Drought, salinity, and other abiotic stresses have serious effects on plant photosynthesis, respiration, and the entire growth and development process. The ways and methods of plants dealing with various abiotic stresses in the environment are complex and orderly, and transcription factor regulation is an important method (Singh et al., 2002). Many transcriptomics studies have shown that regulating genes, especially those encoding transcription factors, such as C2H2, WRKY, bZIP, MYB, SBP, HB, DREB, AP2/EREBP, and NAC, are upregulated significantly after plants undergo adversity. The NAC transcription factor has multiple plant-specific biological functions and plays an important role in many aspects of plant growth and development as well as in biological and abiotic stress responses (Olsen et al., 2005). In 2003, NAC genes from wheat (Triticum aestivum), maize (Zea mays), Arabidopsis thaliana, and rice (Oryza sativa) were divided into two groups, Group I and Group II, by homologous evolution analysis (Ooka et al., 2003). In 2012, the NAC transcription factor was further divided into six functional groups by system evolution analysis: NAM/CUC3, SND, TIP, SNAC, ANAC034, and ONAC4 genes in rice induced by abiotic stress and 26 genes responding to biological stress. For example, the CINAC gene responds to drought, salt, low-temperature, high-temperature, and other abiotic stress (Huang et al., 2012), and the ANAC092 transcription factor in A. thaliana responds to salt stress by promoting plant senescence (Balazadeh et al., 2010). Northern blot analysis showed that the expression of the PenAC1 gene was strongly induced by drought and salt stress. After the PenAC1 gene of Populus euphratica was overexpressed in A. thaliana, the ratio of Na+/K+ in roots and leaves was low, and the expression level of the AthKT1 gene was significantly inhibited, which enhanced the resistance of A. thaliana to salt stress (Wang and Dane, 2013). Overexpression of the TaNAC2 homolog gene TaNAC2a in tobacco (Nicotiana tabacum) proved that TaNAC2a could enhance tolerance to drought in tobacco (Tang et al., 2012). The Rosa RhNAC3 transcription factor is involved in response to drought stress in rose (Rosa hybrida) and participates in the stress response through an ABA-dependent regulatory pathway (Jiang et al., 2014). BrNAC responds to high-temperature and low-temperature stress (Ma et al., 2014), and GhNAC8-GhNAC17 responds to...
abiotic stress, such as ABA, drought, salinity, and high or low temperature (Shah et al., 2013).

*C. grandiflora* has characteristics such as scrubby plants, long flowering period, rich color, abiotic and biotic resistance, and easy cultivation. It is an important groundcover plant material for landscape construction in north China and northeast China. To improve the adaptability of *C. grandiflora*, broadening the application area, especially for use in greening dry and barren areas, is a very meaningful breeding direction. In this study, the ClNAC9 gene was transferred into *C. grandiflora* ‘niu9717’ by the *A. tumefaciens*–mediated method, and corresponding resistance was studied by subjecting it to drought, saline, and alkali stress. This study provides a theoretical basis for expanding the scope of *C. grandiflora* in landscape architecture, thus enriching plant resources for landscape architecture in cities.

**Materials and Methods**

**Materials**

The *C. grandiflora* ‘niu9717’ was cultured in the plant cultivation room in the College of Landscape Architecture in Northeast Forestry University (Haerbin, China). The *C. grandiflora* ‘niu9717’ fully developed young leaves under the apical bud were used as explants and cut into 0.5 × 0.5-cm squares for genetic transformation studies. *A. tumefaciens* GV3101 was obtained from the Beijing Forestry University Landscape Architecture School (Beijing, China). The transgenic vector for overexpression of CINAC9 *A. tumefaciens* culture medium luria-bertani (LB) (pH 7.0) contained 10 g L⁻¹ peptone, 5 g L⁻¹ yeast extract, and 10 g L⁻¹ NaCl.

**Genetic transformation of *C. grandiflora***

The genetic transformation of *C. grandiflora* refers to the method of Liu (2015). After kanamycin sensitivity tests, selection for plant rooting pressure, precultivation, *A. tumefaciens* infection, cocultivation, and delayed cultivation, the resistant seedlings obtained were cultivated in medium containing kanamycin at 10 mg L⁻¹ Murashige and Skoog (Qingda Hope Bio-Technology Co., Qingdao, China) based on rooting screening integrant and PCR detection. Three positive strains (CINAC-5, CINAC-9, and CINAC-13) were selected for further Northern blot detection (Li et al., 2007).

**Salt and alkali tolerance of transgenic plants**

When the root lengths of CINAC-5, CINAC-9, and CINAC-13 strains and WT plants were ≈1.5 cm long, they were transplanted to peat and vermiculite at a ratio of 1:1 in well-mixed cultures. The cultures were placed in an artificial climate chamber under a 16/16-h light/dark cycle, with light intensity of 120 μmol m⁻² s⁻¹, temperature of 24/18 °C (day/night), and salt stress and alkali stress treatments were performed after 40 d. Concentrations of 100, 200, or 300 mmol L⁻¹ of NaCl salt solution and 50, 100, or 150 mmol L⁻¹ of NaHCO₃ base solution were used. Sterile water was used as the control, and plants were irrigated every 5 d. Salt stress was applied five times per day, and alkali stress was applied four times per day. Samples were taken after treatment, and relative water content (RWC), cell membrane permeability, malondialdehyde (MDA) content, superoxide dismutase (SOD) activity, peroxidase (POD) activity, catalase (CAT) activity, and other physiological indexes were detected in plants after salt stress. These physiological indexes were determined following the methods of Zhang et al. (2012). Proline content (Wang et al., 2016b), soluble protein content (Sami et al., 2015), MDA content, and antioxidant enzyme system (SOD, POD, CAT) activity were detected in plants after alkaline stress.

**Detection of drought tolerance in transgenic plants**

When the root lengths of CINAC-5, CINAC-9, and CINAC-13 strains and WT plants were ≈1.5 cm long, they were transplanted to peat soil and vermiculite at a ratio of 1:1 in...
well-mixed cultures. The cultures were placed in a culture room and
drought stress treatments were performed after 40 d, applied by
withholding watering of the plants in the culture room. The water
stress treatment times of 5, 10, and 15 d were set, and the samples
were taken at each time point. Treatment with no water stress (0 d)
served as a control. After treatment, the RWC and chlorophyll
content followed the method of Wang et al. (2015). MDA content,
and antioxidant defense enzyme system (SOD, POD, CAT) activity
indexes were detected.

Statistical methods
A one-way analysis of variance followed by Duncan’s multiple range test \( (P = 0.05) \) was used to test if treatment
means differed statistically from one another. Excel (2007;
Microsoft, Redmond, WA) and SPSS software (version
19.0J; IBM Corp., Armonk, NY) were used for all the
statistical analyses. Three biological replicates were used for each
analysis.

Results

Establishment of genetic transformation system for \textit{C. grandiflora}
Through an \textit{A. tumefaciens}–mediated method, an efficient
genetic transformation system was established for ‘niu9717’.
Kanamycin at 10 mg L\(^{-1}\) is the best antibiotic screening
concentration for leaves, and 8 mg L\(^{-1}\) is the best choice for rooting
transformed plants; leaf preculture time is 2 to 3 d; if optical density at
600 nm (OD600) is \( \approx 0.6 \), the infection time is 15 min; if OD600 is
\( \approx 0.8 \), the infection time is 10 min; and the coculture time is 2 d or a
delayed cultivation of 3 d. Plants
with kanamycin resistance were ob-
tained (Fig. 1).

PCR identification and Northern
blot analysis of transgenic plants
The resistant seedlings were fur-
ther rooted and screened on the
rooting medium containing kana-
mycin at 10 mg L\(^{-1}\). Finally, 15
transgenic \textit{CINAC9} resistant seed-
lings were obtained and numbered
A1 to 15. The genomic deoxyribo-
nucleic acid (DNA) of the trans-
genic resistant seedlings was
extracted, and the extracted geno-
mic DNA was used as a template for
PCR identification using \textit{CINAC9}
gene-specific primers. The results
showed that a \textit{CINAC9} gene-spe-
cific fragment whose length is the
same as the positive control (\( 650 \)
base pairs) was amplified among the
15 detected strains of \textit{CINAC9}
transgenic resistant plants. There
were no bands in the negative con-
trol plants without the transgene, so
that it can be inferred that the \textit{CINAC9} gene has been integrated
into the ‘niu 9717’ genome (Fig. 2). Northern blotting was
performed using total ribonucleic acid (RNA) from \textit{CINAC9}-5,
\textit{CINAC9}-6, \textit{CINAC9}-13, and WT leaves. There were expression
signals at the level of messenger RNA among transgenic
strains, but no hybridization signal in the control WT group
(Fig. 3). These results indicated that the \textit{CINAC9} gene was
expressed at the transcriptional level.

Salt tolerance of transgenic \textit{C. grandiflora}

Salt stress phenotype. The effect of different concentra-
tions of NaCl solution on the morphology of plants under 20 d
of salt stress is shown in Fig. 4. Under mild salt stress (100
mmol·L⁻¹), WT plants showed symptoms of slight harm, and the top leaves of some plants were slightly yellowed. The morphology of transgenic plants was nearly unchanged. Under moderate stress (200 mmol·L⁻¹), all the plants showed different degrees of harm, and the WT plants were the most seriously affected. The shoot tip was withered, the leaves yellowed, and it was dry and drooping. It only had the ability to maintain vitality. The ClNAC9-5 strain was the lightest in the transgenic lines, and the ClNAC9-6 and ClNAC9-13 strains changed significantly. The ClNAC9-6 strain was the most susceptible strain of the transgenic lines, whereas ClNAC9-6 and ClNAC9-13 changed significantly. Leaves yellowed severely and stems withered, but to a lesser extent than WT plants; when severely stressed (300 mmol·L⁻¹), the above-ground parts of the WT plants were all wilted, and individual chlorosis was observed in individual parts of the ClNAC9-6 and ClNAC9-13 plants. Although the ClNAC9-5 strain was not completely wilted, it was severely affected. The overall trend showed that the three transgenic lines were significantly more resistant to salt stress than WT plants, but when the salt stress reached a high concentration of stress (300 mmol·L⁻¹), all of the open pitted chrysanthemums were unable to tolerate the stress.

**Analysis of physiological indexes of transgenic plants under salt stress.** As shown in Fig. 5, the effects of NaCl salt stress for 20 d on the RWC of transgenic and WT plants showed a decreasing trend with the increase in stress concentration.
With the increase in stress, MDA and the relative conductivity of transgenic and WT plants showed an upward trend, but the rate of increase was reduced significantly in transgenic lines. The SOD activity of WT and transgenic lines was basically the same without stress, and the activity of POD and CAT in the transgenic lines was slightly higher than WT plants. With the increase in salt stress, the activity of SOD, POD, and CAT in three transgenic lines and WT plants all tended to increase and then decrease. Under mild salt stress, transgenic plants and WT plants showed a significant increase in CAT activity and reached the highest value. SOD and POD peaked at moderate stress, followed by a downward trend. Under severe stress, SOD, POD, and CAT did not resist the salt stress; although the transgenic lines also decreased, they were still higher than the WT plants.

Alkali tolerance of the transgenic C. grandiflora

**ALKALI STRESS PHENOTYPE.** The effect of different concentrations of alkali solution on the morphology of C. grandiflora at 15 d is shown in Fig. 6. Under mild stress (50 mmol·L⁻¹), WT plants and the three transgenic lines showed no obvious change, indicating that slight alkali stress did not affect the growth of C. grandiflora. Under moderate stress (100 mmol·L⁻¹), the WT plants showed severe leaf chlorosis and wilt sagging, while some of the leaves were withered at the edge of the leaves. The transgenic lines also had yellow leaves, and some dry sagging plant stems appeared in all the lines, but the extent of harm was significantly less than in WT plants. Under severe stress (150 mmol·L⁻¹), the WT plants could not withstand the stress. Most and CAT activities of CINAC9-13 transgenic lines were lower than those in CINAC9-5 lines. The proline content and MDA content of WT plants tended to increase continuously, and the content of soluble protein and the activities of the antioxidant protective enzyme system increased at first and then decreased. Comprehensive analysis showed that transgenic plants increased alkali resistance.

Drought tolerance of transgenic C. grandiflora

**DROUGHT STRESS PHENOTYPE.** During drought stress, the morphological changes in CINAC9-5, CINAC9-6, and CINAC9-13 transgenic lines and WT were observed and photographed. Figure 8 shows the morphological changes of C. grandiflora under drought stress at 0, 5, 10, and 15 d. With the prolongation of drought stress, the WT plants wilted faster than the transgenic plants under the conditions of water loss. WT plants began to lose water and to yellow after 10 d of stress, and the external morphology of the transgenic lines began to change slightly. Under stress at 15 d, the leaves of WT seedlings had severe dehydration, and the external appearance began to turn yellow, curly and drooping, whereas the transgenic lines began to dehydrate and wilt. The CINAC9-13 line was more dehydrated but remained active. These observations indicate that the transgenic lines were significantly improved compared with the WT in terms of drought resistance; from the perspective of morphological changes, the transgenic lines CINAC9-5 and CINAC9-6 have strong drought resistance.

**ANALYSIS OF PHYSIOLOGICAL INDEXES OF TRANSGENIC PLANTS UNDER DROUGHT STRESS.** The changes in RWC, chlorophyll...
content, MDA content, and antioxidant protective enzyme system of the leaves of transgenic and WT *C. grandiflora* under drought stress for 15 d are shown in Fig. 9. With the extension of drought conditions, the RWC and chlorophyll content of WT leaves continued to decrease and gradually lowered compared with the transgenic lines. The content of MDA increased steadily and was always higher than that of the transgenic lines. The activity of the antioxidant protective enzyme system was increased at first and then decreased, and it was lower than that of transgenic plants at the late stage of stress. The content of MDA in the transgenic lines kept increasing, while the chlorophyll content, SOD, POD, and CAT activity increased and then decreased. The transgenic lines *CINAC9*-5 and *CINAC9*-6 showed similar changes, indicating strong drought tolerance. The results showed that transgenic *CINAC9* plants had stronger drought resistance than WT plants.

**Discussion**

Genetic transformation commonly uses antibiotics as a selection marker, with different varieties of antibiotic susceptibility screening. Selecting the correct antibiotics and determining the critical screening concentration of antibiotics is the key to successful genetic transformation. The toxicity of different antibiotics to *C. grandiflora* were chloramphenicol >
rifampin > streptomycin > minomycin > ampicillin > antimicrobial. This research adopts the plant binary expression vector pBI121 containing the neomycin phosphotransferase gene, which produces kanamycin resistance in transformed cells. The effects of different concentrations of kanamycin on the induction of adventitious buds in vitro were studied. The results showed that *C. grandiflora 'niu 9717'* was sensitive to kanamycin. On the differentiation medium supplemented with kanamycin, the explants of leaves gradually became yellow or withered and died due to the increase in the concentration without any differentiation, and the lethality was obvious. In this study, resistant plants were obtained when screened by rooting. Negative reaction plants were obtained when screened by PCR, indicating that pseudotransformants were present in the study. According to the causes of pseudotransformation (Li et al., 2012), appropriate preventive measures include increasing the contact area of the transformation receptor and screening the medium in the process of transformation. The selection pressure in time is added, and after obtaining resistant buds, some methods were used, such as gradually increasing the selective pressure to reduce the occurrence of false positive seedlings. In this study, transgenic plants were detected by PCR and Northern blot analysis. The results showed that the *CINAC9* gene was introduced and integrated into the genome of *C. grandiflora*.

The NAC transcription factor has a great response to different stress conditions (Wang et al., 2013). Many members of the NAC gene family have differential expression characteristics in their response to abiotic stresses. In this experiment, salt, alkali, and drought stress were measured, and the physiological indexes were measured after the transformation of the *CINAC9* gene. The relative conductivity of WT plants increased more than that of transgenic plants, and the damage to the cell membrane system of WT plants was more severe, consistent with the experimental results of Zhou et al. (2013). Under salt, alkali, and drought stress, the water content of WT plants decreased much more than in the transgenic lines. The activities of SOD, POD, and CAT in *C. grandiflora* increased with the prolongation in stress time and concentration and then decreased, and the activity in transgenic lines was higher than in WT plants. However, the content of MDA in WT plants showed a more significant increasing trend, indicating that the membrane peroxidation persists and increases continuously under stress, causing damage to plants. The change trend in enzyme activity in the protective enzyme system was consistent with that of Wang et al. (2016a) and An et al. (2016). With the
increase in alkali stress concentration, the proline content of the transgenic lines was slightly higher than that of WT plants. The content of soluble protein showed an upward trend under mild stress, indicating that the synthesized rate of soluble proteins increases and then declines under short-term and low-concentration stress. However, the transgenic lines were still slightly higher than the WT plants, indicating that introducing the ClNAC9 gene into C. grandiflora enhances the ability to maintain protein synthesis and decomposition ability. The contents of proline and soluble protein in the three transgenic lines did not appear to be significantly higher than in WT plants. It is speculated that the transfer of the ClNAC9 gene does not increase the tolerance of C. grandiflora to alkali stress by increasing the content of free proline and soluble protein, and the specific reason remains to be further studied.

In late drought stress, the content of chlorophyll in WT and transgenic lines was decreased, but the chlorophyll content in transgenic lines was higher than in the WT. In addition, the chlorophyll content of the WT was more sensitive to the loss of water, and the recovery ability was also significantly weaker than the transgenic lines. Studies by Hao et al. (2011) demonstrated that the soybean GmNAC20 transcription factor can regulate stress tolerance by activating the DREB/CBF-COR pathway, and its expression in transgenic A. thaliana enhances tolerance to salt and cold stress. GmNAC11 may enhance the salt tolerance of transgenic A. thaliana by

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Fig. 9. Effects of different times of drought stress on relative water content, chlorophyll content, malondialdehyde (MDA) accumulation and superoxide dismutase (SOD), peroxidase (POD), and catalase activity (CAT) in leaves of Chrysanthemum grandiflora. Different letters indicate significant differences ($P < 0.05$). FW = fresh weight.
regulating DREB1A and other stress-related genes (Hao et al., 2011). The ONAC045 transcription factor is an important regulator of drought resistance in rice. Transient expression in onion epidermal cells indicated that the ONAC045 protein was localized in the nucleus and in transgenic rice overexpressing ONAC045. The results showed that two stress-related genes are expressed, enhancing the tolerance of plants to drought stress (Zhang et al., 2009). In addition, other studies have shown that overexpression of the OsNAC10 gene can significantly enhance the tolerance of rice to drought, high salt, and low temperature at the vegetative stage; and in the reproductive growth stage, the yield of rice can be increased by 25% to 42% under drought conditions or 5% to 14% under normal conditions (Jeong et al., 2010). The resistance of three transgenic lines to salinity, alkalinity, and drought stress were also not completely consistent. The CINAC9-5 strain showed the most superior performance and the strongest resistance, whereas the CINAC9-6 and CINAC9-13 strains had stronger salt, alkaline, and drought stress resistance than the WT but weaker than the ClNAC9 strain. The same gene was transferred into the same plant, but the phenotypes of different strains are not completely consistent. The expression levels of transferred genes in different strains need further study.

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