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Establishment of an efficient alfalfa regeneration system via organogenesis and the co-expression of *Arabidopsis SOS* genes improves salt tolerance in transgenic alfalfa (*Medicago sativa* L.)

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Abstract

The Salt Overly Sensitive (SOS) signal transduction pathway is pivotal in Na⁺ efflux and facilitates ion transport and homeostasis for improved salt tolerance in plants. Ten alfalfa varieties were used as experimental materials, and two alfalfa regeneration systems were established and optimized. Cotyledons and hypocotyls were initially used as explants to induce embryogenic callus via the indirect production of somatic embryos in order to establish a callus acceptor system. Cotyledonary nodes were used as explants to induce adventitious bud formation via direct organogenesis, thereby establishing an *in vitro* regeneration system that could be used for the genetic transformation. *Agrobacterium*-mediated transformation of the cotyledonary nodes of the alfalfa cv. “Golden Empress b” was used to generate 25 independent sources of transformed plants exhibiting herbicide tolerance. Four of the positive transgenic plants were randomly selected for southern blot analysis, and three hybridization signals with one or two copies were detected. Reverse transcription PCR showed that the Bialaphos resistance (*Bar*) and *SOS1* genes were expressed in the transgenic plants and that multiple exogenous salt-tolerant genes were integrated into the transgenic plant genome and expressed at the transcriptional level. The overexpression of *Arabidopsis SOS* genes in alfalfa conferred a high degree of salinity tolerance, enhanced plant growth, a lowered accumulation of Na⁺, increased accumulation of K⁺ in the leaves, and altered physiological and biochemical parameters in response to salt stress.

Keywords alfalfa; *in vitro* organogenesis; regeneration system; Salt Overly Sensitive (SOS); transformation; salt tolerance;

Introduction

Alfalfa (*Medicago sativa* L.) is the most widely grown forage legume worldwide and represents about 2.5 % of the total agricultural hectareage (Osborn et al. 1997). It is a perennial herbaceous plant with a developed root system, and the long-term planting of alfalfa can improve saline soil, prevent soil erosion, and increase soil fertility, thereby promoting ecological sustainability. However, salt stress of 50–200 mM NaCl has been reported to restrict the growth and productivity of alfalfa (Bekki et al. 1987). The selection and breeding of stress-resistant cultivars using traditional breeding methods is challenging. Transgenic and molecular marker-assisted selection methods offer an effective alternative means of improving salt resistance and generating resistant plant strains.

The salt-tolerance traits of plants are quantitative characteristics because they are regulated by a suite of genes and are influenced by the environment (Gao 2000). Conventional plant breeding is time-consuming and the traits are difficult to introgressed, as gene transfer can only occur across the whole genome of the individual and single genes cannot be selected and transferred accurately, and therefore the performance of the offspring cannot be predicted. Moreover, as high saline-alkali tolerance germplasm resources are scarce, the progression in the improvement of salinity tolerance is often slow. Transgenic technology improves specific traits by selecting and transferring genes with distinct functions into an organism, resulting in the accurate prediction of offspring performance. By using genetic engineering tools to breed leguminous forage grass with salt-tolerant characteristics, the genes of wild salt-tolerant resources can be introduced into cultivated varieties, resulting in new transgenic varieties with high salt tolerance (Jin et al. 2010; Liu 2012; Wang et al. 2012).

Abiotic stress factors, such as salt, alkali, drought, and cold, negatively impact plant growth, development, and survival, resulting in reduced yields (Yamaguchi-Shinozaki et al. 2002; Huai et al. 2009; Jin et al. 2010). Saline-alkali soil is generally derived from high sodium ion accumulation, and leads to osmotic stress, water deficit, enzyme inactivation, and even cell death (Zhu 2000). Soil salinization and alkalization are serious abiotic adversities, and also constitute cosmopolitan issues that impact the ecological and agricultural environment.

The Salt Overly Sensitive (*SOS*) signal transduction pathway is pivotal for discharging Na⁺ and maintaining the ionic balance on a cellular level, and confers improved salt tolerance in plants (Zhu

2003). The *SOS* pathway comprises *SOS1*, *SOS2*, and *SOS3*; three key protein components in *Arabidopsis thaliana* (Zhu 2003). The *AtSOS1* gene (6,076 bp) is a cell membrane Na^+/H^+ antiporter that mediates Na^+ efflux at the root epidermis and long-distance transfer from the belowground parts to the aboveground parts while protecting individual cells from Na^+ toxicity (Shi et al. 2000). The *AtSOS2* gene (5,144 bp) encodes a serine/threonine protein kinase that is necessary for endocellular Na^+ and K^+ homeostasis (Liu et al. 2000). The *AtSOS3* gene (2,298 bp), the first key gene associated with plant salt resistance to be cloned, replies to the Ca^{2+} signal by either activating protein phosphatases or inhibiting genes that modulate calcium ions and the sodium ion transfer system (Liu and Zhu 1998). The *SCaBP8/CBL10* (*SOS3*-like calcium binding protein 8/calcineurin B-like 10) gene (2,493 bp) encodes calcineurin B-like proteins belonging to Ca^{2+} -sensor protein families, and is a type of *SOS2* active regulator that operates mainly in *A. thaliana* buds and functions as a sensor relay (Chen et al. 2012).

Plant intracellular Ca^{2+} concentration increases under Na^+ stress and can be sensed by the calcium binding protein *SOS3*, which is located in the cytoplasm. *SOS3* activates *SOS2* kinase and forms *SOS2/SOS3* kinase complexes to regulate K^+ and Na^+ stability. *SCaBP8* is highly sensitive to salt in bud tissues and, together with *SOS3*, regulates the activity of *SOS2* (Du et al. 2011). Following phosphorylation, the *SOS2/SOS3* kinase complexes specifically regulate plasmalemma *SOS1*, maintains K^+ and Na^+ ion balance inside and outside the cell, and improves plant salt resistance (Halfter et al. 2000).

It has previously been demonstrated that alfalfa seedlings genetically modified with *GmDREB1*, a gene associated with improved stress tolerance in plants, exhibit greater salt tolerance (200 mM NaCl) than the non-genetically modified controls (Jin et al. 2010). The overexpression of the *Arabidopsis DREB* gene was found to confer enhanced salt tolerance to transformed alfalfa (Bi 2010). Many studies have investigated the influence of single genes on salt tolerance (Shi et al. 2003; Huai et al. 2009; Jin et al. 2010; Bi 2010; Li et al. 2011), but few have reported on multiple genes. Previously, the *SOS1*, *SOS2-SOS3*, and *SOS1-SOS2-SOS3* genes from the dicotyledonous *Arabidopsis* were transferred into the alfalfa cultivar “Zhongmu No.1”, resulting in transgenic plants that exhibited higher salt tolerance than the controls (Mao 2009). Furthermore, the over-expression of *A. thaliana SOS1+SOS2+SOS3* conferred enhanced salt tolerance in tall fescue (Ma et al. 2014). The co-expression of the *Suaeda corniculata* tonoplast Na^+/H^+ antiporter gene *ScNHX1* and *S. corniculata* tonoplast H^+ -PPase gene

ScVP improved salt and alkali resistance in transgenic alfalfa (Liu et al. 2013). Salt tolerance in higher plants is a quantitative trait that is regulated by polygenes and many signaling pathways, and a complex interaction system exists between the genes and the signaling pathways (Gao 2000).

Our laboratory previously reported that the overexpression of *A. thaliana* *SOS1+SOS2+SOS3* enhances salt tolerance in tall fescue (Ma et al. 2014). Treatment with 350 mM NaCl resulted in the transgenic tall fescue exhibiting healthy growth and lower accumulation of Na⁺ and higher accumulation of K⁺ in the root system. Physiological and biochemical enzyme activity was notably increased in the transgenic plants, and the malondialdehyde (MDA) content was reduced compared to the wild-type. In this study, we compared several factors affecting transgenic efficiency, including explant type, plant culture medium, and hormonal regulation. We established two alfalfa regeneration systems and aimed to develop an effective *in vitro* regeneration system that could be used for the genetic transformation of salt sensitive genes in alfalfa. Furthermore, the *A. thaliana* *SOS1+SOS2+SOS3+SCaBP8* genes were successfully introduced into the alfalfa genome, conferring enhanced salt tolerance to the alfalfa.

Materials and methods

Plant materials

Mature alfalfa (*Medicago sativa* L.) seeds of 10 different cultivars, including the American cultivars “Jacklin”, “Golden Empress”, “Golden Empress b”, “Queen 2000”, and “Sanditi”, the Canadian cultivar “Algonquin”, the German cultivar “Plato”, and the Chinese cultivars “Zhongmu No.1”, “Aohan”, and “Longdong”, were kindly provided by Dr. Ma (Ningxia University Grass Research Institute, Ningxia, China). The seeds of the 10 cultivars were surface-sterilized in 75% ethyl alcohol for 1 min and in 0.1% mercuric chloride solution for 10 min, and then washed with sterilized water five times. After surface disinfection, the seeds were germinated on Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 30 g·L⁻¹ sucrose and 8 g·L⁻¹ agar (pH 5.8) for germination and grown in the light for 7 d at 25°C (Ma et al. 2014). After 7 d, the cotyledons, hypocotyls, and cotyledonary nodes of the aseptic alfalfa seedlings were collected as explants. The cotyledonary node explants were ultimately selected for genetic transformation (Hinchee et al. 1988).

Callus regeneration system

Callus induction

The cotyledons, leaves, petioles, hypocotyls (2–3 mm), roots, and seeds were induced to generate embryonic calli on sterilized callus induction medium supplemented with different concentrations of auxins and cytokinins, including 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (6-BA), and kinetin (KT), with 30 g·L⁻¹sucrose and 8 g·L⁻¹agar at pH 5.8 in sterilized Petri dishes (autoclaved at 0.1 Mpa and 121°C for 20 min) under dark conditions for 2 weeks at 25 ± 1°C. The medium lacking hormones was set as the control group. The callus induction medium was supplemented with different concentrations of 2,4-D (0.0 mg·L⁻¹, 1.0 mg·L⁻¹, 2.0 mg·L⁻¹, 3.0 mg·L⁻¹, 4.0 mg·L⁻¹, 5.0 mg·L⁻¹, 6.0 mg·L⁻¹, and 7.0 mg·L⁻¹), 6-BA (0.0 mg·L⁻¹, 1.0 mg·L⁻¹, 2.0 mg·L⁻¹ and 3.0 mg·L⁻¹), and KT (0.0 mg·L⁻¹, 0.1 mg·L⁻¹, 0.15 mg·L⁻¹, 0.2 mg·L⁻¹, and 0.25 mg·L⁻¹). The callus induction media consisted of 57 different hormone levels (Table 1). Each medium consisted of three repeated experimental treatments, and each treatment was performed with 50 explants. The subcultivation lasted for 15 d and was repeated twice. After 30 d, the callus induction rate was calculated as follows: callus induction rate (%) = number of calli induced in explants/ total inoculated explants number × 100%.

Callus differentiation

After subculturing twice, bright yellow and compact embryogenic calli were placed onto MS callus differential culture medium supplemented with different concentrations of cytokinins, including 6-BA, KT, and 1-naphthaleneacetic acid (NAA), with 30 g·L⁻¹sucrose and 8 g·L⁻¹agar at pH 5.8 for callus differentiation in the light for 2 weeks at 25 ± 1°C. Medium lacking any hormones was set as the control group. For the treatment groups, the 6-BA concentrations were 0.0 mg·L⁻¹, 0.3 mg·L⁻¹, 0.5 mg·L⁻¹, and 1.0 mg·L⁻¹; the KT concentrations were 0.0 mg·L⁻¹, 1.0 mg·L⁻¹, 1.5 mg·L⁻¹, 2.0 mg·L⁻¹, 2.5 mg·L⁻¹, and 3.0 mg·L⁻¹; and the NAA concentrations were 0.0 mg·L⁻¹, 0.05 mg·L⁻¹, 0.1 mg·L⁻¹, 0.15 mg·L⁻¹, and 0.2 mg·L⁻¹. The callus differentiation media consisted of 31 different hormone levels (Table 3). Each medium consisted of three repetitive experimental treatments, and each treatment included 50 explants. The subcultivation lasted for 20 d, and was repeated twice. After 40 d, the callus differentiation rate was calculated as: callus differentiation rate (%) = callus number of differentiated

buds / total inoculated callus number $\times 100\%$.

Rooting of regenerated plants

Well-grown differentiated shoots approximately 2 cm in height were harvested from the calli and placed on $\frac{1}{2}$ MS rooting medium supplemented with $1.0 \text{ mg}\cdot\text{L}^{-1}$ indole-3-butyric acid (IBA), $20 \text{ g}\cdot\text{L}^{-1}$ sucrose, and $8 \text{ g}\cdot\text{L}^{-1}$ agar (pH 5.8), and maintained in the light for 2 weeks at $25 \pm 1^\circ\text{C}$.

In vitro organ regeneration system

Adventitious bud induction

The cotyledonary nodes of 7–8-d-old aseptic seedlings grown on the seed germination medium were selected as explants. The two cotyledons were not separated, but two-thirds of the two cotyledon pieces were removed and 2 mm of hypocotyl was kept. The cotyledonary nodes were incubated on adventitious bud induction medium supplemented with different concentrations ($0.5 \text{ mg}\cdot\text{L}^{-1}$, $1.0 \text{ mg}\cdot\text{L}^{-1}$, $1.5 \text{ mg}\cdot\text{L}^{-1}$, and $2.0 \text{ mg}\cdot\text{L}^{-1}$) of 6-BA with $30 \text{ g}\cdot\text{L}^{-1}$ sucrose and $8 \text{ g}\cdot\text{L}^{-1}$ agar at pH 5.8 for adventitious bud induction in the light for 2 weeks at $25 \pm 1^\circ\text{C}$. The cotyledonary node explants were selected for genetic transformation (Hinchee et al. 1988). Each treatment was assessed in triplicate, and each treatment contained 100 inoculated explants (Table 4). After 10 d, the budding rate was calculated as budding rate (%) = No. of budding explants / No. of inoculated explants $\times 100\%$.

Adventitious bud elongation

After being cultured on the adventitious bud induction medium for about 15 d, the cotyledonary nodes appeared as multiple shoot clumps. They were then transferred into shoot elongation medium supplemented with different concentrations ($0.5 \text{ mg}\cdot\text{L}^{-1}$, $1.0 \text{ mg}\cdot\text{L}^{-1}$, $1.5 \text{ mg}\cdot\text{L}^{-1}$, and $2.0 \text{ mg}\cdot\text{L}^{-1}$) of 6-BA, with $30 \text{ g}\cdot\text{L}^{-1}$ sucrose and $8 \text{ g}\cdot\text{L}^{-1}$ agar at pH 5.8 where they continued elongating in the light for 2 weeks at $25 \pm 1^\circ\text{C}$ (Table 4). After 15 d, the budding differentiation rate was calculated as: budding differentiation rate (%) = No. of budding explants / No. of inoculated explants $\times 100\%$.

Adventitious bud radication

When the adventitious buds had reached 2–3 cm, healthy adventitious buds were cut from the base of the explants and placed on adventitious bud radication medium supplemented with different

concentrations ($0.5 \text{ mg}\cdot\text{L}^{-1}$, $1.0 \text{ mg}\cdot\text{L}^{-1}$, $1.5 \text{ mg}\cdot\text{L}^{-1}$, and $2.0 \text{ mg}\cdot\text{L}^{-1}$) of IBA, $30 \text{ g}\cdot\text{L}^{-1}$ sucrose, and $8 \text{ g}\cdot\text{L}^{-1}$ agar at pH 5.8. Rooting was allowed to continue in the light for 2 weeks at $25 \pm 1^\circ\text{C}$ (Table 4). After 15 d, the adventitious bud rooting rate was calculated as follows: rooting rate of adventitious buds (%) = No. of adventitious bud roots/total number of inoculated adventitious buds $\times 100\%$.

Transplantation of the regenerated plantlets

After 15 d of culturing on the rooting medium, the regenerated plantlets had produced a large number of adventitious roots. The bottle cap was removed for 2–3 d and distilled water was applied each day to irrigate the seedlings. The aseptic regenerated seedlings were removed with forceps and then rinsed with water and placed into sterile pots (soil: nutrient soil = 1:1) and cultivated at $25 \pm 1^\circ\text{C}$ under a 16-h light cycle in the greenhouse.

Plasmid construction

The recombinant plant multi-gene binary expression vector pSOS (kindly provided by Dr Yan Guo, State Key Laboratory of Plant Biochemistry and Molecular Biology, China Agricultural University, Beijing, China) contained the *SOS1* (700 bp), *SOS2* (550 bp), *SOS3* (683 bp), and *SCaBP8* (460 bp) genes, and the stress-inducible *Rd29A* promoter (Quan et al. 2007; Yang et al. 2009) (Fig. 1). pSOS also carried a Bialaphos resistance (*Bar*) gene (463 bp) as a selectable marker, which was introduced into the GV3101 strain using a conductivity meter (Berres et al. 1992). TM1, TM2, and TM220 are matrix attachment region sequences of tobacco, and were used to help overcome transgene silencing and improve the transgene expression level (Allen et al. 2000; Xue et al. 2005).

Alfalfa genetic transformation and regeneration

The cotyledonary nodes of the aseptic alfalfa seedlings were collected as explants and infected with a cell suspension of *A. tumefaciens* strain GV3101 with an A_{600} of 0.6 for 20 min. Forceps were used to place the infected explants into Petri dishes that had been lined with several sheets of sterile filter paper to absorb any excess water. They were then transferred onto co-culture medium (MS medium supplemented with $30 \text{ g}\cdot\text{L}^{-1}$ sucrose and $8 \text{ g}\cdot\text{L}^{-1}$ agar, pH 5.8) and maintained in the dark at 25°C for 3 d. The co-cultivated explants were inoculated onto adventitious bud induction and selection

medium (MS medium supplemented with 1.0 mg·L⁻¹ 6-BA, 500 mg·L⁻¹ cefotaxime, 20 mg·L⁻¹ hygromycin, 30 g·L⁻¹ sucrose, and 8 g·L⁻¹ agar, pH 5.8) and cultured in the light for 2 weeks at 25 ± 1°C. Subsequently, the formed *hyg*-resistant adventitious buds were transferred onto adventitious bud elongation medium (MS medium plus 0.5 mg·L⁻¹ 6-BA, 300 mg·L⁻¹ cefotaxime, 20 mg·L⁻¹ hygromycin, 30 g·L⁻¹ sucrose, and 8 g·L⁻¹ agar, pH 5.8) until the buds had reached approximately 3–4 cm. After 15 d, the obtained *hyg*-resistant adventitious buds were rooted onto the adventitious bud radication medium (½ MS medium plus 1.0 mg·L⁻¹ IBA, 300 mg·L⁻¹ cefotaxime, 20 mg·L⁻¹ hygromycin, 20 g·L⁻¹ sucrose, and 7 g·L⁻¹ agar, pH 5.8). Finally, the regenerated plants were then transplanted into potted soil and placed in the greenhouse where they were watered and allowed to grow for 14 d under natural illumination at 25 ± 1°C.

PCR analysis

Genomic DNA from the transgenic plants and un-transformed plants (UT/WT) was isolated using a genomic DNA extraction kit (AxyPrep, China). The plasmid was used as a positive control, and the DNA isolated from UT plants was used as a negative control. The PCR reaction mixture system (50 µL) was as follows: 2×*EcoTaq* PCR SuperMix 25 µL, PI (10 µmol/L) 2.0 µL, PII (10 µmol/L) 2.0 µL, template DNA 2.0 µL, and ddH₂O 19 µL. The PCR reactions were run for 30 cycles of pre-denaturation at 94.0°C for 5 min, denaturation at 94.0°C for 30 s, annealing at 52.0–59.0°C for 30 s, and elongation at 72.0°C for 1 min, followed by a final extension of 72.0°C for 5 min. The PCR amplification products were separated on a 1.0% agarose gel (Table 5).

Southern blot analysis

About 10 µg genomic DNA was extracted from the UT plants and PCR positive plants and digested with *EcoRI* and electrophoresed on 1% agarose gel, following which the gel was transferred to a Hybond N⁺ nylon membrane. The nylon membrane was hybridized with a 463 bp *Bar* gene fragment as a probe, which was labeled using the DIG kit (Roche, Switzerland). The transgenic plant gene copy number was tested by non-radioactive kit.

Reverse transcription (RT)-PCR analysis

The UT and transgenic plants were treated with 300 mM NaCl for 7 d, and the soil at the roots of these plants was watered with a prepared NaCl solution daily. The total RNA of four biological replicates of the UT and transgenic lines was extracted using a TRIzol kit (Invitrogen, USA) according to the manufacturer's instructions. First-strand cDNA synthesis was synthesized with 2 µg total RNA using cDNA Synthesis Supermix (Transgen, China). *Bar* and *SOS1* gene expression was amplified using RT-PCR. Amplified fragments were separated on 1% agarose gel electrophoresis.

Salt tolerance identification analysis

Phenotypic determination

The WT and T₀ transgenic alfalfa plants were cultivated in flower pots containing a perlite, vermiculite, and soil mixture (1:1:1) in a phytotron. The plants were treated with 0, 100, 200, and 300 mM NaCl solution for 7 d, respectively. The height, leaf area, and fresh weight of all plants were measured after treatment with 0 mM and 300 mM NaCl solution, and each treatment was measured in triplicate.

Changes in antioxidant enzyme activity, chlorophyll, proline, MDA, and ion content

Leaves were collected from the WT and T₀ transgenic lines (P1) after 0 mM and 300 mM NaCl salt stress treatment. Chlorophyll content was determined using a TYS-A handheld chlorophyll meter (Hinotek, Ningbo, China). Superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) activities and proline and MDA content were measured using an assay kit according to the manufacturer's instructions (Solarbio, Shanghai, China). Ion content was measured by atomic absorption spectrophotometry according to the method of Xu et al. (2006). The physiological and biochemical data measurements were repeated three times.

Statistical Analyses

Analysis of variance (ANOVA) was performed in SPSS (SPSS Statistics 19.0, Inc. Chicago, IL, USA) to assess the differences in rates of callus induction, callus differentiation, rooting of regenerated plants, adventitious bud induction, adventitious bud differentiation, and adventitious bud radication between the different treatments. Values within a row followed by lowercase letters are significantly different at $P < 0.05$. Values within a row followed by capital letters are significantly different at

$P < 0.01$.

Results

Establishment of two regeneration systems

Germination rates of the different varieties

Ten alfalfa seed cultivars were germinated on MS medium (Fig. 2), and the germination rate under light conditions was higher than under dark conditions. Under light, the seeds mainly grew cotyledons, while in the dark they primarily grew buds. The germination rates of the seeds under light conditions were ranked as follows from high to low: “Sanditi”, “Golden Empress b”, “Jacklin”, “Plato”, “Algonquin”, “Golden Empress”, “Queen 2000”, “Zhongmu No.1”, “Aohan”, “Longdong” (Fig. 3). The low germination rate of some of the seeds may be attributed to genotypic differences.

Callus induction rate of different explants in the callus regeneration system

The cotyledons, leaves, petioles, hypocotyls, roots, and seeds of the 10 varieties of aseptic seedlings were selected as the explants to induce callus formation on callus induction medium (CIM). The calli induced by the cotyledons and leaves were yellow/green in color with a rough surface, while the calli developed by the petioles and hypocotyls were cream/white in color and with a smooth surface. The root and seed explants of the aseptic seedlings did not form calli, so the root and seed induction rate of the 10 varieties of aseptic seedlings were 0 %. The callus induction rate of the hypocotyl explants was relatively higher than the cotyledons, leaves, and petioles. The hypocotyls exhibited strong differentiation, and the callus induction rate of the hypocotyls of “Jacklin” was the highest, reaching 100 % (Fig. 4).

Effect of different media and hormone concentration ratios on callus induction

Effect of different 2, 4-D concentration on callus induction

Hypocotyls from the different varieties were inoculated onto the callus induction medium supplemented with different concentrations ($1.0 \text{ mg}\cdot\text{L}^{-1}$, $2.0 \text{ mg}\cdot\text{L}^{-1}$, $3.0 \text{ mg}\cdot\text{L}^{-1}$, $4.0 \text{ mg}\cdot\text{L}^{-1}$, $5.0 \text{ mg}\cdot\text{L}^{-1}$, $6.0 \text{ mg}\cdot\text{L}^{-1}$, and $7.0 \text{ mg}\cdot\text{L}^{-1}$) of 2, 4-D. The callus induction rate of the 10 varieties increased initially and then decreased with increasing 2, 4-D concentration. At a 2,4-D concentration of $2.0 \text{ mg}\cdot\text{L}^{-1}$, the callus induction rate was at its maximum, and was thus considered to be the optimal

concentration for callus induction (Table 1 and Table 2).

Effect of different types and ratios of cytokinin concentrations on callus induction

Cytokinins are important for callus induction in alfalfa and function together with auxins at the callus induction phase. The callus induction rate was optimal at a 6-BA concentration of $2.0 \text{ mg}\cdot\text{L}^{-1}$. In order to promote callus growth, Uchimiya and Murashige (UM) (1974) vitamin medium was added to the MS medium containing $2.0 \text{ g}\cdot\text{L}^{-1}$ casein acid hydrolysate as the basic culture medium. The callus induction rate was optimal at a KT concentration of $0.25 \text{ mg}\cdot\text{L}^{-1}$ (Table 1). We discovered that following the addition of 6-BA, the calli become loose in texture, had a rough surface, were watery and white in color, and the non-embryonic callus number had increased over the subsequent callus differentiation and growth of the plantlets. Thus, the embryonic callus induction effect of $0.25 \text{ mg}\cdot\text{L}^{-1}$ KT was considered to be better than $2.0 \text{ mg}\cdot\text{L}^{-1}$ 6-BA.

Effect of different hormone concentrations on callus differentiation

After culturing under a 16 h daylight cycle for 30 d, the calli differentiated into green sprouts, fibrous roots, or green shoots. MS medium supplemented with different concentrations and combinations of 6-BA, NAA, and KT significantly affected the frequency of the callus differentiation.

The results indicated that when only 6-BA was added at concentrations ranging between 0.3 and $1.0 \text{ mg}\cdot\text{L}^{-1}$, the callus differentiation rate increased gradually compared with the control group. When the 6-BA concentration exceeded $1.0 \text{ mg}\cdot\text{L}^{-1}$, the callus differentiation rate began to decline and the calli started browning (Table 3).

Callus differentiation was promoted when 6-BA was used synergistically with KT. However, callus differentiation was fairly low, and the calli appeared loose in texture, watery, white in color, and struggled to differentiate into green seedlings when 6-BA was used in conjunction with NAA. The results demonstrated that $1.0 \text{ mg}\cdot\text{L}^{-1}$ 6-BA + $0.5 \text{ mg}\cdot\text{L}^{-1}$ KT was optimal for callus differentiation. After 30 d under this condition, the calli had differentiated into green sprouts, and the hypocotyl explants differentiation rate of “Zhongmu No.1” was the highest, reaching 69.75%, while the calli in the other media only differentiated into a few green shoots (Table 3).

In vitro organ regeneration system

Effect of different 6-BA concentrations on adventitious bud induction

The cotyledonary node explants were inoculated into adventitious bud induction medium under aseptic conditions and the number of plants exhibiting more than 2 cm of growth after 15 d were counted. Notably, when the 6-BA concentration was $1.0 \text{ mg} \cdot \text{L}^{-1}$, the budding rate was 75%, which was significantly higher than the budding rates of the other concentrations (Table 4). Thus, the optimal adventitious bud induction medium was determined to be MS+ $1.0 \text{ mg} \cdot \text{L}^{-1}$ 6-BA + 3% sucrose + 0.8% agar at pH 5.8.

Effect of different 6-BA concentrations on adventitious bud elongation

When the concentration of 6-BA reached $1.0 \text{ mg} \cdot \text{L}^{-1}$, the cotyledonary node explants appeared weak and broke easily, grew slowly, and started to vitrify. The bud differentiation rate of the cotyledonary nodes on adventitious bud elongation medium supplemented with $0.5 \text{ mg} \cdot \text{L}^{-1}$ 6-BA was comparatively higher (Table 4). The adventitious buds grew robustly under this 6-BA concentration and did not display vitrification.

Effect of different IBA concentrations on adventitious bud rooting

The adventitious shoots were able to root and regenerate plants on the MS medium lacking hormones. In order to improve the rooting rate and reduce the rooting time, the divergent and elongated buds were transferred into $\frac{1}{2}$ MS medium with different concentrations of IBA. Our findings indicated that when the IBA concentration was $1.0 \text{ mg} \cdot \text{L}^{-1}$, the rooting rate increased by up to 90%, the rooting time shortened, and numerous fibrous roots could be easily generated (Fig. 5, Table 4). When the IBA concentration was $2.0 \text{ mg} \cdot \text{L}^{-1}$, adventitious shoots also took root, but displayed delayed rooting time and an obviously lower rooting rate (70%). MS medium supplemented with $1.0 \text{ mg} \cdot \text{L}^{-1}$ IBA thus improved the rooting rate of the regenerating plants.

Two types of alfalfa regeneration systems were established and optimized. Firstly, the cotyledons were used as explants to induce embryogenic callus formation via indirect somatic embryogenesis to establish a callus regeneration system (Fig. 6). Secondly, the cotyledonary nodes were used as explants to induce adventitious bud formation via direct organogenesis to establish an *in vitro* regeneration system (Fig. 7).

After an extensive genotype screening, the commonly cultivated variety “Golden Empress b” was

selected as the experimental material for the transformation. Following pretreatment, the mature seeds developed into aseptic seedlings on the MS medium in 7 d (Fig. 7a). The cotyledonary nodes of the aseptic seedlings were explanted and placed into adventitious bud induction medium (Fig. 7b and c). The multiple genes carried by the pSOS vector were integrated into the cotyledonary node explants using *Agrobacterium*-mediated genetic transformation. The infected cotyledonary nodes were co-cultured with *Agrobacterium* for 3 d (Fig. 7d). The adventitious buds were placed into adventitious bud elongation medium containing 5 mg·L⁻¹ glufosinate for 2 weeks (Fig. 7e). Glufosinate-resistant buds continued to grow in the rooting medium (Fig. 7f). After 15 d, the supposed transgenic alfalfa plants developed rapidly and produced well-developed root systems (Fig. 7g). Following this, the transformed alfalfa plants were transplanted into soil in the greenhouse where they were acclimated (Fig. 7h and i).

Molecular analysis of transgenic plants

To confirm whether the foreign multi-genes had integrated into the alfalfa genome, we used PCR with suitable primers for amplifying the DNA sequences of the *Bar*, *SOS1*, *SOS2*, *SOS3*, and *SCaBP8* genes separately. We obtained 25 *Bar*-resistant lines. Six strains (24%) were identified as positive transgenic plants. The PCR data showed that the *SOS1* (700 bp), *SOS2* (550 bp), *SOS3* (683 bp), and *SCaBP8* (460 bp) genes had all integrated into the alfalfa genome (Fig. 8a and d).

The *Bar* gene was integrated into the alfalfa genome and further verified for transgene integration using Southern blot hybridization. Genomic DNA was digested with restriction endonuclease *EcoRI* and hybridized with the *Bar* probe. No hybridization signals were observed in the UT plants. Three of the four tested transgenic lines showed one to two strong hybridization signals (Fig. 8e).

RT-PCR analysis was used to confirm *Bar* gene and *SOS1* gene expression. The expected size of the *Bar* band (463 bp) and *SOS1* band (700 bp) was amplified from the transgenic lines, whereas no band was detected in the non-transformed plants (Fig. 8f).

Enhanced salt tolerance in the transgenic plants

Phenotypic determination

After 7 days, the P1 transgenic lines exhibited increases in plant height, leaf area, and fresh weight in response to salt stress (300 Mm NaCl) relative to WT plants (Fig. 9a-c). These results indicate that

the growth of the transgenic alfalfa under salt stress was superior to WT.

Changes in antioxidant enzyme activity, chlorophyll, proline, MDA, and ion content

The P1 transgenic lines exhibited higher chlorophyll content under the 300 mM NaCl stress treatment (Fig. 10a). In addition, the SOD, POD, and CAT activities and Pro content in all the plants increased under both normal and salt treatment conditions. The activity of the three antioxidant enzymes and the accumulation of osmotic adjustment substances in *Arabidopsis SOS* genes-overexpressing alfalfa increased significantly compared to the WT (Fig. 10b–e). Lipid peroxidation and MDA content were reduced in the P1 transgenic lines, whereas the MDA content increased in WT plants after salt stress (Fig. 10f).

In the presence of 300 mM NaCl, the Na⁺ ion content in the leaves of all the plants increased. However, the P1 transgenic lines accumulated less Na⁺ in the leaves than WT plants (Fig. 11a). The K⁺ content in the leaves of WT plants decreased as the NaCl concentration increased; however, the K⁺ content in the leaves of the P1 transgenic lines increased, and the transgenic lines also contained higher K⁺ contents in the leaves than WT under salt treatment (Fig. 11b).

Discussion

Although both cotyledons and hypocotyls can be used as explants to regenerate complete plants, these explants differ with regards to callus induction and differentiation, as well as the development ability of the regenerated plants. In addition to the findings of this study, previous studies have showed that the hypocotyls constitute the best explant material for alfalfa callus induction (David et al. 1992; Yang et al. 2002). From the experimental results in this study, the regeneration ability of the hypocotyl explants was better than the cotyledons due to their higher rate of callus differentiation, which corroborates several earlier studies (Liu et al. 1993; Shu et al. 1993; Liu 1996).

A previous study found that the callus induction rate of MS salt and Uchimiya and Murashige (1974) vitamin medium was higher than that of other media under different concentrations of various hormones and callus induction medium combination ratios (Jin et al. 2010). Additionally, the 2,4-D concentration was determined to be the primary factor influencing the alfalfa callus induction rate in the aforementioned study. It has been suggested that 2,4-D is essential in alfalfa callus induction on MS medium and that the appropriate concentration is 2.0 mg·L⁻¹ (Wang et al. 2010). We also discovered

that a 2,4-D concentration of $2.0 \text{ mg}\cdot\text{L}^{-1}$ was associated with optimal induction. In fact, the combination of 2,4-D with a low concentration of cytokinin resulted in even better embryonic callus development, particularly when combined with a low concentration of KT. When the hormone concentration was too high, the number of non-embryonic calli increased. The optimal concentration for callus induction was determined to be $0.25 \text{ mg}\cdot\text{L}^{-1}$ KT, corroborating the findings of Wang et al. (Wang et al. 2008). In the past, low differentiation rates have caused "bottlenecks" in the process of callus differentiation and regeneration. We found that the callus differentiation rate was very low in the callus differentiation medium supplemented with 6-BA. In the absence of 6-BA, the calli were loose in texture and poorly formed, but the addition of KT and NAA resulted in a 90% increase in callus differentiation. A combination of auxins and cytokinins improved the induction and differentiation rate more than their single use (Lv and Qu 2004). In a previous study, the addition of 2,4-D and KT resulted in a 54.5% callus differentiation rate in alfalfa (Ana et al. 2006).

In the process of plant *in vitro* organogenesis, explants can be directly regenerated into plantlets (direct organogenesis), which is achieved by varying the hormone concentrations in the media. Alternatively, the callus can be formed from the explant and the organs can then be produced from the callus (indirect organogenesis) (Colby et al. 1991; Huang and Li 1995; Gaba et al. 1999). Cotyledonary node explants can be directly induced from adventitious buds via *in vitro* organogenesis, and the application of different cytokinin concentrations facilitates the elongation and growth of the adventitious buds into plantlets (Zheng et al. 2008; Hua et al. 2009). *In vitro* regeneration systems have the advantages of easy development, a short regeneration cycle, and simple operation (Meurer et al. 1998). Many studies have indicated that a high 6-BA concentration results in browning and even death in explants, as 6-BA stimulates the activity of polyphenol oxidase, and the phenolic substances of the cells oxidize to quinones that are toxic to the explants (Shu et al. 1993; Huang et al. 1995; Lv et al. 2004; Wang et al. 2010). Our results showed that the addition of $1.0 \text{ mg}\cdot\text{L}^{-1}$ 6-BA to the adventitious bud induction medium benefitted adventitious bud formation, while the addition of $0.5 \text{ mg}\cdot\text{L}^{-1}$ 6-BA to the adventitious bud elongation medium improved adventitious bud elongation. Additionally, less vitrification and browning were observed than that at higher 6-BA concentrations in the bud elongation phase (Zheng et al. 2008).

Plant salt tolerance is a quantitative trait that is controlled by multiple genes and signaling pathways that are interrelated. While several studies of alfalfa have focused on single gene qualitative

traits, fewer reports have evaluated multigene quantitative traits. In a previous study, the *SOS1*, *SOS2-SOS3*, and *SOS1-SOS2-SOS3* genes of *A. thaliana* were introduced into wheat, and the resulting transgenic plants exhibited greater salt tolerance than the wild-type (Wang 2008). Similarly, these genes were also introduced into alfalfa “ZhongMu No.1”, and the resulting transgenic strain demonstrated higher salt tolerance than the wild-type (Mao 2009). Quintero et al. (2002) reported that the SOS system is reconstituted and co-expression of *SOS1*, *SOS2*, and *SOS3* confers more salt tolerance than expression of one or two SOS proteins in yeast cells. Yang et al. (2009) tested overexpression of multiple genes to improve salt tolerance in *Arabidopsis*. They produced six different transgenic *Arabidopsis* plants overexpressing *AtNHX1*, *SOS1*, and *SOS3* alone or in different combinations (*AtNHX1+SOS3*, *SOS2+SOS3*, *SOS1+SOS2+SOS3*). Surprisingly, the *AtNHX1* alone did not show significant salt tolerance. In 220 mM NaCl treatment for 3 days, less than 20% of the control and transgenic plants overexpressing only *AtNHX1* survived, but over 80% of the transgenic plants overexpressing *SOS1*, *SOS3*, *SOS2+SOS3*, *AtNHX1+SOS3*, or *SOS1+SOS2+SOS3* survived, and treatment with 220 mM NaCl resulted in the transgenic *A. thaliana* of *AtNHX1+SOS3*, *SOS2+SOS3* and *SOS1+SOS2+SOS3* exhibited healthy growth and greater salt tolerance than the transgenic single *SOS1* gene or *SOS3* gene. The salt tolerance of plants is associated with the ability to extrude Na^+ from the root and maintain a low Na^+/K^+ ratio in the cell. Transgenic plants overexpression *SOS1* (driven by the 35S promoter) apparently reduced Na^+ accumulation (Shi et al. 2003). In tall fescue, the *SOS1+SOS2+SOS3+CBL10*-overexpressing transgenic lines accumulated less Na^+ and more K^+ than the wild-type plants, as well as significantly increased enzyme activities and reduced MDA contents compared to the wild-type under 350 mM NaCl stress treatment (Ma et al. 2014). In our study, we were able to establish an efficient alfalfa regeneration system via organogenesis that could also be used for transformation. The *SOS1+SOS2+SOS3+SCaBP8+Bar* genes were for the first time introduced into the genome of alfalfa cv. “Golden Empress b”. Our findings indicated that the co-expression of *Arabidopsis* SOS genes and the *SCaBP8* gene could maintain Na^+ homeostasis and improve salt tolerance in transgenic alfalfa, and the *Bar* gene could improve the herbicide resistance of the transgenic plants. The SOD, POD, and CAT were the important antioxidation enzymes for decreasing ROS harm and maintaining the integrity of the cellular membrane construction (Li et al. 2011). In our report, the SOD, POD, and CAT activities and Pro content of wild-type were inferior to transgenic alfalfa, but the MDA content was higher than transgenic alfalfa. These results implied that the Na^+

content of cytoplasm in transgenic alfalfa could be kept at a low level and the *SOS* pathway genes and *SCaBP8* gene accelerated a large amount of Na^+ efflux. The co-expression of *SOS* pathway genes and *SCaBP8* gene made the transgenic plants decrease accumulation of Na^+ and mitigate the toxic effects of Na^+ and could efficiently maintain Na^+ homeostasis of cytoplasm and improve salt tolerance of transgenic plants (Ma et al. 2014). Results from the phenotypic traits and contents of Na^+ and K^+ and the physiological traits illustrated that this improvement of salt tolerance in transgenic plants was caused by the functional co-expression of all of these *SOS* pathway genes and *SCaBP8* gene. Our findings should inform the future breeding of salt-resistant alfalfa cultivars.

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Figure Captions

Fig. 1. T-DNA structure schematic diagram of the binary vector pSOS used for the alfalfa transformation. left border of T-DNA, LB; nuclear matrix combination sequence, *TM220*; Bialaphos resistance gene, *Bar*; nuclear matrix combination sequence, *TM2*; inducible promoter, *rd29A*; SOS3-like Calcium binding protein gene, *SCaBP8*; a NOS terminator, NOS; nuclear matrix combination sequence, *TM1*; Salt Overly Sensitive genes, *SOS2*, *SOS3*, *SOS1*; right border of T-DNA, RB; *TM220*. *TM1*, *TM2*, and *TM220* are the MAR sequences.

Fig. 2. Aseptic alfalfa seedlings. (a) Alfalfa seeds germinated on MS medium. (b) Aseptic seedlings.

Fig. 3. The seed germination rate of alfalfa.

Fig. 4. Effect of the different explants of the 10 cultivars on callus induction. (The root and seed induction rate of the 10 varieties of aseptic seedlings were not detected)

Fig. 5. The fibrous roots of the replanted alfalfa.

Fig. 6. The callus regeneration system of alfalfa. (a) Aseptic seedlings. (b) Hypocotyls. (c) Callus. (d) Callus differentiation (e) Green buds. (f, g) Regenerated seedlings. (h) Roots of regenerated plants. (i) Regenerated plants.

Fig. 7. *Agrobacterium*-mediated transformation of alfalfa. (a) Aseptic seedlings. (b) Cotyledonary node. (c) Adventitious bud induction. (d) Co-culture. (e) Adventitious bud elongation. (f) Transgenic plants. (g) Roots of transgenic plants. (h) Transplantation. (i) Transgenic plants in the greenhouse.

Fig. 8. Molecular identification of WT and transgenic alfalfa. (a) PCR amplification of the *SOS1* gene (700 bp). M, DL2000 marker; +, Plasmid as positive control; -, DNA of WT plants as the negative control; 1–6: positive transgenic plants. (b) PCR amplification of the *SOS2* gene (550 bp). (c) PCR amplification of the *SOS3* gene (683 bp). (d) PCR amplification of the *SCaBP8* gene (460 bp). (e)

Southern hybridization of transgenic alfalfa. DNA with *Eco*I and probing with labeled *Bar*. (f) RT-PCR of the *Bar* and *SOS1* gene of WT and transgenic alfalfa. M, DL2000 marker; lanes 1 and 2 represent the *Bar* amplicon from lines P1 and P2; Lane 3 represents the WT; Lanes 4 and 5 represent *SOS1* PCR amplicons from lines P1 and P2.

Fig. 9. Phenotypic determination of WT and transgenic alfalfa at 0 mM and 300 mM NaCl. (a) Plant height. (b) Leaf area. (c) Fresh weight.

Fig. 10. Physiological and biochemical determination of WT and transgenic alfalfa at 0 mM and 300 mM NaCl. (a) Chlorophyll content. (b) SOD activity. (c) POD activity. (d) CAT activity. (e) Proline content. (f) MDA content.

Fig. 11. Na⁺ and K⁺ content in the leaves of WT and transgenic alfalfa at 0 mM and 300 mM NaCl. (a) Na⁺ content. (b) K⁺ content.

Table 1. Effects of different cytokinins on callus induction.

NO	Hormone level	Callus induction rate/%
1	2,4-D 0.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	0.00±0.00
2	2,4-D 1.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	23.75±1.71 ^{fE}
3	2,4-D 1.0mg·L ⁻¹ +6-BA 1.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	33.55±2.98 ^{dD}
4	2,4-D 1.0mg·L ⁻¹ +6-BA 2.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	50.00±0.87 ^{aAB}
5	2,4-D 1.0mg·L ⁻¹ +6-BA 3.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	48.00±1.00 ^{bB}
6	2,4-D 1.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.1 mg·L ⁻¹	24.50±0.66 ^{fE}
7	2,4-D 1.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.15 mg·L ⁻¹	32.00±1.00 ^{eD}
8	2,4-D 1.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.2 mg·L ⁻¹	40.25±1.51 ^{cC}
9	2,4-D 1.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.25 mg·L ⁻¹	51.45±0.87 ^{aA}
10	2,4-D 2.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	50.00±0.87 ^{aAB}
11	2,4-D 2.0mg·L ⁻¹ +6-BA 1.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	52.35±0.61 ^{dCD}
12	2,4-D 2.0mg·L ⁻¹ +6-BA 2.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	68.50±1.80 ^{aA}
13	2,4-D 2.0mg·L ⁻¹ +6-BA 3.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	65.00±3.61 ^{bA}
14	2,4-D 2.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.1 mg·L ⁻¹	34.50±0.50 ^{hG}
15	2,4-D 2.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.15 mg·L ⁻¹	42.00±1.73 ^{gF}
16	2,4-D 2.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.2 mg·L ⁻¹	57.00±2.65 ^{cB}
17	2,4-D 2.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.25 mg·L ⁻¹	69.00±1.73 ^{aA}
18	2,4-D 3.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	55.00±1.00 ^{cdBC}
19	2,4-D 3.0mg·L ⁻¹ +6-BA 1.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	45.00±1.73 ^{fEF}
20	2,4-D 3.0mg·L ⁻¹ +6-BA 2.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	48.50±1.50 ^{eDE}

21	2,4-D 3.0mg·L ⁻¹ +6-BA 3.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	38.00±4.58 ^{fgEF}
22	2,4-D 3.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.1 mg·L ⁻¹	39.50±1.32 ^{efE}
23	2,4-D 3.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.15 mg·L ⁻¹	40.00±1.00 ^{eE}
24	2,4-D 3.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.2 mg·L ⁻¹	46.00±1.73 ^{cC}
25	2,4-D 3.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.25 mg·L ⁻¹	57.00±4.58 ^{aA}
26	2,4-D 4.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	38.50±1.80 ^{efgEF}
27	2,4-D 4.0mg·L ⁻¹ +6-BA 1.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	39.50±1.32 ^{efE}
28	2,4-D 4.0mg·L ⁻¹ +6-BA 2.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	49.00±2.00 ^{bB}
29	2,4-D 4.0mg·L ⁻¹ +6-BA 3.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	42.65±0.61 ^{dD}
30	2,4-D 4.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.1 mg·L ⁻¹	37.00±1.00 ^{efF}
31	2,4-D 4.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.15 mg·L ⁻¹	39.00±1.00 ^{bcBCD}
32	2,4-D 4.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.2 mg·L ⁻¹	41.00±1.73 ^{bB}
33	2,4-D 4.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.25 mg·L ⁻¹	45.50±5.77 ^{aA}
34	2,4-D 5.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	38.00±2.65 ^{cdCD}
35	2,4-D 5.0mg·L ⁻¹ +6-BA 1.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	39.50±1.32 ^{bcBC}
36	2,4-D 5.0mg·L ⁻¹ +6-BA 2.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	40.00±2.00 ^{bcBC}
37	2,4-D 5.0mg·L ⁻¹ +6-BA 3.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	39.00±2.00 ^{bcBCD}
38	2,4-D 5.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.1 mg·L ⁻¹	36.50±2.65 ^{dD}
39	2,4-D 5.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.15 mg·L ⁻¹	38.75±0.62 ^{cBCD}
40	2,4-D 5.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.2 mg·L ⁻¹	39.00±0.87 ^{bcBCD}
41	2,4-D 5.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.25 mg·L ⁻¹	42.50±2.60 ^{aA}
42	2,4-D 6.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	34.00±1.73 ^{efDE}

43	2,4-D 6.0mg·L ⁻¹ +6-BA 1.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	36.50±0.50 ^{bcdBCD}
44	2,4-D 6.0mg·L ⁻¹ +6-BA 2.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	37.50±4.09 ^{bcBC}
45	2,4-D 6.0mg·L ⁻¹ +6-BA 3.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	35.00±2.00 ^{defCDE}
46	2,4-D 6.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.1 mg·L ⁻¹	34.50±1.32 ^{defCDE}
47	2,4-D 6.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.15 mg·L ⁻¹	35.50±1.50 ^{cdeBCDE}
48	2,4-D 6.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.2 mg·L ⁻¹	36.75±1.39 ^{bcdBCD}
49	2,4-D 6.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.25 mg·L ⁻¹	38.50±2.78 ^{bB}
50	2,4-D 7.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	32.75±1.32 ^{fE}
51	2,4-D 7.0mg·L ⁻¹ +6-BA 1.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	33.50±1.32 ^{bcABC}
52	2,4-D 7.0mg·L ⁻¹ +6-BA 2.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	35.00±2.00 ^{aA}
53	2,4-D 7.0mg·L ⁻¹ +6-BA 3.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹	33.75±1.09 ^{abcABC}
54	2,4-D 7.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.1 mg·L ⁻¹	31.50±1.50 ^{eD}
55	2,4-D 7.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.15 mg·L ⁻¹	32.00±1.00 ^{deCD}
56	2,4-D 7.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.2 mg·L ⁻¹	33.00±2.60 ^{cdBCD}
57	2,4-D 7.0mg·L ⁻¹ +6-BA 0.0 mg·L ⁻¹ +KT 0.25 mg·L ⁻¹	34.75±0.50 ^{abAB}

NOTE: Values within a row followed by lowercase letters are significantly different at $P<0.05$. Values within a row followed by capital letters are significantly different at $P<0.01$.

Table 2. Effects of 2,4-D concentrations on callus induction.

2, 4-D/ mg.L ⁻¹	Callus induction rate of ten alfalfa varieties/%									
	“Jacklin”	“Algonquin”	“GoldenEmpress a”	“GoldenEmpress b”	“Queen 2000”	“Plato”	“Suntory”	“Zhongmu No.1”	“Aohan”	“Longdong”
1.0	56.00±1.00 ^{dD}	55.00±1.73 ^{cC}	58.00±1.00 ^{eE}	60.00±0.00 ^{cC}	50.00±1.00 ^{deDE}	60.00±3.61 ^{cCD}	62.00±1.73 ^{FD}	50.00±1.00 ^{cE}	52.00±1.73 ^{cE}	40.00±1.00 ^{eE}
2.0	100.00±0.00 ^{aA}	100.00±3.00 ^{aA}	100.00±0.00 ^{aA}	100.00±0.00 ^{aA}	100.00±0.00 ^{aA}	100.00±0.00 ^{aA}	100.00±0.00 ^{aA}	100.00±0.00 ^{aA}	100.00±0.00 ^{aA}	100.00±0.00 ^{aA}
3.0	80.00±0.00 ^{hB}	70.00±5.29 ^{hB}	89.00±1.00 ^{hB}	74.00±3.46 ^{hB}	70.00±2.65 ^{hB}	100.00±0.00 ^{aA}	96.00±2.00 ^{hA}	83.00±1.73 ^{hB}	77.00±1.00 ^{hB}	85.00±1.00 ^{hB}
4.0	72.00±1.73 ^{cC}	67.00±2.64 ^{hB}	83.00±1.73 ^{cC}	60.00±0.00 ^{cC}	65.00±1.73 ^{cC}	75.00±4.36 ^{hB}	80.00±2.65 ^{cB}	80.00±3.61 ^{hB}	70.00±2.00 ^{cC}	83.00±1.00 ^{hB}
5.0	70.00±2.00 ^{cC}	65.00±4.58 ^{hB}	75.00±1.73 ^{dD}	50.00±1.00 ^{dD}	53.00±2.65 ^{dD}	71.00±1.00 ^{hB}	74.00±2.00 ^{dC}	69.00±1.73 ^{cC}	61.00±1.73 ^{dD}	60.00±0.00 ^{cC}
6.0	57.00±6.24 ^{dD}	52.00±1.73 ^{cC}	55.00±1.00 ^{eE}	42.00±1.73 ^{eE}	47.00±2.65 ^{eE}	64.00±2.00 ^{cC}	66.00±1.00 ^{eD}	58.00±2.00 ^{dD}	49.00±1.00 ^{eE}	53.00±2.65 ^{dD}
7.0	45.00±3.00 ^{eE}	40.00±1.00 ^{dD}	43.00±2.64 ^{eF}	31.00±1.73 ^{eF}	33.00±1.00 ^{eF}	55.00±1.00 ^{dD}	56.00±1.73 ^{eE}	42.00±1.73 ^{eF}	37.00±1.00 ^{eF}	42.00±1.73 ^{eE}

NOTE: Values within a row followed by lowercase letters are significantly different at $P<0.05$. Values within a row followed by capital letters are

significantly different at $P<0.01$.

Table 3. Effects of different concentrations of 6-BA and KT on the callus differentiation rate.

NO	Hormone level	Callus differentiation rate/%
1	6-BA 0.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹ +NAA 0.0 mg·L ⁻¹	12.00±1.73 ^{gG}
2	6-BA 0.3 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹ +NAA 0.0 mg·L ⁻¹	31.50±2.41 ^{fF}
3	6-BA 0.3 mg·L ⁻¹ +KT 0.5 mg·L ⁻¹ +NAA 0.0 mg·L ⁻¹	59.50±0.87 ^{aA}
4	6-BA 0.3 mg·L ⁻¹ +KT 1.0 mg·L ⁻¹ +NAA 0.0 mg·L ⁻¹	53.00±1.00 ^{bB}
5	6-BA 0.3 mg·L ⁻¹ +KT 1.5 mg·L ⁻¹ +NAA 0.0 mg·L ⁻¹	45.75±1.52 ^{cC}
6	6-BA 0.3 mg·L ⁻¹ +KT 2.0 mg·L ⁻¹ +NAA 0.0 mg·L ⁻¹	38.75±0.43 ^{eE}
7	6-BA 0.3 mg·L ⁻¹ +KT 2.5 mg·L ⁻¹ +NAA 0.0 mg·L ⁻¹	33.50±0.43 ^{fF}
8	6-BA 0.3 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹ +NAA 0.1 mg·L ⁻¹	33.00±3.61 ^{fF}
9	6-BA 0.3 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹ +NAA 0.2 mg·L ⁻¹	43.00±2.65 ^{dD}
10	6-BA 0.3 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹ +NAA 0.3mg·L ⁻¹	58.75±1.15 ^{aA}
11	6-BA 0.3 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹ +NAA 0.4 mg·L ⁻¹	52.50±2.18 ^{bB}
12	6-BA 0.5 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹ +NAA 0.0 mg·L ⁻¹	43.50±0.50 ^{dD}
13	6-BA 0.5 mg·L ⁻¹ +KT 0.5 mg·L ⁻¹ +NAA 0.0 mg·L ⁻¹	61.50±2.18 ^{aA}
14	6-BA 0.5 mg·L ⁻¹ +KT 1.0 mg·L ⁻¹ +NAA 0.0 mg·L ⁻¹	54.00±2.00 ^{bB}
15	6-BA 0.5 mg·L ⁻¹ +KT 1.5 mg·L ⁻¹ +NAA 0.0 mg·L ⁻¹	46.50±0.50 ^{cC}
16	6-BA 0.5 mg·L ⁻¹ +KT 2.0 mg·L ⁻¹ +NAA 0.0 mg·L ⁻¹	40.75±0.75 ^{eE}
17	6-BA 0.5 mg·L ⁻¹ +KT 2.5 mg·L ⁻¹ +NAA 0.0 mg·L ⁻¹	35.75±0.43 ^{fF}
18	6-BA 0.5 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹ +NAA 0.1 mg·L ⁻¹	34.00±1.00 ^{fF}
19	6-BA 0.5 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹ +NAA 0.2 mg·L ⁻¹	44.75±0.50 ^{dCD}

20	6-BA 0.5 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹ +NAA 0.3 mg·L ⁻¹	60.75±1.09 ^{aA}
21	6-BA 0.5 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹ +NAA 0.4 mg·L ⁻¹	53.00±2.65 ^{eE}
22	6-BA 1.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹ +NAA 0.0 mg·L ⁻¹	45.75±5.02 ^{gG}
23	6-BA 1.0 mg·L ⁻¹ +KT 0.5 mg·L ⁻¹ +NAA 0.0 mg·L ⁻¹	69.75±0.43 ^{aA}
24	6-BA 1.0 mg·L ⁻¹ +KT 1.0mg·L ⁻¹ +NAA 0.0 mg·L ⁻¹	58.75±2.95 ^{cC}
25	6-BA 1.0 mg·L ⁻¹ +KT 1.5 mg·L ⁻¹ +NAA 0.0 mg·L ⁻¹	49.00±4.58 ^{fF}
26	6-BA 1.0 mg·L ⁻¹ +KT 2.0 mg·L ⁻¹ +NAA 0.0 mg·L ⁻¹	41.50±3.77 ^{hH}
27	6-BA 1.0 mg·L ⁻¹ +KT 2.5 mg·L ⁻¹ +NAA 0.0 mg·L ⁻¹	36.75±1.09 ^{iI}
28	6-BA 1.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹ +NAA 0.1 mg·L ⁻¹	35.75±1.64 ^{iI}
29	6-BA 1.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹ +NAA 0.2 mg·L ⁻¹	45.00±1.89 ^{gG}
30	6-BA 1.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹ +NAA 0.3 mg·L ⁻¹	61.75±0.25 ^{bB}
31	6-BA 1.0 mg·L ⁻¹ +KT 0.0 mg·L ⁻¹ +NAA 0.4 mg·L ⁻¹	55.50±0.50 ^{dD}

NOTE: Values within a row followed by lowercase letters are significantly different at $P<0.05$. Values within a row followed by capital letters are

significantly different at $P<0.01$.

Table 4. Effects of different 6-BA concentrations on adventitious bud induction and elongation, and the effects of different IBA concentrations on adventitious bud rooting.

Medium	Benzyladenine	Indole-3-butyric	No. of inoculated	Budding	Differentiation	Root rate/%
type	/mg·L ⁻¹	acid/mg·L ⁻¹	explants	rate/%	rate/%	
MS	0.5	0.5	100	60.00±2.00 ^{cB}	70.00±1.00 ^{aA}	86.00±2.00 ^{bA}
MS	1.0	1.0	100	75.00±2.65 ^{aA}	60.00±1.73 ^{bB}	90.00±1.00 ^{aA}
MS	1.5	1.5	100	70.00±1.73 ^{bA}	50.00±2.65 ^{cC}	80.00±2.65 ^{cB}
MS	2.0	2.0	100	50.00±3.61 ^{dC}	45.00±1.00 ^{dD}	70.00±1.00 ^{dC}

NOTE: Adventitious bud induction and elongation media supplemented with different concentrations (0.5 mg·L⁻¹, 1.0 mg·L⁻¹, 1.5 mg·L⁻¹ and 2.0 mg·L⁻¹) of 6-BA. Adventitious bud radication medium supplemented with different concentrations (0.5 mg·L⁻¹, 1.0 mg·L⁻¹, 1.5 mg·L⁻¹ and 2.0 mg·L⁻¹) of IBA. Values within a row followed by lowercase letters are significantly different at *P*<0.05. Values within a row followed by capital letters are significantly different at *P*<0.01.

Table 5. PCR primer information.

Gene	Forward primer	Reverse primer	Product size
<i>Bar</i>	5' GCG GTC TGC ACC ATC GTC A 3'	5' GTA CCG GCA GGC TGA AGT CCA 3'	463bp
<i>SOS1</i>	5' CCT GCC AAA GGA AAT CAT C 3'	5' GCT TGC TAC ATT TCT GCT G 3'	700bp
<i>SOS2</i>	5' TTA GTG GAC AGG GTT ACG A 3'	5' GCT GTA GCG AAC TCA ATG G 3'	550bp
<i>SOS3</i>	5'ACCACCGAGCTCTAGGAAGATACG 3'	5' GGGATG GGC TGCTCT GTA TCG AAG 3'	683bp
<i>SCaBP8</i>	5'GCAATTCTGCGCCGTCTTTAT ACC 3'	5' CAT TTG TTG CAC CTC TTC TCG CTC 3'	460bp

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

Fig. 7.

Fig. 8.

Fig. 9.

Fig. 10.

Fig. 11.