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Expression of the Ced-9 (Cell Death abnormal-9) gene in transgenic Chrysanthemum morifolium can enhance drought and salt tolerances

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Abstract

Salt and drought are important abiotic stresses of *Chrysanthemum morifolium*(Asteraceae). Limited information is available with regard to molecular management strategies for salt and drought. *Ced-9* (*Cell Death abnormal*-9) is already known to enhance the tolerance of plants to abiotic stresses. Asteraceae plants overexpressing *Ced-9* have showed improved tolerance to stresses - they kept freshness for longer time, and were fast to grow, and had higher tolerance to salt.

Additional analyses revealed that transgenic plants generally show lower transpiration rate and higher chlorophyll content when subjected to salt and drought stresses. These results suggest that *Ced-9* inhibits the activity of a cysteine protease with caspase-like activity and that *Ced-9* also enhances salt and drought tolerances by maintaining intracellular ion balance by apparently inhibiting the external efflux of K^+ induced by salt stress. *Ced-9* has been proved to improve the low temperature tolerance of asteraceae plants by controlling 2,4-D signal and other pathways responding to stresses.

1. Introduction

Drought and Salt limits the normal growth and consequently their productivity and quality by preventing the *Chrysanthemum* from expressing their full genetic potential. Making an improvement in these characteristics under salt and drought stresses is a major challenge for flower breeders (Liu et al. 2019).

Different breeding strategies for stress resistance have emerged, all with their merits and demerits. Conventional breeding approaches have attempted through the use of genetic variability available in diverse germplasm and somaclonal variation, interspecific hybrids and induced mutations (Ashraf 2010; Badu-Apraku and Yallou 2019; Kindiger et al. 2006).

Current methods applied in chrysanthemum breeding include cross-breeding, mutation, radiation breeding, satellite breeding, tissue culture, and single cell breeding and so on (Eva María Gómeza et al. 2019; Takashi Nakatsuka, Kanae Koishi 2018; Tsuyoshi Motohashi et al. 2014; Y.H. Qua et al. 2009).

However, this approach has low success rates due to complex adaptive mechanisms of cells that induce abiotic stress tolerance involved in various metabolic pathways(Shinozaki et al. 2003; Yamaguchi-Shinozaki and Shinozaki 2006). Another approach to increase the tolerance to abiotic stress including drought has been the use of genetic engineering to increase the tolerance to various stresses in many transgenic plants. Transgenic methods used in chrysanthemum include *A. tumefaciens* infection and direct transfer. The *A. tumefaciens* infection mainly uses leaf slices. Several genes have been transferred to chrysanthemum by gene shotgun method and leaf slice method, both of which were successful(ARLAL.BUSH and STEVENG. PUEPPKE 1991; S. Mahadtanapuka et al. 2014; Patelm et al. 2013; M.F.Van Wordragen et al. 1992).

The transgenic system of different chrysanthemum species was established through the transfer of the target gene(Daniela Pavingerova et al. 1994; Adnane Hitmil et al. 2000; Mitiouchkina T Y et al. 2000).

Exogenous genes mainly include flower color modifiers (LC, CHS), resistance genes (Bt, Mp_1, TAWV), flowering regulatory genes (LFY), and plant morphological modifiers (Gai, Rolc, PHYB). The genetic modification controlled flower color, enhanced resistance (pests, cold), improved plant morphology, and controlled flowering time. Some of them expressed target traits in genetic modified plants and were tested in the field, but some showed gene silencing(LoriA. Urban et al. 1994).

Overexpression of the CBF family gene *ClCRF1* in chrysanthemum enhanced its tolerance to salt and drought(Wenjie Gao, Miao He et al. 2018), and overexpression of COL16 was found to increase the biosynthetic accumulation of chlorophyll II in petunia (Akemi Ohmiya et al. 2018).

In addition, chrysanthemum transformed with rice chitinase gene (RCC2) was found to improve resistance to gray mold (Y. Takatsu, Y. Nishizawa 1999).

Ced-9 is an apoptotic inhibitor gene found in nematodes, consisting of the *Bcl-2/Ced-9* gene superfamily because of its structural and functional conservation.

The Ced-9 gene was found in *n1950* mutant of PCD-suppressed nematodes(Yun Wang et al. 2014). During nematode development, *ced-3, ced-4, Ced-9* and *egl-1* genes are involved in apoptosis. Expression of *Bcl-xl* and *Ced-9* genes in tobacco could inhibit cell death induced by UV radiation and hypersensitivity to herbicides(Ichiro Mitsuhara et al. 1999).

It is known that overexpression of *Ced-9* and *Bcl-xl* genes in tobacco enhances plant resistance to high salinity and low temperature through the protection of organelles(Mitsuhara et al. 1999).

Tomato plants transformed with *Bcl-xl* and *Ced-9* by Agrobacterium-mediated infection acquire resistance to cucumber mosaic virus (CMV) and the inhibitor gene of apoptosis completely inhibits cell death due to hypersensitivity reactions, which can confer protection against pathogen invasion at the individual level and resistance to unfavorable environments such as low temperature, high temperature, high osmotic pressure, and salt stress (Xu. P et al. 2004).

The most interesting thing is that the rice and tobacco transformed with *Bcl-2* and *Ced-9, the* apoptosis inhibitors of animal origin, acquired resistance to salt stress.

A new research result has been reported, saying that in rice transformed with *Bcl-2*, the content of chlorophyll of mesophyll cells increased and death of shoot tip cells of rice root was inhibited. This proves that the expression of this gene inhibits the external efflux of K^+ which occurs under salt stress, and suppresses the expression of OsVPEs, which consequently inhibits apoptosis(Kim yongho et al. 2014).

By real-time measurements of K^+ flow in tobacco mesophyll cells transformed with *Ced-9* under salt stress, it was found that *Ced-9* significantly inhibited the external efflux of K^+ induced by salt stress, maintained intracellular ion balance, and significantly suppressed the external efflux of K + induced by oxidative stress, thereby attenuating plant damage by oxidative stress (Uta Anschütz et al. 2014).

In addition, it has been shown that Arabidopsis and tobacco plants transformed with *Ced-9* and *Bcl-2* genes are tolerant to Al^{3+} and salt and promote Al tolerance by inhibiting Al-induced apoptosis (Wenzhe Wang et al. 2009).

Chrysanthemum (*Chrysanthemum morifolium*) is a perennial rooted fallow flower and is widely cultivated worldwide because of its large flower size, good flower color and flower shape and long period of flowering(Rinaldi Sjahril et al. 2022).

There is little information on the tolerance of chrysanthemum to the drought and salt in relation with the overexpression of *Ced-9 .*

2. Materials And Methods

2.1. Chrysanthemum morifolium variety and Agrobacterium strain

We chose *Chrysanthemum morifolium* and transformed it with *Agrobacterium tumefasiens* EHA105(pCAMBIA13011-*Ced-9*) provided by College of Life, Zhejiang University, People's Republic of China.

The plasmid pCAMBIA13011 contains the kanamycin resistance gene (*nptII*) under the control of the pnos promoter, the hygromycin resistance gene *(hph*), a gene for GUS activity (*uidA*)and the intron-*uidA*. Both, of *hph* and *uidA* genes, were under the control of the 35S promoter of cauliflower mosaic virus.

2.2. Tissue culture and Agrobacterium-mediated transformation of leaf slices

Healthy plants of chrysanthemum grown in the greenhouse were selected and the nodal with lateral buds for tissue culture was sterilized with 70% ethanol for 10s, 0.2% mercuric chloride ($HgCl_2$) for 15 min, washed five times with sterile water, and transfected. The infectious fluid of the leaf slices was removed on the sterile filter paper and dried for 30–40 min and then co-cultured for 2–5 days in the dark at 25°C.

As an infection and co-culture medium, MS (BA 3.0 mg/L + NAA 0.1 mg/L) supplemented with 100μmol/L AS was used. The concentration was A_{600} = 0.01 and it was infected for 5 min.

2.3. Selection and regeneration of transgenic plants

After co-cultured with Agrobacterium, leaf slices were inoculated on MS solid medium supplemented with Hyg 50 /mL, Cef 500 /mL, and 2.4-D 2 mg/L and transferred to primary selection at 28℃ for 60 days under light. Antibiotic-resistant shoots were transferred to secondary selection in nodal selection medium.

Regenerated plants, of which root, stem and leaf were almost differentiated, were removed from the test tube (or strengthened in the greenhouse for 4 to 5 days after opening the mouth) and washed thoroughly

with water. In vitro plantlets were planted at 2×2 cm intervals on acclimatization medium and covered with the plastic membrane for 3–7 days under light. After removing the membrane, it was acclimatized for 20 to 25 days under light which increased gradually, and transferred to a greenhouse to grow mature plants (Patel and Qu 2013; Ramírez-Mosqueda et al. 2017).

2.4. Assay for GUS activity

In vitro plantlets were transferred to the coupling solution and stained at 37°C for 12h to detect GUS expression. The composition of the coupling solution was 0.1 M sodium phosphate buffer (pH 7.0), 10 mm EDTA, 0.1% (W/V) Triton, and 0.1% (W/V) X-Gluc.

Blue spots were recorded through a stereoscopic microscopy(Leica, Switzerland). Controls were samples without *A. tumefaciens* co-culture.

2.5. Genomic DNA extraction and PCR analysis

Genomic DNA was extracted according to an already known method (Dellaporta et al., 1983). Polymerase chain reaction (PCR) was followed in a C1000 Touch TM thermal cycler (BIORAD) with Taq polymerase (Invitrogen, USA). The following parameters were used: 94°C for 5 min, followed by 30 cycles of 94°C for 30s, 58°C for 30s and72°C for 1min. Final extension time of 6min at 72°C was used.

For identification of *Ced-9* transgenic plants, primers P35S F (5'-ACC ACG TCT TCA AAG CAA GTGG-3'), P35S R (5'CCT CTC CAA ATG AAA TGA ACT TCC – 3'), Tnos F (5'-TTG AAT CCT GTT GCC GGT CTTG-3'), Tnos R (5'-ACA CCGCGC GCG ATA ATT TATC-3'), *Ced-9* F (5’–ATGACACGCTGCACGGCG–3’) and *Ced-9* R (5’–TTACTTCAAGCTGAACATCATCCGC–3’) were used.

These primers amplify 123bp fragment of 35S promotor sequence, 199bp fragment of nos terminator sequence and 869bp fragment of the *Ced-9*. PCR products were separated by electrophoresis on a 2% agarose Gel.

2.6. Evaluation of transgenic plant for Drought tolerance

To determine the drought tolerance, acclimatized plantlets of transformed and non-transformed (8th micropropagation plants) were planted on 15cm-diameter pots containing 300g of substrate (coal ashes : humus soil : field soil = 1 : 1 : 1). They were maintained until 8 to 10 leaves were grown, and the leaf wilting index of the plants was investigated after 10 days of drought stress treatment.

2.7. Salt tolerance evaluation

We used in vitro plantlets to evaluate the salt tolerance. NaCl of different concentrations (0, 0.2, 0.4, 0.6, 0.8, and 1.0%) were added to the nodal medium (MS + NAA 0.1 mg/L) to prepare NaCl medium. The axillary buds of 30-day-old chrysanthemum plantlets were excised into two segments, inoculated on the NaCl medium of different concentrations and incubated at 28°C in the light for 30 days.

After that, growth was observed to compare transgenic and non-transgenic plants.

2.8. Determination of chlorophyll content

Chlorophyll was extracted from a wet sample(0,2g) using 90% ethanol for 20h in the dark. Absorbance was measured at 664 and 649 nm and the total chlorophyll concentration (µg/mL) of the samples was determined according to a document (Lichtenthaler and Wellburn 1983).

2.9. Statistical analysis

Each treatment replicated three times. Treatment means and the standard error of the means were calculated using SAS (Statistical Analysis System, Cary, NC) software. Statistical analysis was performed with the Student's *t* test.

3. Results

3.1. Plant transformation and assay of transformed plants

The evaluation of the Hyg susceptibility of chrysanthemum leaf slices.

Table 1 The evaluation of the Hyg susceptibility of *Chrysanthemum morifolium* leaf slices

Concentration (mg/L)	No of inoculation (piece)	Rate of shoot induction (%)	Rate of necrosis (%)
0	30	82	0
10	30	15	33.3
15	30	0	100
20	30	0	100
25	30	0	100

Medium : MS BA3.0 /L NAA0.1 /L

hown in Table 1, shoot differentiation and callus formation were inhibited when the Hyg concentration was increased. And it was completely inhibited at the Hyg 15 mg/L. This indicates that Hyg-tolerant (Hyg^r) callus can be selected on the medium supplemented with Hyg 15 mg/L.

3.2. The evaluation of the Hyg susceptibility of *Chrysanthemum* nodes

The Hyg susceptibility of *Chrysanthemum* nodes was determined for the transformation. As shown in Table 2, the growth rate and rooting rate decreased when the Hyg concentration was increased, and the former was completely inhibited at Hyg 30 mg/L and did not survive. This indicates that using the mediumsupplemented with Hyg 30 mg/L (Hyg^r) *chrysanthemum* nodes can be selected.

Table 2 The evaluation of Hyg susceptibility of *Chrysanthemum* nodes

Concentration (mg/L)	No of inoculation (piece)	Rate of growth (%)	Rate of rooting (%)	Rate of necrosis (%)
0	30	100	100	0
10	30	100	100	0
20	30	26	60	0
30	30	0	0	100
40	30	0	0	100

medium:MS NAA 0.1 /L

3.3. Effect of pre-culture and co-culture for transformation

Pre-culture and co-culture have a very important effect on the target gene transfer by leaf slice infection. After Agrobacterium infection, leaf slices were selected under the above conditions.

Table 3
Effect of pre-culture and co-culture for transformation of leaf slices

№	Pre-culture (d)	Co-culture (d)	No of Inoculation (piece)	Damage rate after co-culture (%)	No of Shoot induction (piece)	No of selection (piece)
1	2(control)	2(control)	200	0	0	0
2	2	2	230	2	5	5
3	2	3	210	3	3	3
4	2	4	200	10	1	0
5	4	2	240	12	2	2
6	4	3	200	12	2	2
7	4	4	200	21	0	0

MS + NAA 0.1mg/L + BA 1.0mg/L + Hyg 15 mg/L + Cef 300 mg/L + scurose 3 + Agar 0.8%, pH 5.8, *The control refers to pre-cultured and uninfected leaf slices.

As shown in Table 3.7 and 5 plants were chosen for the selection of Hyg-resistant plants from leaf slices during the pre-culture of 2 to 4 days and the co-culture of 2 to 3 days, respectively.

This shows that during those days, plant cells were activated by the effects of growth regulators on the wound parts of leaf slices, and the susceptibility to the target gene increased the number of selected plants.

In addition, the rate of fungal damage of leaf slices on the selection medium after pre-culture and co-culture showed that it tended to increase along the pre-culture and co-culture.

More correctly, the transformant selection was preferable until 4 days of pre-culture and 3 days of co-culture, but not so good after those days. The selected Hyg-resistant plants were re-selected on nodal selection medium.

3.4. Assay for GUS Activity

We have examined the plant transformation by assaying the expression of the T-DNA with *uidA* that encodes β-glucuronidase (GUS) in shoot tip and is formed in plants cultured from multiple shoots (Fig. 1). The staining patterns of the upper stem by X-gluc, a substrate of GUS, showed the integration of T-DNA.

3.5. PCR Analysis

Plants derived from Hyg^r calli were tested by PCR to check the integration of the *35S* promoter and *nos* terminator sequence. The PCR was carried out using genome DNA from the control and Hyg^r plants to check the integration of T-DNA of the selected 12 kinds of chrysanthemum in vitro plantlets. Of the putative transgenic T_0 plants tested, selected plantlets showed fragments of 123bp and 199bp (Fig. 3).

The check of the integration of 869bp fragment of the transgene by PCR showed that the *Ced-9* was successfully integrated(Fig. 4).

3.6. Effect of NaCl on Chrysanthemum plantlet transformed with Ced-9 gene

The expression of Ced-9 gene in chrysanthemum was compared with NaCl tolerance of transgenic and non-transgenic plants.

Table 4 Effect of Nacl concentration on the growth of in vitro plantlets

NaCl Concentration (%)		Length (cm)	No of leaf (piece)	No of root (piece)	Length of root (mm)
1	Transgenic plantlets	3.3±0.3	4.1±0.5	1.7±0.2	1.8±0.2
	Non-transgenic plantlets	1.5±0.2	3.4±0.3	1.0±0.1	1.0±0.2
0.8	Transgenic plantlets	3.5±0.3	4.4±0.5	1.9±0.3	2.0±0.3
	Non-transgenic plantlets	2.5±0.2	3.9±0.4	1.3±0.2	1.6±0.1
0.6	Transgenic plantlets	3.5±0.4	4.7±0.3	2.1±0.2	2.2±0.2
	Non-transgenic plantlets	3.3±0.2	4.3±0.4	1.7±0.1	1.9±0.1
0.4	Transgenic plantlets	4.1±0.3	4.9±0.5	2.2±0.3	2.5±0.3
	Non-transgenic plantlets	3.9±0.4	4.7±0.3	2.1±0.2	2.4±0.2
0.2	Transgenic plantlets	4.7±0.3	5.3±0.3	2.4±0.4	2.7±0.2
	Non-transgenic plantlets	4.6±0.4	5.2±0.4	2.5±0.3	2.8±0.2
0	Transgenic plantlets	5.2±0.4	5.5±0.5	2.7±0.3	2.8±0.3
	Non-transgenic plantlets	5.1±0.5	5.6±0.6	2.8±0.4	2.9±0.3

As shown in Table 4 and Fig. 5, the growth of in vitro plantlets was inhibited by the increase of NaCl concentration in the medium. When NaCl concentration was 1%, the weight and number of leaves of the non-transgenic plantlets were twice when compared to transgenic plantlets. Therefore, it is possible that transgenic plants have higher Nacl tolerance than non-transgenic plants.

3.7. Effect of the expression of the *Ced-9* on Chlorophyll Content of Chrysanthemum under Salt Stress

When the length of root of the non-transgenic and transformed plants with *Ced-9* grew 10 cm long, they were transferred on the culture medium with NaCl of different concentrations and incubated for 3 days, and leaf slices were cut to measure the chlorophyll content as shown in Fig. 6.

As shown in Fig. 6, under normal conditions, there was no difference in the chlorophyll content of non-transgenic and transgenic plantlets, but it decreased significantly when the concentration of NaCl increased.

However, when the concentration of salt was between 0.4-1.0%, the chlorophyll content of transgenic plantlets was significantly higher than the non-transgenic plantlets. This suggests that the expression of

the *Ced-9* has a beneficial effect on the inhibition of chlorophyll synthesis by salt stress in chrysanthemum.

3.8. Determination of drought tolerance in transgenic chrysanthemum

Figure 6 Growth of the transgenic and non-transgenic plantlets under drought. Leaf wilting index after the drought treatment in 10 days from control(red)and transgenic plants (blue). Non-transgenic plants started to wilt on the 6th day after the cessation of watering, but the transgenic plants started to wilt on the 9th day. Transgenic plants showed significantly high leaf wilting index than the control ($P < 0.05$)

Under drought stress, the leaf wilting index of the control plantlets on 20th day was 0.7 times higher than that of the transgenic plantlets. This indicates that the drought stress resistance of the selected plantlets is higher than that of the control plantlets.

3.9. Low temperature tolerance of transgenic chrysanthemum

The plantlets were planted on pots in a non-heated greenhouse and we investigated the charateristics of their low temperature-tolerant growth. The results are shown in Fig. 9.

As shown in Fig. 8, the plant height and number of leaves in the non-heated greenhouse were significantly higher at 60th (3.1cm) and 90th (2.8cm)days when the temperature was the lowest compared to the control, and at 120th days, the plant height was shorter than the control by 17.4cm.

This indicates that the transgenic plantlets were more cold tolerant than the control, so that the vegetative growth was accelerated and transferred to the reproductive stage in March.

Table 5
Flowering characteristics of experimental and control plants in the greenhouse

Date Group	Floral bud emergence (month/day)	Flowering time (month/day)	Rate of flowering (%)	Size of flowers (cm)
Transgenic plants	3/1 ± 3	4/15 ± 3	56.7	12.6 ± 1.3
Non-transgenic plants	3/8 ± 5	4/20 ± 6	33.3	10.5 ± 1.2
Difference	7	5	23.4	2.1
Research period ; December, 2018 ~ March, 2019				

As shown in Table 5, you can see that in the greenhouse, the floral bud emergence in the control plots was about 7 days compared to the control, the flowering time was about 5 days earlier, and the flowering rate was 56.7%, which was 23.4% higher than the control.

3.10. Rust infection of transgenic and non-transgenic plantlets on pots

Table 6
Rust infection of transgenic and non-transgenic plantlets on pots

Term Group	No of leaf (piece)	No of rust cases per plant (piece)	Rust infection (per plant)		
			1 ~ 10 (cases)	11 ~ 20 (cases)	21 ~ 30 (cases)
Transgenic plantlets	28.0 ± 3.8	9.9 ± 0.9	5.9 ± 0.7	2.7 ± 0.3	1.3 ± 0.2
Non-transgenic plantlets	30.0 ± 2.6	18.6 ± 2.1	2.6 ± 0.2	4.9 ± 0.4	11.1 ± 1.4

As shown in Table 6, the total rust cases on leaves of the transgenic plantlets are half as many as the non-transgenic plantlets. In addition, the rust cases in transgenic plantlets occurred more by 3.3 than the non-transgenic plantlets when there were 1–10 leaves. It decreased to 9.8 when there were 21–30 leaves.

This indicates that *Ced-9* has disease resistance, and thus the *Ced-9* is expressed in cold-tolerant transgenic chrysanthemum.

4. Discussion

Responses to various stresses like drought or salinity, have been studied in many ways such as molecular approaches. Genes encoding transcription factors, protein kinases, chaperones, metabolic enzymes, epigenetic proteins, and detoxifying enzymes are known to protect cells from stress (Hu and Xiong, 2014). In this study, we used the *Ced-9* which plays an important role in the abiotic stress to plants. We found that drought, salinity and cold tolerances of the introduced transgenic chrysanthemum were high under stress and recovery conditions. This suggests that *Ced-9* is conserved in plants and that *Ced-9* plays a physiological role of enhancing drought, salt and cold tolerances of chrysanthemum.

Overexpression of *Ced-9* in chrysanthemum showed no morphological changes under normal growth conditions, but better growth of transgenic plants under drought, salinity and low temperature conditions (Figs. 7 and 9).

After various stresses in the pots, the transgenic plants were bluish but the control withered (Figs. 8, 10 and 11). *Ced-9* transgenic chrysanthemum has a higher chlorophyll content than the control (Fig. 6).

In terms of rust infection, the treatment had a lower total leaf rust cases than the control, indicating that the *Ced-9* has disease resistance as discussed previously (Desjardins P and Ledoux S, 1998), confirming that overexpression of the gene enhances rust resistance (Fig. 11). The acclimatization ability of the in vitro plantlets showed that the transgenic plantlets had a higher number of roots and root mass than the control plantlets. This shows that the transgenic plants had higher rooting ability and higher drought resistance.

Our results demonstrate for the first time that the transfer of the *Ced-9* into evergreen chrysanthemum enhances various stress tolerances of the evergreen chrysanthemum.

The expression of the Ced-9 is thought to suppress the peroxidation of membrane lipids by significantly inhibiting the production of ROS in plant cells caused by unfavorable environmental stresses. These results have already been reported for tobacco and rice.

Furthermore, it is possible that the expression of Ced-9 inhibits the production of ROS by modulating the membrane permeability changes in unfavourable environmental conditions through interactions with transmembrane proteins, when compared to previous studies which said that the expressin of *Ced-9* inhibits the disruption of intracellular ion balance.

Declarations

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References

1. Anschütz, U., Becker, D., Shabala, S., 2014. Going beyond nutrition: Regulation of potassium homoeostasis as a common denominator of plant adaptive responses to environment. J. Plant. Physiol. 171, 1–18.
2. Badu-Apraku, B., Talabi, A.O., Fakorede, M.A.B., Fasanmade, Y., Gedil, M., Magorokosho, C., Asiedu, R., 2019. Yield gains and associated changes in an early yellow bi-parental maize population following genomic selection for *Striga* resistance and drought tolerance. BMC. Plant. Biol. 19, 129.
3. BUSH, A.L., PUEPPKE, S.G., 1991. Cultivar-strain specificity between *Chrysanthemum morifolium* and *Agrobacterium tumefaciens*. Physiol. Mol. Plant. Pathol. 39, 309–323.
4. Desjardins, P., Ledoux, S., 1998. Expression of *ced-3* and *Ced-9* homologs in Alzheimer's disease cerebral cortex. Neuroscience Letters. 244, 69–72.
5. Dellaporta, S., Wood, J., Hicks, J., 1983. A plant DNA minipreparation, version II. Plant. Mol. Bio. Rep. 1, 19–21.
6. Gao, W., He, M., Liu, J., Ma, X., Zhang, Y., Dai, S., Zhou, Y., 2018.Overexpression of *Chrysanthemum lavandulifolium ClCBF1* in *Chrysanthemum morifolium* 'White Snow' improves the level of salinity and drought tolerance. Plant. Physiol. Biochem. 124, 50–58.
7. Gómez, E.M., Dicent, F., Batlle, I., Romero, A., Ortega, E., 2019. Cross-incompatibility in the cultivated almond (*Prunus dulcis*): Updating, revision and correction. Scientia Horticulturae. 245, 218–223.
8. Hitmil, A., Coudret, A., Barthomeuf, C., Sallanon, H., 2000. Role of intracellular water retention strength in freezing tolerance of *Chrysanthemum cinerariaefolium* Vis. cell cultures. J. Plant. Physiol. 157, 47–53.
9. Hu H, Xiong L (2014) Genetic engineering and breeding of drought resistant crops. Annu. Rev. Plant. Biol. 65, 715–741.
10. Karimi, S., Karami, H., Mokhtassi-Bidgoli, A., Tavallali, V., Vahdati, K., 2018. Inducing drought tolerance in greenhouse grown *Juglans regia* by imposing controlled salt stress: The role of osmotic adjustment. Scientia Horticulturae. 239, 181–192.
11. Kim, Y., Wang, M., Bai, Y., Zeng, Z., Guo, F., Han, N., Bian, H., Wang, J., Pan, J., Zhu, M., 2014. Bcl-2 suppresses activation of *VPEs* by inhibiting cytosolic Ca^{2+} level with elevated K^{+} efflux in NaCl-induced PCD in rice. Plant. Phys. Biochem. 80, 168–175.
12. Liu, L., Ji, H., An, J., Shi, K., Ma, J., Liu, B., Tang, L., Cao, W., Zhu, Y., 2019. Response of biomass accumulation in wheat to low-temperature stress at jointing and booting stages. Environmental and Experimental Botany. 157, 46–57.
13. Lichtenthaler, H.K., Wellburn, A.R., 1983. Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochem. Soc. Trans. 11, 591–592.
14. Mahadtanapuk, S., Teraarusiri, W., Nanakorn, W., Yu, L.D., Thongkumkoon, P., Anuntalabhochai, S., 2014. A novel ion-beam-mutation effect application in identification of gene involved in bacterial antagonism to fungal infection of

15. ornamental crops. Nucl. Inst. Meth. Phys. Rese. B. 326, 209–213.
16. Mitsuhara, I., Malik, K.A., Miura, M., Ohashi, Y., 1999. Animal cell-death suppressors Bcl-x_L and Ced-9 inhibit cell death in tobacco plants. Curr. Biol. 9, 775–778.
17. Motohashi, T., Umemuro, H., Takyu, T., Sato, Y and Kondo, K., 2014. Mutation breeding of *Glebionis coronalia* by exposing gamma irradiation.Chromosome Botany. 9, 55–56.
18. Nakatsuka, T., Koishi, K., 2018. Molecular characterization of a double-flower mutation in *Matthiola incana*. Plant Science. 268, 39–46.
19. Ohmiya, A., Oda-Yamamizo, C., Kishimoto, S., 2018. Overexpression of CONSTANS-like 16 enhances chlorophyll accumulation in petunia corollas. Plant Science. 280, 1–18.
20. Pavingerova, D., Dostal, J., Biskova, R., Benetka, V., 1994. Somatic embryogenesis and *Agrobacterium*-mediated transformation of Chrysanthemum. Plant Science. 97, 95–101.
21. Qu, Y.H., Lin, C., Zhou, W., Li, Y., Chen, B., Chen, G.Q., 2009. Effects of CO_2 concentration and moisture content of sugar-free media on the tissue-cultured plantlets in a large growth chamber. Commun. Nonlin. Sci. Numer. Simul. 14, 322–330.
22. Ramírez-Mosqueda, M.A., Iglesias-Andreu, L.G., Luna-Sánchez, I.J., 2017. Light quality affects growth and development of in vitro plantlet of Vanilla planifolia Jacks. South African Journal of Botany. 1–6.
23. Sjahril, R., Jamaluddin, I., Nadir, M., Asman, Haring, F., Riadi, M., Larekeng, S.H., Mariani, T.S., Trisnawaty, A.R.,Panga, N.J., Tambung, A., Chin, D.P., Mii, M., 2022. Performance of transgenic Chrysanthemum harbouring Wasabi defensing gene for White rust disease resistance. Journal of Biosciences. Vol.29, 621–631.
24. Shinozaki, K., Yamaguchi-Shinozaki, K., Seki, M., 2003. Regulatory network of gene expression in the drought and cold stress responses. Curr. Opin. Plant. Biol. 6, 410–417.
25. Takatsu, Y., Nishizawa, Y., Hibi, T., Akutsu, K., 1999. Transgenic chrysanthemum (*Dendranthema grandiflorum*(Ramat.) Kitamura) expressing a rice chitinase gene shows enhanced resistance to gray mold (*Botrytis cinerea*). Scientia Horticulturae. 82, 113–123.
26. Urban, L.A., Sherman, J.M., Moyer, J.W., Daub, M.E., 1994. High frequency shoot regeneration and *Agrobacterium*-mediated transformation of chrysanthemum (*Dendranthema grandiflora*). Plant Science. 98, 69–79.
27. Van Wordragen, M.F., Jong, J.D., Schornagel, M.J., Dons, H.J.M., 1992. Rapid screening for host-bacterium interactions in Agrobacterium-mediated gene
28. transfer to chrysanthemum, using the GUS-intron gene. Plant Science. 81, 207–214.
29. Wang, W., Pan, J., Zheng, K., Chen, H., Shao, H., Guo, Y., Bian, H., Han, N., Wang, J., Zhu, M., 2009. Ced-9 inhibits Al-induced programmed cell death and promotes Al tolerance in tobacco. Bioc. Biop. Res. Com. 383, 141–145.
30. Wang, Y., Wang, S., Luo, X., Yang, Y., Jian, F., Wang, X., Xie, L., 2014. The roles of DNA damage-dependent signals and MAPK cascades in tributyltin-induced germline apoptosis in Caenorhabditis

elegans. Chemosphere. 108, 1–8.

Figures



Figure 1

Resistant shoots state after selection culture(Hyg^r, 60d).

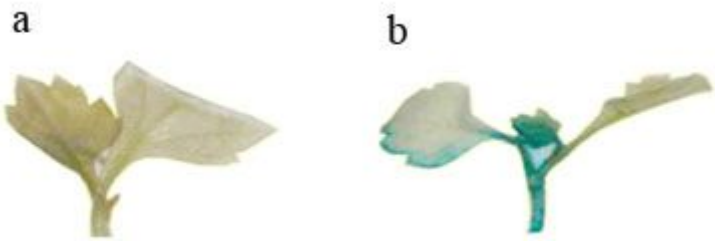


Figure 2

Plant transformation and essay of transgenic plants. a; Plant in 60 days culture(control). b; GUS expression in regenerated plant in 60 days culture from Hyg^r leaf slices after *Agrobacterium* inoculation

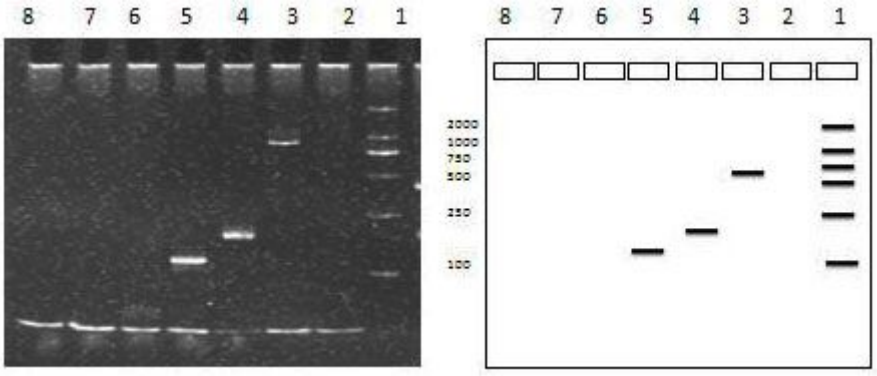


Figure 3

PCR analysis in transgenic plantlets. Lane 1. Marker of the molecular weight(DL 2 000), Lane 2. Negative control, Lane 3. PCR analysis of transgenic plant using *Ced-9* primers, Lane 4. PCR analysis of transgenic plant using *nos*terminator primers(199bp), Lane 5. PCR analysis of transgenic plant using *35S* promoter primers(123bp), Lane 6. PCR analysis of non-transgenic negative control using *Ced-9* primers(869bp), Lane 7. PCR analysis of non-transgenic negative control using *nos* terminator primers, Lane 8. PCR analysis of non-transgenic negative control using *35S* promoter primers

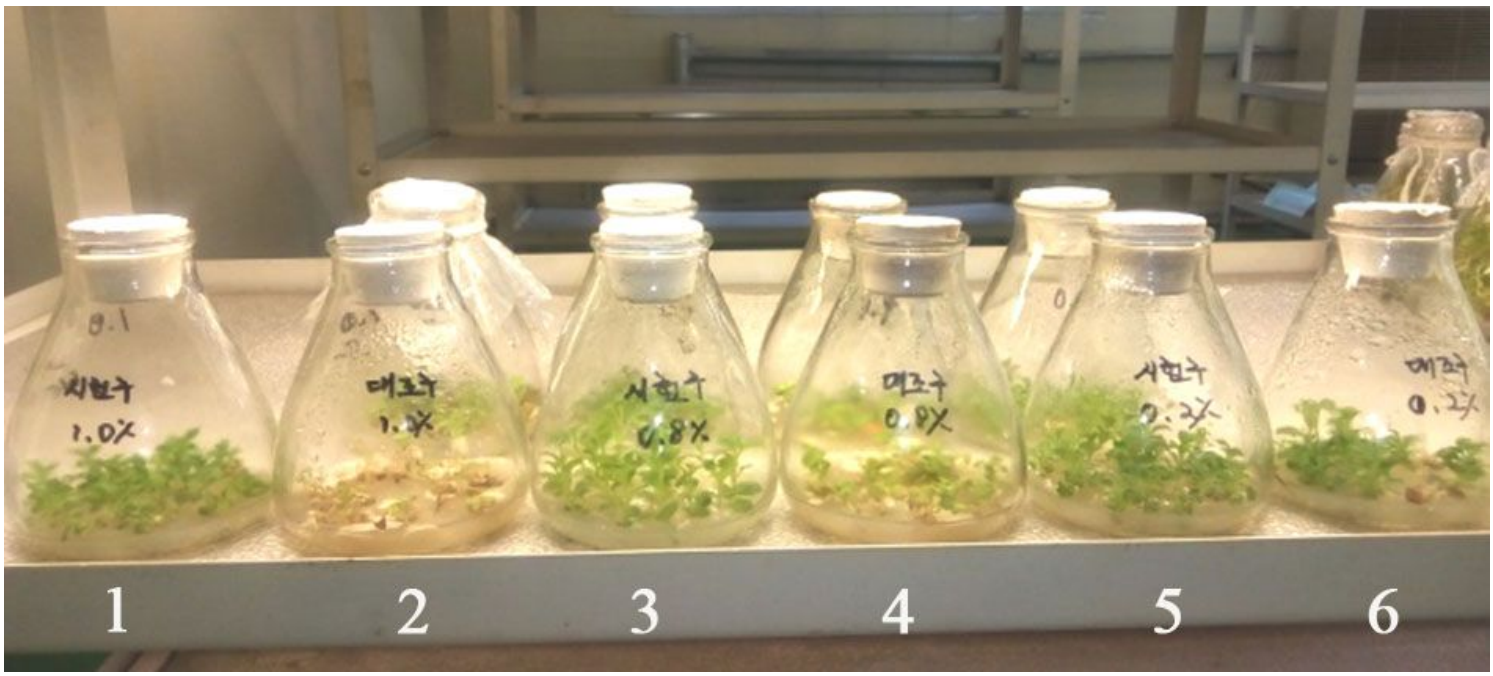


Figure 4

Characterization of different salt-treated transgenic plantlets. 1. Transgenic plantlets(1.0%), 2. Non-transgenic plantlets(1.0%), 3. Transgenic plantlets(0.8%), 4. Non-transgenic plantlets(0.8%), 5. Transgenic plantlets(0.2%), 6. Non-transgenic plantlets(0.2%)

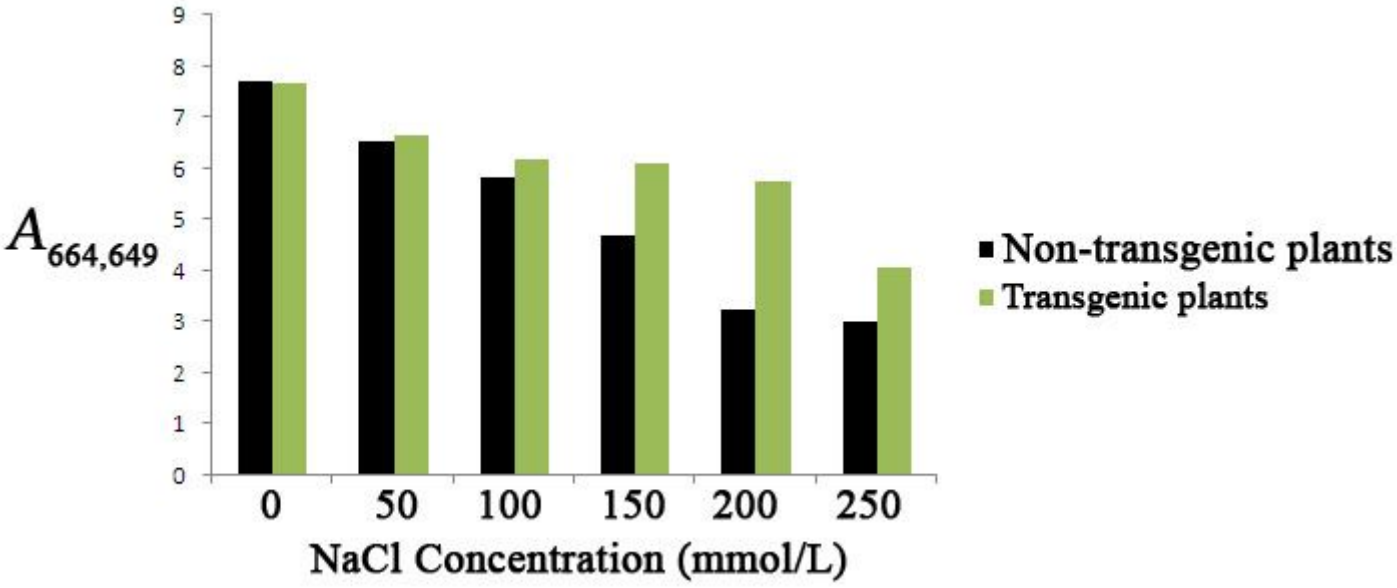


Figure 5

Chlorophyll content of transgenic and non-transgenic plant leaves. Chlorophyll contents after the drought treatment in 10 days from control(black)and transgenic plants (green). Transgenic plants showed significantly high chlorophyll contents than the control ($P < 0.05$)

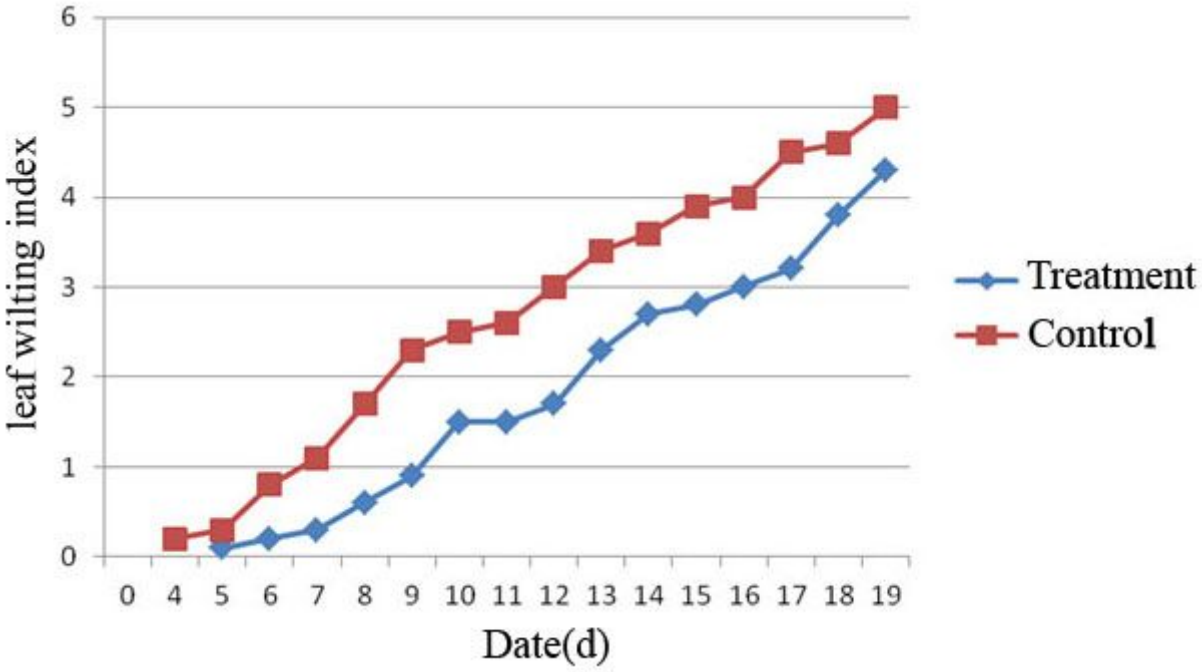


Figure 6

Growth of the transgenic and non-transgenic plantlets under drought. Leaf wilting index after the drought treatment in 10 days from control(red)and transgenic plants (blue). Non-transgenic plants started to wilt

on the 6th day after the cessation of watering, but the transgenic plants started to wilt on the 9th day. Transgenic plants showed significantly high leaf wilting index than the control ($P < 0.05$)



Figure 7

Characterization of drought-treated transgenic plants(black; control, green; transgenic plants). Leaf wilting Characterization after the drought treatment in 10 days from control(black)and transgenic plants (green). Under drought stress, the leaf wilting index of the control plantlets on 20th day was 0.7 times higher than that of the transgenic plantlets

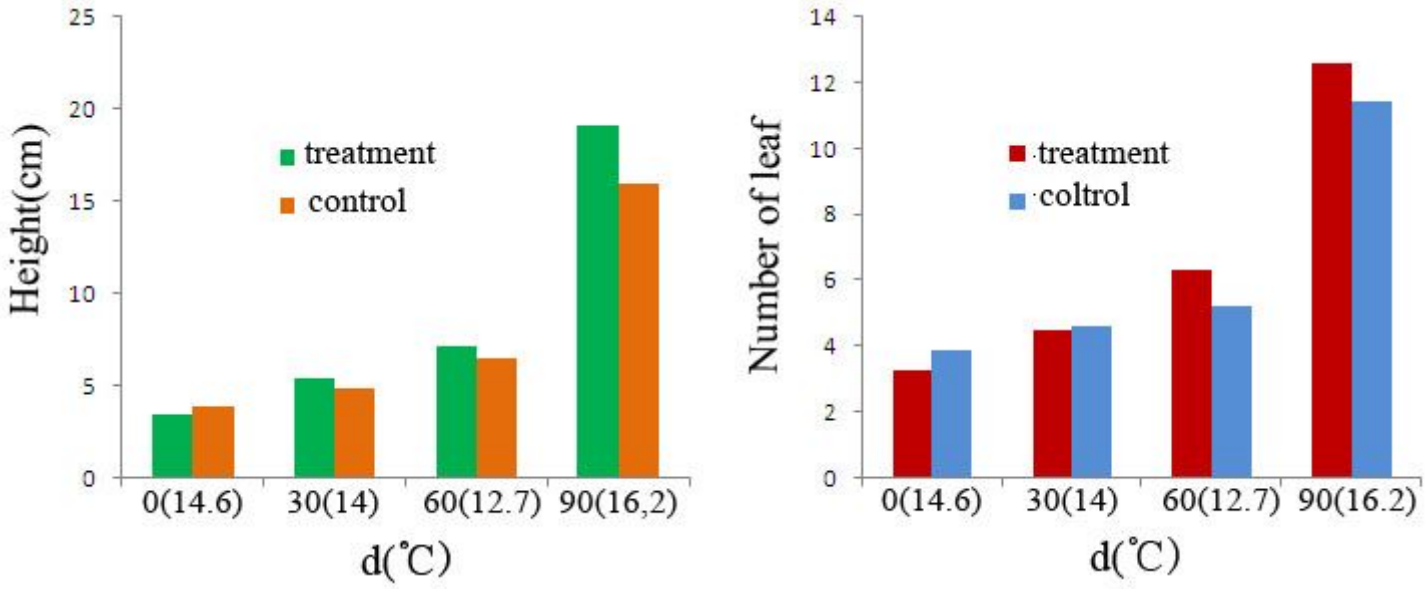


Figure 8

Growth characteristics of transgenic plantlets and control plantlets at low temperature. Research period; December, 2018 ~March, 2019. Growth characteristics under the low temperature condition in 60 days

from control(black)and transgenic plants (green). Transgenic plants showed significantly high growth than the control ($P < 0.05$)



Figure 9

Flowering status of transgenic and non-transgenic plants(left; non-transgenic plant, right; transgenic plant).



Figure 10

Rust infection of transgenic and non-transgenic plantlets(black; transgenic plants, grey; control). Rust infection under pot culture condition in 60 days from control(grey) and transgenic plants (black).

Transgenic plants showed significantly high rust resistance than the control