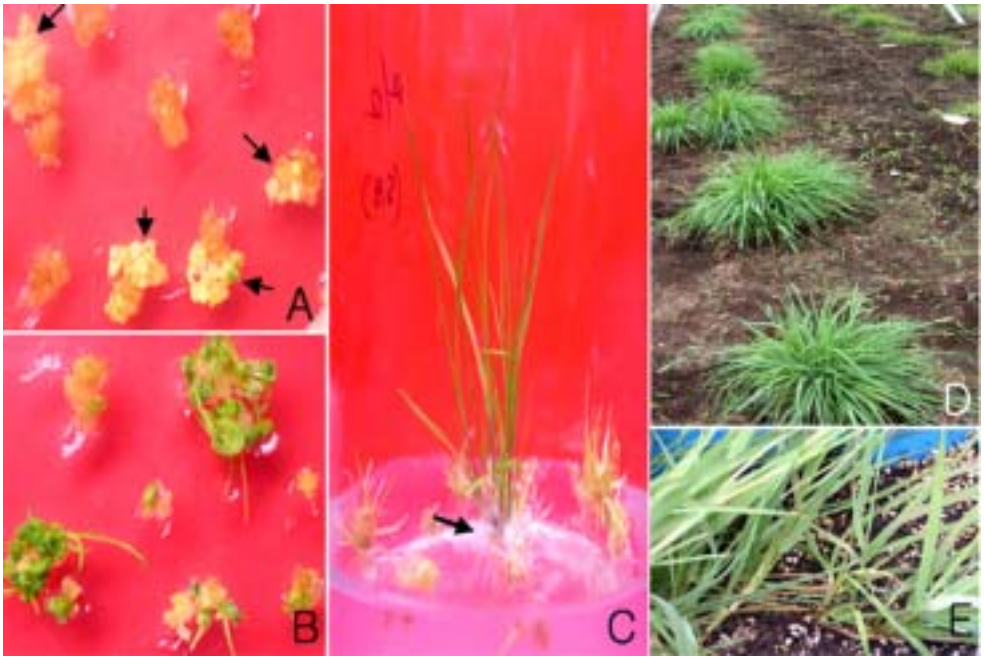

Fig. 34. Regeneration and selection of transgenic *A. mongolica* plants. A: Hygromycin-resistant calli were generated after 2 weeks of selection. B: Hygromycin-resistant shoots regenerated from these calli after 5 weeks of selection. C: Roots induced from the resistant shoots. D: Transgenic plants growing in the open field. E: The stolons were arisen from 3-year-old transgenic plants.

Fig. 35. Histochemical GUS expression in callus (A) and seedling (B) of wild-type *A. mongolica*. Blue staining indicates GUS expression. Arrows indicate GUS expression part.

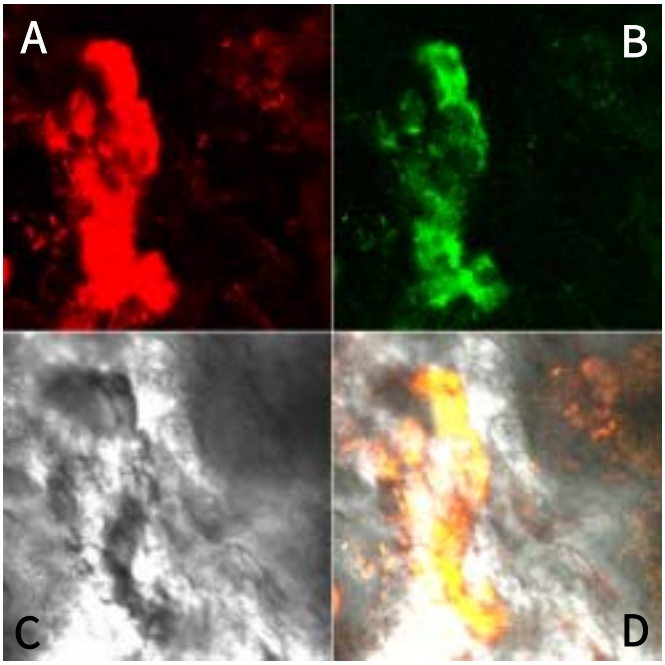

Fig. 36. The GFP expression in callus of wild-type *A. mongolica*. Confocal images were obtained from a Zeiss LSM510 confocal microscope with red (A), green (B), and transmission (C). Image D is the superimposed image of red and green images. Co-localization areas are displayed with false color yellow in (A and B) are merged in D.

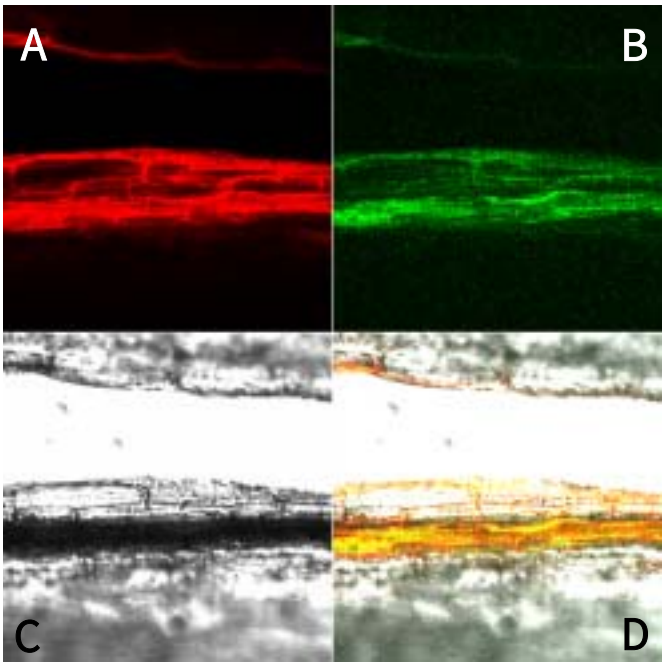

Fig. 37. The GFP expression in lower part of stem of wild-type *A. mongolica* plants. Confocal images were obtained from a Zeiss LSM510 confocal microscope with red (A), green (B), and transmission (C). Image D is the superimposed image of red and green images. Co-localization areas are displayed with false color yellow in (A and B) are merged in D.

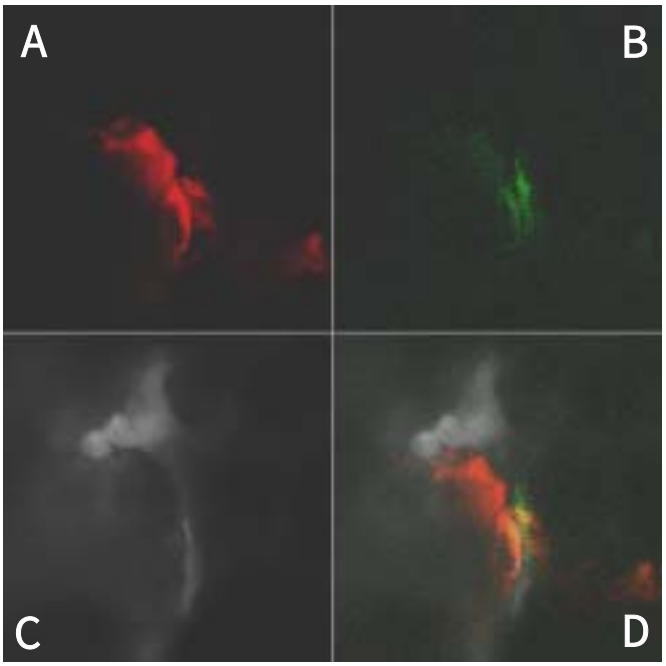

Fig. 38. The GFP expression in hygromycin-resistant callus of *A. mongolica*. Confocal images were obtained from a Zeiss LSM510 confocal microscope with red (A), green (B), and transmission (C). Image D is the superimposed image of red and green images. Co-localization areas are displayed with false color yellow in (A and B) are merged in D.

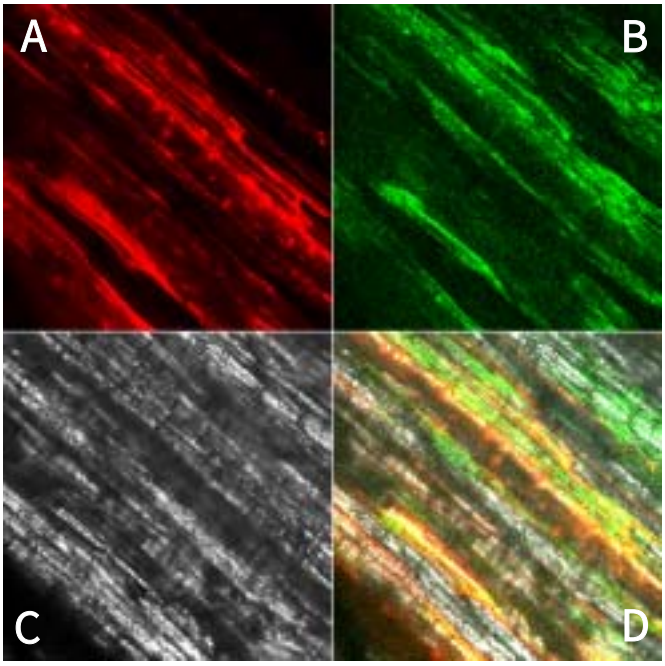

Fig. 39. The GFP expression in lower part of stem of putative transgenic *A. mongolica* plants. Confocal images were obtained from a Zeiss LSM510 confocal microscope with red (A), green (B), and transmission (C). Image D is the superimposed image of red and green images. Co-localization areas are displayed with false color yellow in (A and B) are merged in D.

4.3. Confirmation of *ABF3* gene integration and expression in transgenic plants

The hygromycin-resistant plants were analyzed by PCR and Southern blot analysis to determine and confirm the integration of transgenes. Gel electrophoresis of the PCR amplification products revealed in all cases the presence of a 480-bp *ABF3*, a 1080-bp *hph* and a 966-bp *gus* band of the expected size in the hygromycin-resistant plants and its absence in the wild-type plants (Fig. 40), indicating that the selection was stringent. These plants were further confirmed by Southern blot analysis. A genomic DNA from the five lines of hygromycin-resistant plants and wild-type plants was digested with *Hin*dIII to confirm the transgene integration, and with *Bam*HI to detect the gene copy number, and then hybridized with the ABF3 probes, respectively. The wild-type plants showed no bands (Fig. 41A and B, WT lanes), whereas all the resistant plants showed expected bands at 2 kb (Fig. 41A, lanes T1-5) indicating the gene was successfully integrated into the genome of transgenic plants. Since the T-DNA of pCUMB has a single *Bam*HI site (Fig. 14), the numbers of hybridizing band reflects the number of T-DNA integration unless repeats have been integrated. The copy number of integrated *ABF3* gene was one in the transgenic plant lines T1 and T3 (Fig. 41B, lane 1 and 3), and two in the transgenic plant lines T2, T4 and T5 (Fig. 41B, lane 2, 4, and 5), respectively. In order to confirm the expression of *ubi::ABF3*, RT-PCR and Northern blot analysis with the hygromycin-resistant plants were performed. RT-PCR analysis demonstrated that

expression levels of ABF3 gene were similar in five independent transgenic lines (Fig. 42, lanes 2-6), whereas it was not detected in wild-type plant (Fig. 42, WT lane). Thus, ABF3 gene expression also was not detected in wild-type plant, while it was detected at high levels in transgenic lines T1, T2, and T4 and at lower levels in transgenic lines T3 and T5 (Fig. 43A). Therefore, T3 and T4 were chosen as representative lines of lower and higher expression level, respectively, and they were used for the stress test.

4.4. Tolerances of transgenic plants to heat stress test under water deficit condition

The stress test was carried out in two steps. First, water deficit test, the 3-year-old transgenic and wild-type plants were withheld from water supply for 20 days. At the end of 20 days of water deficit treatment, transgenic plants kept about 3-4 fold higher water content compared with the wild-type plants (Fig. 43B). Consistent with this result, the stomata of the transgenic plants also opened smaller than did the wild-type plants (Fig. 43C). Thus, it can be postulated that the overexpression of ABF3 was resulted in enhanced stomatal closure and water deficit tolerance. After the water deficit treatment, 47% of wild-type, 85.5% of T3 and 93.2% of T4 transgenic plants were survived (Fig. 43D). Second, already stressed plants were treated under heat stress condition. After the test 65.7% of T3 and 75.9% of T4 transgenic plants, but only 23.2% of the wild-type plants were survived (Fig. 43D). The result indicates that the transgenic plants are

approximately 2-3 fold more tolerant to heat stress under water deficit condition than wild-type plants without affecting plant growth (Fig. 43E).

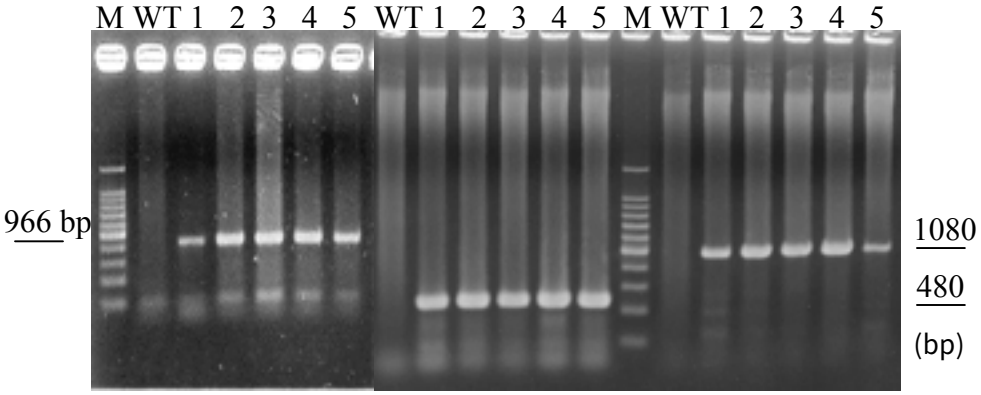

Fig. 40. PCR products amplified with GUS, ABF3, and hygromycin gene primers. M: DNA marker, WT: wild-type plants, Lines (T1-5): putative transgenic *A. mongolica* lines.

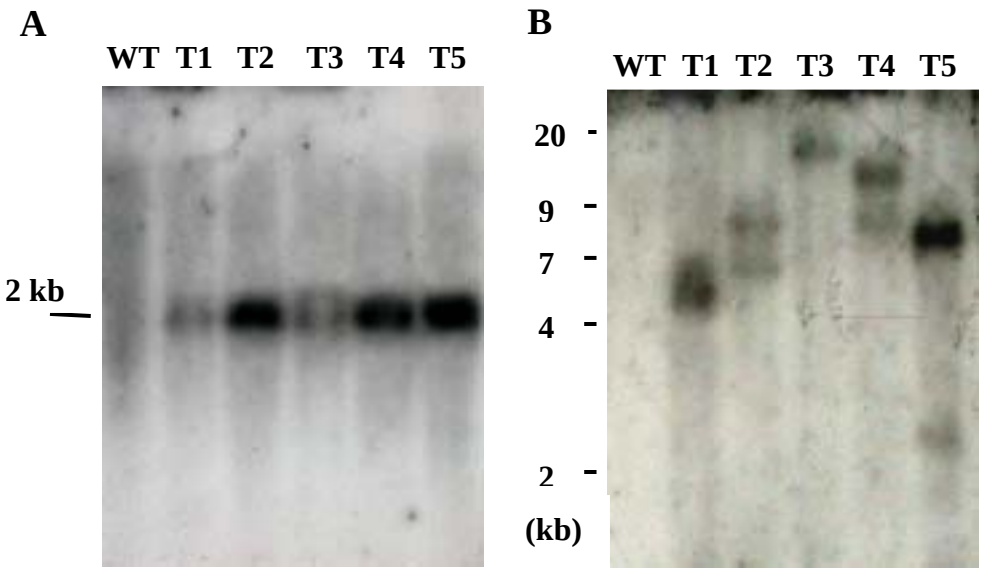

Fig. 41. Southern blot analysis of wild-type and transgenic *A. mongolica* plant lines T1, T2, T3, T4, and T5. About 25 μg of *Hin*dIII (A) or *Bam*HI (B)-digested genomic DNA samples were probed with ^{32}P labeled ABF3 probes.

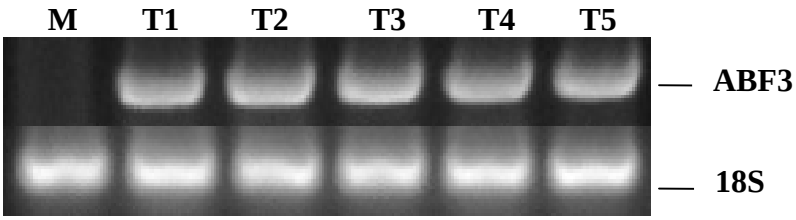

Fig. 42. RT-PCR analysis of *ABF3* gene expression in wild-type (WT) and five independent transgenic *A. mongolica* plant lines (T1-5).

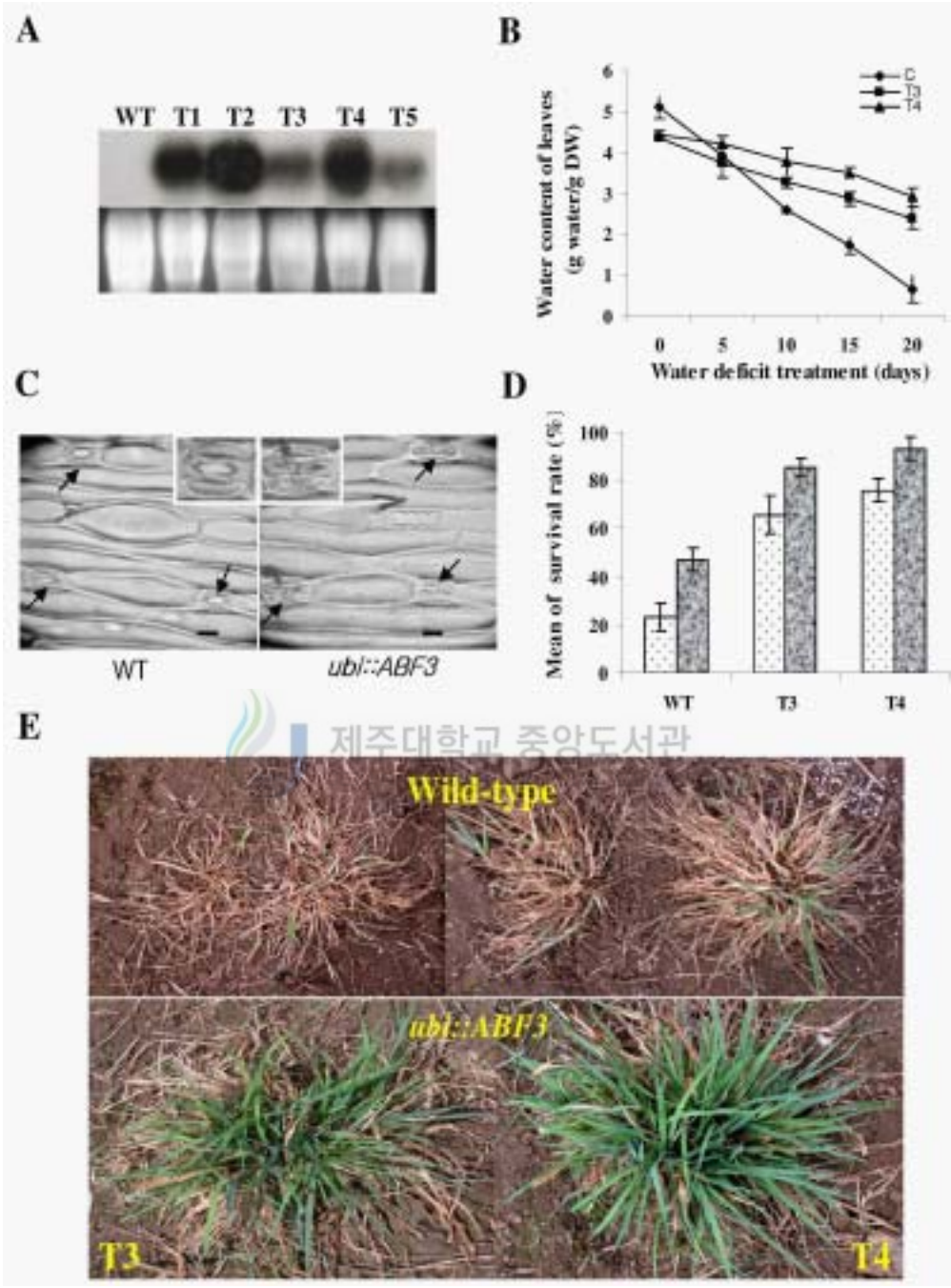

Fig. 43. Heat stress test under water deficit condition in wild-type and *ubi::ABF3* transgenic *A. mongolica*. A: Northern blot analysis of wild-type

and transgenic plant lines T1, T2, T3, T4, and T5. About 30 μg of total RNA samples were probed with ^{32}P labeled ABF3 probe. The bottom panel shows ethidium bromide staining of the RNA gel. B: Water content of leaves of wild-type and transgenic plant lines T3 and T4 was measured at 0, 5, 10, 15, and 20 days after water deficit treatment. The values indicate the means of five individual leaves. Bars refer to standard error. C: Stomatal aperture of wild-type and transgenic plant line T4. Stomatal guard cells were observed at 10 days of water deficit treatment. Arrows indicate guard cells, and the inserts show representative stomata (bars=0.01 mm). D: Mean of survival rate of wild-type and transgenic plant lines T3 and T4 after water deficit test (gray column) and heat test under water deficit condition (spotted column). Data were collected after 14 days of recovering under normal conditions with irrigating, respectively. Columns indicate the mean of three replicates. Bars refer the standard error. E: The plants after heat test under water deficit condition.

5. DISCUSSION

According to the previous studies in *Agrobacterium*-mediated transformation of bentgrass, transformation efficiencies of 7.4% (Yu et al., 2000), 36.7% (Chai et al., 2004), 36.2% (Luo et al., 2004) and 19.4% (Han et al., 2005) have been reported. In this study, 49.2% of calli showed hygromycin resistance, however only 23.4% of them regenerated green shoots. The higher transformation efficiency obtained in this study was probably caused by the application of highly regenerable and reproducible callus cultured from $CaCl_2$ free medium. In the previous studies, gene escape from hygromycin selection has reported. For instance, only 12 of 62 of the plants that have been survived at the concentration of 75 mg/L hygromycin for selection were showed positive in the PCR and Southern blot analysis, suggesting that the selection pressure of 75 mg/L hygromycin was not seem to be enough for the selection of true transformants (Han et al., 2005). Moreover, Xiao and Ha (1997) reported that they obtained 19 of 21 putative transformants during selection of creeping bentgrass transformants on the medium containing 200 mg/L of hygromycin following particle bombardment. But in this study, we did not find any gene escape even on 50 mg/L hygromycin-containing medium. Based on these results, it seems that there are different responses of calli in hygromycin concentrations. We agree with Han et al. (2005) that it was due to the different physiological status

between the calli obtained through different methods, and besides calli grown from seeds will also be of different genotype.

The analysis of the gene transmission to the progeny was impossible because all the plants, including wild-type plants were highly sterile. Because *A. mongolica* requires a field vernalization period for flowering, they did not flower during over the 3 years growth in the open field in subtropical Jeju Island. As many turfgrasses, *A. mongolica* can grow with vegetative propagation, and the sterility itself does not preclude their use. Furthermore, the sterility could be a great advantage, because it prevents transgene escape through cross-pollination with nontransgenic plants, and it also decreases environmental concerns due to potential transfer of genes through intra- and interspecific hybridization since *A. mongolica* is a self-sterile, wind pollinated, outcrossing species (Belanger et al., 2003). Therefore, we think *A. mongolica* is an ideal candidate for the genetic modification using valuable genes in Korea.

The loss of plant productivity caused by high temperature is often serious when combined with the other environmental stresses such as water deficit. Plant hormone abscisic acid (ABA) is essential for the adaptive responses of plants to water and heat stresses. We have shown here that the overexpression of *ubi::ABF3* in transgenic bentgrass enhances tolerance to heat stress under water deficit condition without affecting plant growth. The result demonstrates that *ABF3* gene, which is from *Arabidopsis*, is functional in monocotyledonous plant *Agrostis mongolica* Roshev. Therefore, we

concluded that *ABF3* gene can be used to improve multiple stress tolerances in many important crops.

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