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Regulation of A 9-*cis*-epoxycarotenoid Dioxygenase (*NCED*) Gene from Raspberry on High Salinity and Cold Tolerance

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Abstract: Abscisic acid (ABA) is a plant hormone that plays an important role in plant development and abiotic stress. The 9-*cis*-epoxycarotenoid dioxygenase (NCED) is a key rate-limiting enzyme in the ABA biosynthetic pathway. The physiological and molecular mechanisms of NCED regulating plant development and abiotic stress tolerance have been reported in many plant species, but gene function of *RiNCEDs* in *Rubus idaeus* L. is rarely reported. In this study, the open reading frame (ORF) sequence of *RiNCED*2 in red raspberry fruit was isolated and the function of this gene under abiotic stress was investigated. While *RiNCED*2 was induced by cold, high salinity, drought and ABA, it was highly expressed in new leaves as measured by real-time qPCR. Overexpression of *RiNCED*2 in *Arabidopsis* under both high-salt and cold stress increased ABA content, demonstrating that *RiNCED*2 was involved in ABA biosynthesis. Meanwhile, the leaf wilting degree of transgenic *Arabidopsis* was less, while the content of malondialdehyde (MDA) was significantly reduced, and the chlorophyll content, proline content, peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD) activities were significantly increased. These results indicated that overexpression of *RiNCED*2 enhanced the resistance of transgenic *Arabidopsis* to high salt and cold.

Key words: 9-*cis*-epoxycarotenoid dioxygenase, raspberry, high salt, cold

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Introduction

Abscisic acid (ABA) is a plant hormone regulating the essential physiological processes of plants and plant responses to various environmental stresses (Chen *et al*., 2020; Brookbank *et al*., 2021). It was reported to involve in many plant growth and development processes, such as promoting stomatal closure, inhibiting growth, promoting dormancy and promoting the formation of potato tubers (Raghavendra *et al*., 2010; Yoshida *et al*., 2014; de Zelicourt *et al*., 2016; Ha *et al*., 2018). ABA also plays important roles in coping with abiotic stress, participating in signal transduction pathway and promoting gene expression (Danquah *et al*., 2014; Huang *et al*., 2016; Ma *et al*., 2018). A large number of studies show that plants enhance their resistance to external stress environments by increasing ABA levels (Lee and Luan, 2012). Under stress, the content of ABA in plants increases rapidly, and the expression of many stress-related genes that induced by ABA changes significantly. This indicates

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that ABA is involved in the signal transmission of plants under stress (Vishwakarma *et al*., 2017). In recent years, the cloning and functional identification of key genes in ABA biosynthesis have attracted more and more attention.

Previous studies have shown that ABA synthesis in higher plants is mainly through an indirect pathway of carotenoid cleavage, including the synthesis of epoxide carotenoid precursors in plastids, the formation and cleavage of lutein in plastids, and the final synthesis of ABA through cytoplasmic reactions (Nisar *et al*., 2015). The 9-*cis*-epoxycarotenoid dioxygenase catalyzes the reaction of 9-*cis*-violaxanthin and 9-*cis*-neoxanthin cleaves to produce the luteal aldehyde, which is a precursor for ABA synthesis (Verslues, 2016). A large number of studies have proved that *NCED* plays a very important role in the biosynthesis of ABA. Under the same culture conditions, the expression of *SlNCED*1 and *SlNCED*2 in tomato is inhibited, and the expression level of ABA is only 20% to 50% of that in normal tomato. Whereafter, the carotenoid content of transgenic tomato fruits during the ripening period is analyzed. And the results showed that the contents of lutein, zeaxanthin, lycopene, *β*-carotene and other carotenoids in the pulp and peel of transgenic tomato are 30%-45% and 34%-50% higher than those of wild tomato, respectively (Sun *et al*., 2012). This proved that *NCED* genes play a role in carotenoid decompose and ABA synthesis in plants. When the activity of NCED is inhibited, the synthesis of ABA is hindered, which is manifested by a decrease in ABA content and a decrease in the accumulation of carotenoid precursors. Because NCED-mediated processes are critical in ABA synthesis, it is considered a key rate-limiting enzyme in ABA synthesis (Melhorn *et al*., 2008). At present, *NCED* genes of many species have been identified and reported, such as cowpea, cassava, grape, sweet cherry, strawberry, begonia, etc (Iuchi *et al*., 2000; Arango *et al*., 2010; Priya and Siva, 2015; Zhang *et al*., 2015). Previous studies have shown that ABA synthesis is regulated by different *NCED* genes at different stages of plant tissue growth and development. For example, *AtNCED*3 in *Arabidopsis* is mainly expressed in leaves and responds to water stress (Endo *et al*., 2008), while *AtNCED*6 and *AtNCED*9 are mainly expressed in germinating seeds and regulate ABA synthesis (Lefebvre *et al*., 2006). There are also a large number of studies on the function of *NCED* genes in different plants. For example, the mRNA level of *PtNCED*1 decreases during the germination of mature seeds of *Phaius tankervilleae* accompanied by a significant decrease in endogenous ABA content, after seed germination, the expression level of *PtNCED*1 in protocorm increases with the increasing of ABA content under dehydration stress, indicating that *PtNCED*1 is involved in the development of protocorm and seed dormancy and germination by regulating endogenous ABA content (Lee *et al*., 2018).

In recent years, researchers have paid more and more attention to the role of *NCED* genes of plant in response to abiotic stress. The *OsNCED*3 mutants *nced*3-1 and *nced*3-2 are obtained by CRISPR/cas9 technology in rice. Compared with the control, the two *OsNCED*3 mutants with earlier germination under water stress, have a longer growth period after seedling germination, and are sensitive to water stress and H_2O_2 stress. Further analysis showed that the content of ABA in rice mutants *nced*3-1 and *nced*3-2 is significantly lower than that of the control, proving that *OsNCED*3 regulates seed dormancy, plant growth, abiotic stress tolerance and leaf senescence (Huang *et al*., 2018). Overexpression of *VaNCED*1 from Zuoshan-1 (a drought resistant grape cultivar) in Thompson seedless (a drought sensitive grape cultivar) increases the endogenous ABA content, and enhances drought resistance of Thompson seedless. At the same time, overexpression of *VaNCED*1 also induces the accumulation of jasmonic acid (JA) and upregulation of JA biosynthesis-related genes. In addition, *ABF*2 (ABRE binding factors 2), *PIP*2 (plasma membrane intrinsic proteins 2), *VvCBF*4 (DRE-binding factor 4) and some other drought-responsive genes are also increased to varying degrees in transgenic plants (He *et al*., 2018). These studies showed that *NCED*

genes not only affect the synthesis of ABA in plants, but also play an important role in plant growth, development and resistance to external stresses.

Raspberry (*Rubus idaeus* L.) is an important commercial crop due to its rich in nutritional value and organoleptic characteristics (Probst, 2015). Abiotic stress is one of the main limiting factors for raspberry yield. High salt and cold cause the economic loss of raspberry. This is the primary limiting factors for the expansion of raspberry production in Northeast China. Therefore, in this study, the functions of *RiNCED*2 in raspberry were investigated and the regulation of overexpression of *RiNCED*2 in *Arabidopsis* increasing high salt and cold stress tolerance through the accumulation of ABA was found. These results demonstrated that *RiNCED*2 was served as an important candidate gene for improving high salt and cold stress tolerance in raspberry.

Materials and Methods

Plant materials and treatments

The red raspberry (*Rubus idaeus* L.cv. Heritage) collected in Binxian County, Harbin City, Heilongjiang Province was used as the test material in this study. The planting row spacing was 1 m×2 m, and the collection time was August, 2017. The *Arabidopsis* ecotype Columbia (Col-0) was used. The *Arabidopsis* seeds were surface-sterilized and then planted on MS (Murashige and Skoog, 1962). The plates were kept in darkness at 4℃ for 3 days to germination synchronously, then under a 16 light/8 h dark photoperiod.

The rooted tissue culture plantlets of Heritage (*Rubus idaeus* L.cv.) were transferred to Hoagland nutrient solution at a relative humidity of 80%. The nutrient solution should be replaced every 3-4 days. When the leaves were fully unfolded, seedlings were divided into four parts and treated with salt stress (200 mmol·L^{-1} NaCl solution), cold stress (4℃ growth incubator), drought stress (15% PEG solution) and ABA treatment (100 μmol·L^{-1}) (Yang *et al*., 2021). Seedlings cultured with normal Hoagland nutrient solution were used as control. After treatment for 0, 1, 5, 9, 12, and 24 h, the plant materials were immediately obtained and frozen with liquid nitrogen and stored at −80℃ for RNA extraction.

Gene cloning and sequence analysis

The total RNA was extracted using OminiPlant RNA Kit (CWBIO, Beijing, China) according to the manufacturer's protocols. The quality and quantity of extracted RNA were detected by UV spectrophotometer and 1.0% agarose gel electrophoresis, respectively. For each sample, 1 μg RNA was reverse transcribed into the first-strand cDNA using the Reverse Transcriptase Kit (TaKaRa Bio, Dalian, China). The sequence was queried with the coding sequences (CDS) of strawberry *NCED*1, and BLASTN was performed in Genome Database for Rosaceae (GDR: (https://www.rosaceae.org/) (Jung, 2013), and the open reading frame (ORF) of *RiNCED*2 was acquired. A pair of gene-specific primers was used to amplify the ORF of *RiNCED*2 using raspberry fruit cDNA as a template (Table 1).

Table 1 List of primers

Primer name	Primer sequence	Purpose
NCED2-F	GGGGTACCATGGCTACTCCTTCTAATAGT	Full-length cDNA of *RiNCED*2
NCED2-R	GCTCTAGACTATGCCTGATTTTTCAAGTC	Full-length cDNA of *RiNCED*2
NCED2-qF	AATGCTCGTAACGCCGAC	qPCR
NCED2-qR	CCAGCAGGTGGTGTTCAA	qPCR
Actin-F	CTACCTATTGTAAGGAATGGTGCCT	qPCR
Actin-R	TTCTGCATCCGAGATATCAAGTAGT	qPCR

Multiple sequence alignments among the amino acid sequences of *RiNCED*2 and *NCED* coded proteins from other plant species were analyzed using DNAMAN (version 5.2.2, LynnonBiosoft, San Ramon, CA,

America). Protein evolution tree was obtained using MEGA 5.0 (MEGA, Auckland City, New Zealand) software (neighbor joining method). Homologous proteins of *RiNCED*2 were obtained through BLASTN in the NCBI database.

Vector construction and plant transformation

Primers containing *Kpn* I (forword) and *Xba* lI (reverse) enzyme sites were designed. After double digestion and purification, the amplifiers were inserted into the digested linear pCAMBIA1300s by T_4 DNA Ligase (TaKaRa Bio, Dalian, China) to form 35S::*RiNCED*2. In order to obtain stable transgenic lines of *RiNCED*2 and empty vector, the recombinant vector 35S:: *RiNCED*2 and empty vector pCAMBIA1300s were transformed into *Agrobacterium tumefaciens* GV3101, respectively, then transformed into *Arabidopsis* by the floral-dip transformation method (Clough and Bent, 1998). The seeds of T_0 were selected in 1/2 MS medium supplemented with 50 mg·L^{-1} kanamycin. The selfing offsprings were further detected by PCR. Finally three homozygous of T_3 positive lines were randomly selected for further analysis.

Stress treatments

For stress treatment, 25 plants of the transgenic *Arabidopsis* lines and Col-0 were grown in soil under normal conditions for three weeks, then treated with 200 mmol·L^{-1} NaCl for 7 days or 4℃ for 10 h, next watered for 7 days to recover, and then calculated the survival rates. The plants in normal conditions were used as controls.

Physiological measurements

ABA content measurement was performed as previously described with some slight changes (Huang *et al*., 2018). Leaves (300 mg) were collected from three-leaf stage seedlings and ground in liquid nitrogen. ABA content was quantified using HPLC with Water LC-MS Xevo TQ liquid mass spectrometry. The mobile phase was 75% methanol. The column temperature was 30℃. The chlorophyll content was determined using UV2400 UV/VIS spectrophotometer by detecting the absorbance at 663 nm and 645 nm. The determination of free proline content, malondialdehyde (MDA) content, superoxide dismutase (SOD) activity, catalase (CAT) activity and peroxidase (POD) activity were based on previous studies (Dong *et al*., 2003; Ozkur *et al*., 2009; Zhang *et al*., 2011; Ranieri *et al*., 2000).

Data processing and analysis

All experiments were repeated at least three biological replicates for each treatment. The data were analyzed with SPSS 13.0 software. Differences determined using Student's *t*-test were considered statistically significant at $P \leqslant 0.05$ and $P \leqslant 0.01$. Data were shown as the mean ± SD from three independent experiments.

Results

Isolation and sequence analysis of *RiNCED*2

The *RiNCED*2 (Ro03_G17438) was identified in the GDR database (https://www.rosaceae.org/). The ORF sequence of *RiNCED*2 was cloned from the ripening raspberry (*R. idaeus* L. cv. Heritage) fruit by PCR amplification. The ORF of *RiNCED*2 was 1 821 bp long and encoded a protein that might contain 606 amino acid residues (Fig. 1A). Calculated by the online software tool ProtParam, the isoelectric point of the protein was 6.24 and the molecular weight was 66.478 ku. By comparing the amino acid sequences of RiNCED2 protein with other species with higher homology, it was found that RiNCED2 protein had a conserved domain of NCED protein (Fig. 1A).

The full length of RiNCED2 protein and some previously reported NCED proteins from some plants (Hao *et al*., 2009; Jia *et al*., 2011; Chernys and Zeevaart, 2000; Tung *et al*., 2008; Qin and Zeevaart, 2002; Huang *et al*., 2018) were manually analyzed using multiple sequence alignment to analyze the phylogeny of NCED proteins in plants. As revealed by multiple sequence alignment, RiNCED2 was most homologous to RcNCED2 of *Rosa chinensis* (Fig. 1B).

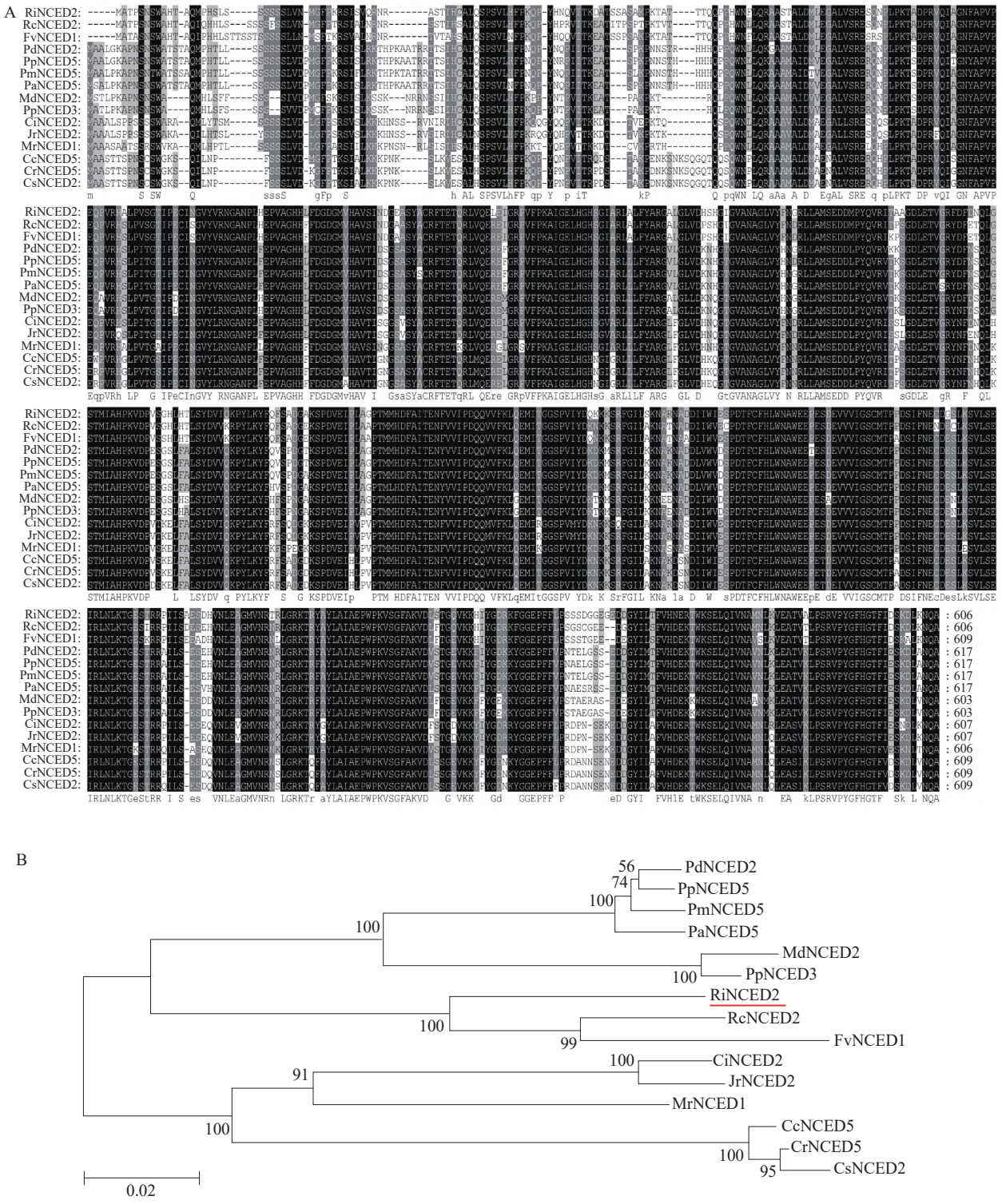

Fig. 1 Multiple sequence alignments and phylogenetic relationship of RiNCED2 with other plant NCED proteins

A, Multiple sequence alignments of RiNCED2 protein with other plant NCED proteins; B, Phylogenetic tree of RiNCED2 (indicated by a red line) and other plant NCED proteins. The accession numbers are as follows: PdNCED2 (*Prunus dulcis*, XP_034213444.1), PpNCED3 (*Pyrus pyrifolia*, AJO53633.1), PpNCED5 (*Prunus persica*, XP_020418325.1), PmNCED5 (*Prunus mume*, XP_008225495.1), PaNCED5 (*Prunus avium*, XP_021834179.1), MdNCED2 (*Malus domestica*, XP_008371610.2), RcNCED2 (*Rosa chinensis*, XP_024161283.1), FvNCED1 (*Fragaria vesca* subsp. *vesca*, XP_004293530.1), CiNCED2 (*Carya illinoinensis*, XP_042939831.1), JrNCED2 (*Juglans regia*, XP_018820621.1), MrNCED1 (*Morella rubra*, KAB1222596.1), CcNCED5 (*Citrus clementina*, XP_006449708.1), CrNCED5 (*Citrus reticulata*, ASK51187.1) and CsNCED2 (*Citrus sinensis*, NP_001275868.1).

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Expression analysis of *RiNCED*2 in red raspberry

Under control conditions, the expression level of *RiNCED*2 in new leaves was higher than that in old leaves, roots and stems (Fig. 2A). Under cold and drought conditions, the expression of *RiNCED*2 increased rapidly, reaching a maximum at 5 h in the new leaves and then showing a downward trend (Fig. 2B). Under high salinity stress and ABA treatment, the expression level of *RiNCED*2 gradually increased after 9 h and then decreased gradually (Fig. 2B). The results showed that cold, high salt, drought and ABA induced *RiNCED*2 expression in new leaves.

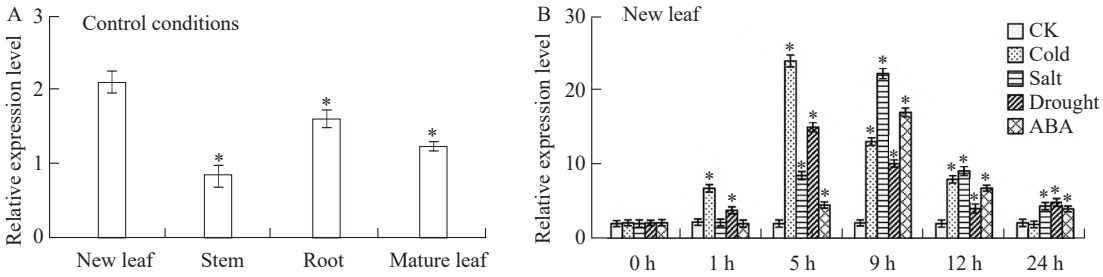

Fig. 2 Expression of *RiNCED*2 gene in different tissues and organs of raspberry and under different abiotic stress

A, Expression of *RiNCED*2 gene in different tissues and organs of raspberry. B, Expression of *RiNCED*2 gene under control condition (CK), low temperature, high salt, drought and ABA for different time. Data represent means and standard errors of three replicates. Asterisks above columns indicate significant difference compared to that in control condition (*$P \leqslant 0.05$).

*RiNCED*2 increasing endogenous ABA biosynthesis and enhancing cold tolerance

To confirm the function of *RiNCED*2 in response to cold, transgenic *Arabidopsis* lines with overexpression of *RiNCED*2 under the control of the CaMV 35S promoter and the empty vector were generated. Among all the T_2 generation transformed lines, the target fragments could be detected in seven transformed lines (strain 1-strain 7, indicated by S1-S7) by RT-PCR with a wild-type (WT, Columbia-0) *Arabidopsis* line as a negative control (Fig. 3A). When the T_3 generation of transgenic lines (S3, S5, S7, randomly selected), transgenic of empty vector (VL) lines and WT plants were cultured under control conditions, both transgenic plants and WT plants grew well, and there was no significant difference in phenotype (Fig. 3B). The plants were treated at 4℃ for 10 h and transferred to a normal condition after 7 days for recovery. The leaves of WT and VL exhibited enhanced yellowing than transgenic lines (Fig. 3B). The average survival rate of *RiNCED*2 overexpressing lines was 29.9%, but the survival rate of VL was just 18.7% and WT was only 14.7% (Fig. 3C). To understand whether endogenous ABA was involved in cold tolerance, the ABA levels in leaves of *Arabidopsis* seedlings were analyzed before and after cold treatment. The results showed that the ABA content of the control group was relatively lower before and after the 4℃ treatment. After treatment at 4℃ for 10 h, the expression level of ABA in the WT was about 50% of that in transgenic *Arabidopsis* plants. Interestingly, it was observed that the ABA content in transgenic plants approximately increased to 5 times that of the control level, which was about 1.8 times higher than that of WT and VL (Fig. 3D).

Furthermore, some physiological indicators related to salt stress were measured to further explore the function of *RiNCED*2. In the control condition, there were no significant differences in chlorophyll content, proline content, MDA content, POD, CAT and SOD enzyme activities between transgenic lines and the WT. After 4℃ treatment, chlorophyll content, proline content, SOD, POD and CAT activities of transgenic lines were significantly higher than those of the WT and VL (Fig. 4A, B, D-F), while MDA content was significantly lower than that of the WT and VL

(Fig. 4C). These results showed that the WT and VL plants suffered more serious membrane damage than transgenic plants. Hence, the results suggested that overexpressing *RiNCED*2 gene could scavenge the intracellular reactive oxygen species (ROS) by increasing the enzyme activities of SOD, POD and CAT. These results demonstrated that *RiNCED*2 played a positive role in cold tolerance in raspberry.

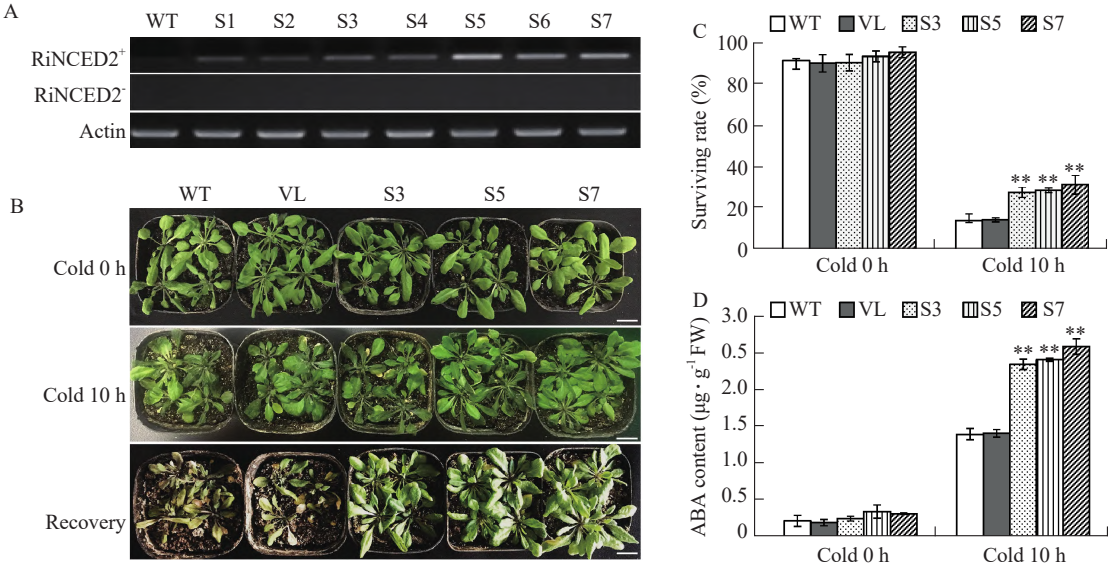

Fig. 3 Overexpression of *RiNCED*2 in *Arabidopsis* improved cold tolerance

A, Expression levels of *RiNCED*2 in wild-type (WT) and T_2 transgenic *Arabidopsis* lines visualized by semi-quantitative RT-PCR using *RiNCED*2 specific primer (RiNCED2$^+$) and *RiNCED*2 non-specific primer (RiNCED2$^-$). Actin is used as control; B, Phenotypes of *RiNCED*2 transgenic *Arabidopsis* lines: strain 3 (S3); strain 5 (S5); strain 7 (S7), wild type (WT) plants and empty vector (VL) transgenic *Arabidopsis* line under cold stress for 0 h, 10 h and recovery. Scale bar corresponds to 1 cm; C, Survival rate of WT, VL and transgenic lines after recovery with or without cold treatment; D, ABA content in transgenic lines and WT before and after cold treatment (**$P \leqslant 0.01$).

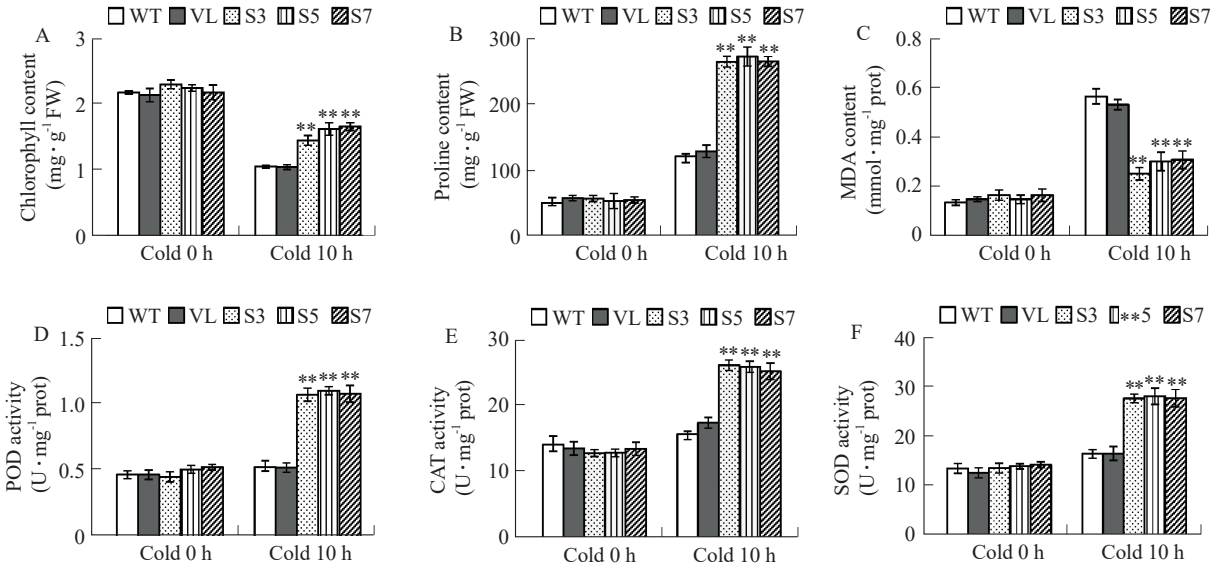

Fig. 4 Physiological indexes of *Arabidopsis* overexpressing of *RiNCED*2 under cold stress

A, Chlorophyll content; B, Proline content; C, Malondialdehyde (MDA) content; D, Peroxidase (POD) activity; E, Catalase (CAT) activity; F, Superoxide dismutase (SOD) activity. Data represent means and standard errors of three replicates. Asterisks above columns indicate significant difference compared to WT (**$P \leqslant 0.01$).

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RiNCED2 contribution to ABA accumulation under high salt stress

To confirm the function of *RiNCED*2 in response to high salt, 3-week-old seedlings of transgenic lines (S3, S5, S7 and VL) and wild type plants (WT) were treated with 200 mmol·L^{-1} NaCl for 7 days and transferred to a normal condition after 7 days for recovery, and the overexpression plants exhibited higher resistance than wild type plants and empty vector transgenic *Arabidopsis* lines (VL). The leaves of all transgenic lines remained green after NaCl treatment, but some of the leaves of WT and VL exhibited enhanced yellowing (Fig. 5A). The average survival rate of three overexpressing lines of *RiNCED*2 was 70.6%, but the survival rate of VL was 46.0%, and WT was only 44.7% (Fig. 5B). Moreover, the ABA levels of transgenic lines with *RiNCED*2 were significantly higher than those in the WT and VL (Fig. 5C).

In agreement with the results of salt stress, the transgenic plants also had higher chlorophyll content, proline content, MDA content, POD, CAT and SOD enzyme activities and lower MDA content after cold treatment (Fig. 5D-I). These results demonstrated that *RiNCED*2 played a positive role in the cold tolerance of raspberries by the same mechanism as salt tolerance.

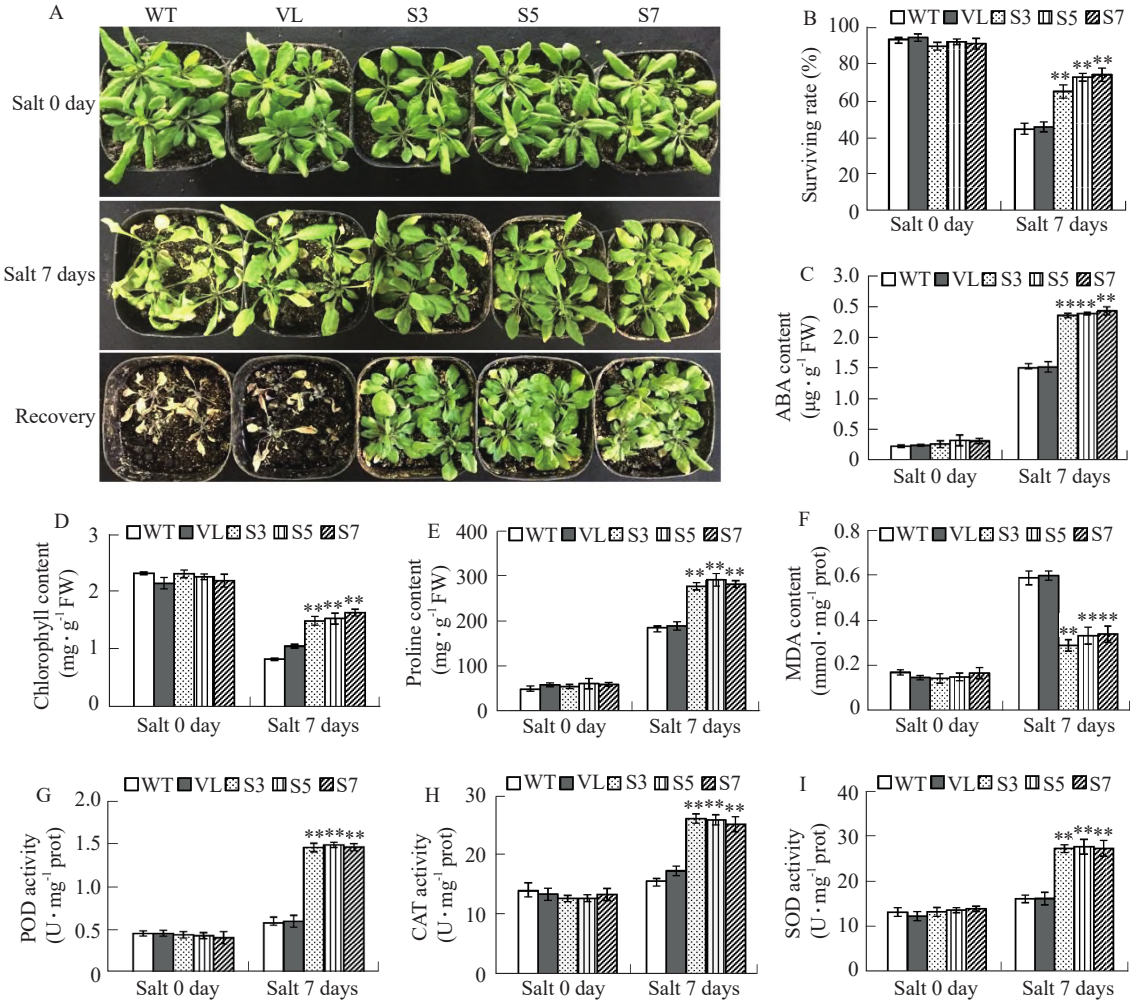

Fig. 5 Overexpressing of *RiNCED*2 improved salt tolerance in *Arabidopsis*

A, Phenotypes of *RiNCED*2 transgenic *Arabidopsis* lines (S3, S5 and S7), wild type plants (WT) and empty vector transgenic *Arabidopsis thaliana* lines (VL) under salt stress for 0 day, 7 days and recovery. Scale bar corresponds to 1 cm. B, Survival rate of WT, VL and transgenic lines after recovery with or without salt treatment. C, ABA content in transgenic and WT *Arabidopsis* before and after salt treatment. D, Chlorophyll content; E, Proline content; F, MDA content; G, POD activity; H, CAT activity and I, SOD activity. Data represent means and standard errors of three replicates. Asterisks above columns indicate significant difference compared to WT (**$P \leqslant 0.01$).

Discussion

Sequence analysis of NCED proteins

ABA was an essential hormone for seed dormancy, stomata aperture, plant development and abiotic stress tolerance. The levels of ABA in plants were regulated by its *de novo* biosynthesis. NCED was the critical rate-limiting enzyme in the ABA biosynthetic pathway (Huang *et al*., 2018). In this research, *RiNCED*2 was a member of the NCED family cloned from raspberry (*Rubus idaeus* L.cv. Heritage), although the partial function of NCED had been reported, its native functional characteristics in raspberry were still unclear. The *NCED* gene (*RiNCED*1) in raspberry had been reported (Yang *et al*., 2021). The similarity of amino acid sequences between RiNCED1 protein and RiNCED2 protein was 69.14%. The same as *RiNCED*2, overexpression of *RiNCED*1 in *Arabidopsis* also increased high salt and cold stress tolerance. It was found that there was a double NCED regulatory mechanism to ensure ABA synthesis in peach, and both PpNCED1 and PpNCED5 isozymes promoted ABA biosynthesis, which was likely to accelerate cell senescence through activating ROS signals (Melhorn *et al*., 2008). So, it was deduced that *RiNCED*1 and *RiNCED*2 in raspberry might be the isozymes increasing the tolerance to abiotic stresses in ABA biosynthesis.

Previous studies had shown that increased *NCED* transcript levels could promote ABA biosynthesis and increase ABA accumulation in plants (Martinez-Andujar *et al*., 2011; Lee *et al*., 2021). This study showed that RiNCED2 protein had high homology with the NCED proteins found in other species. The tissue-specific expression analysis showed that *RiNCED*2 was expressed in the leaves, root and stem of raspberry, and the expression level in the new leaves was the highest. The expression of *RiNCED*2 was also detected by treating the tissue culture seedlings of raspberry with various stresses. The results showed that *RiNCED*2 was induced by drought, cold, salt and ABA, and the response to cold and salt was the most intense. These results showed that *RiNCED*2 might be involved in abiotic stress response to plant ABA dependent pathway.

NCED genes response to high salt and cold stress

The *NCED* genes contributed to the elevation of ABA levels, which promoted abiotic stress tolerance in plants (Bang *et al*., 2013). In this study, the overexpression vector of *RiNCED2* was constructed, and the transgenic *Arabidopsis* material was obtained by *Agrobacterium* mediated genetic transformation. This study showed that the expression of *RiNCED*2 was significantly induced by cold and NaCl stress. Proline was an osmolyte that could facilitate adaptation to water deficient conditions (Khalil *et al*., 2016; Hussain *et al*., 2022). The results suggested that the *RiNCED*2-overexpresing plants were resistance to cold and salt stress, which could possibly be attributed to the fact that they had a high proline content. Similar observations were observed in *BnNCED*3 transgenic plants (Xu and Cai, 2017). Furthermore, ABA was an important hormone to promote stomatal closure, it reduced water loss by regulating stomatal closure in order to acclimate to some abiotic stresses (Edel and kudla, 2016; Finkelstein, 2013). The endogenous ABA content of *RiNCED*2-overexpresing transgenic plants increased after salt and cold, and the degree of leaf wilting decreased, and its MDA content was lower than that of wild type, which was consistent with the observation that *AtNCED*3 and *AtNCED*5 regulated ABA accumulation under water stress (Frey *et al*., 2012), indicating that its resistance was enhanced. These results showed that the overexpression of *RiNCED*2 might reduce the stomatal aperture and reduce the transpiration water loss of leaves, thus enhancing salt and cold resistance of transgenic materials.

Conclusions

In summary, the overexpression of *RiNCED*2 in *Arabidopsis* improved ABA content, which proved

that *RiNCED*2 was involved in ABA biosynthesis. Under high salt and low temperature stress, the transgenic *Arabidopsis* showed higher chlorophyll content, proline content, POD activity, CAT activity, SOD activity and lower MDA content than those in wild-type and empty vector transgenic plants. These results indicated that the overexpressing of *RiNCED*2 gene increased plant resistant to high salt and low temperature.

References

Arango J, Wüst F, Beyer P, *et al*. 2010. Characterization of phytoene synthases from cassava and their involvement in abiotic stress-mediated responses. *Planta*, **232**(5): 1251-1262.

Bang S W, Park S H, Jeong J S, *et al*. 2013. Characterization of the stress-inducible *OsNCED*3 promoter in different transgenic rice organs and over three homozygous generations. *Planta*, **237**(3): 211-224.

Brookbank B P, Patel J, Gazzarrini S, *et al*. 2021. Role of basal ABA in plant growth and development. *Genes* (*Basel*), **12**(12): 1936.

Chernys J T, Zeevaart J A. 2000. Characterization of the 9-*cis*-epoxycarotenoid dioxygenase gene family and the regulation of abscisic acid biosynthesis in avocado. *Plant Physiol*, **124**(1): 343-353.

Chen K, Li G J, Bressan R A, *et al*. 2020. Abscisic acid dynamics, signaling, and functions in plants. *J Integr Plant Biol*, **62**(1): 25-54.

Clough S J, Bent A F. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J*, **16**(6): 735-743.

Danquah A, de Zelicourt A, Colcombet J, *et al*. 2014. The role of ABA and MAPK signaling pathways in plant abiotic stress responses. *Biotechnol Adv*, **32**(1): 40-52.

De Zelicourt A, Colcombet J, Hirt H. 2016. The role of MAPK modules and ABA during abiotic stress signaling. *Trends Plant Sci*, **21**(8): 677-685.

Dong J X, Chen C H, Chen Z X. 2003. Expression profiles of the *Arabidopsis* WRKY gene superfamily during plant defense response. *Plant Mol Biol*, **51**(1): 21-37.

Edel K H, Kudla J. 2016. Integration of calcium and ABA signaling. *Curr Opin Plant Biol*, **33**: 83-91.

Endo A, Sawada Y, Takahashi H, *et al*. 2008. Drought induction of *Arabidopsis* 9-*cis*-epoxycarotenoid dioxygenase occurs in vascular parenchyma cells. *Plant Physiol*, **147**(4): 1984-1993.

Finkelstein R. 2013. Abscisic acid synthesis and response. *Arabidopsis Book*, **11**: e0166.

Frey A, Effroy D, Lefebvre V, *et al*. 2012. Epoxycarotenoid cleavage by NCED5 fine-tunes ABA accumulation and affects seed dormancy and drought tolerance with other NCED family members. *Plant J*, **70**(3): 501-512.

Ha Y M, Shang Y, Yang D, *et al*. 2018. Brassinosteroid reduces ABA accumulation leading to the inhibition of ABA-induced stomatal closure. *Biochem Bioph Res Co*, **504**(1): 143-148.

Hao G P, Zhang X H, Wang Y Q, *et al*. 2009. Nucleotide variation in the NCED3 region of *Arabidopsis thaliana* and its association study with abscisic acid content under drought stress. *J Integr Plant Biol*, **51**(2): 175-183.

He R, Zhuang Y, Cai Y, *et al*. 2018. Overexpression of 9-*cis*-epoxycarotenoid dioxygenase cisgene in grapevine increases drought tolerance and results in pleiotropic effects. *Front Plant Sci*, **9**: 970.

Huang Y C, Niu C Y, Yang C R, *et al*. 2016. The heat-stress factor HSFA6b connects ABA signaling and ABA-mediated heat responses. *Plant Physiol*, **172**(2): 1182-1199.

Huang Y, Guo Y M, Liu Y T, *et al*. 2018. 9-*cis*-epoxycarotenoid dioxygenase 3 regulates plant growth and enhances multi-abiotic stress tolerance in rice. *Front Plant Sci*, **9**: 162.

Hussain N, Sohail Y, Shakeel N, *et al*. 2022. Role of mineral nutrients, antioxidants, osmotic adjustment and PSII stability in salt tolerance of contrasting wheat genotypes. *Sci Rep*, **12**(1): 12677.

Iuchi S, Kobayashi M, Yamaguchi-Shinozaki K, *et al*. 2000. A stress-inducible gene for 9-*cis*-epoxycarotenoid dioxygenase involved in abscisic acid biosynthesis under water stress in drought-tolerant cowpea. *Plant Physiol*, **123**(2): 553-562.

Jia H F, Chai Y M, Li C L, *et al*. 2011. Abscisic acid plays an important role in the regulation of strawberry fruit ripening. *Plant Physiol*, **157**(1): 188-199.

Khalil F, Rauf S, Monneveux P, *et al*. 2016. Genetic analysis of proline concentration under osmotic stress in sunflower (*Helianthus annuus* L.). *Breed Sci*, **66**(4): 463-470.

Lee S C, Luan S. 2012. ABA signal transduction at the crossroad of biotic and abiotic stress responses. *Plant Cell Environ*, **35**(1): 53-60.

Lee S U, Mun B G, Bae E K, *et al*. 2021. Drought stress-mediated transcriptome profile reveals NCED as a key player modulating drought tolerance in *Populus davidiana*. *Front Plant Sci*, **12**: 755539.

Lee Y I, Chen M C, Lin L, *et al*. 2018. Increased expression of 9-*cis*-epoxycarotenoid dioxygenase, *PtNCED*1, associated with inhibited

seed germination in a terrestrial orchid, *Phaius tankervilliae*. *Front Plant Sci*, **9**: 1043.

Lefebvre V, North H, Frey A, *et al*. 2006. Functional analysis of *Arabidopsis NCED*6 and *NCED*9 genes indicates that ABA synthesized in the endosperm is involved in the induction of seed dormancy. *Plant J*, **45**(3): 309-319.

Ma H, Liu C, Li Z, *et al*. 2018. ZmbZIP4 contributes to stress resistance in maize by regulating ABA synthesis and root development. *Plant Physiol*, **178**(2): 753-770.

Martínez-Andújar C, Ordiz M I, Huang Z, *et al*. 2011. Induction of 9-*cis*-epoxycarotenoid dioxygenase in *Arabidopsis thaliana* seeds enhances seed dormancy. *Proc Natl Acad Sci USA*, **108**(41): 17225-17229.

Melhorn V, Matsumi K, Koiwai H, *et al*. 2008. Transient expression of *AtNCED*3 and *AAO*3 genes in guard cells causes stomatal closure in *Vicia faba*. *J Plant Res*, **121**(1): 125-131.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plantarum*, **15**(3): 473-497.

Nisar N, Li L, Lu S, *et al*. 2015. Carotenoid metabolism in plants. *Mol Plant*, **8**(1): 68-82.

Ozkur O, Ozdemir F, Bor M, *et al*. 2009. Physiochemical and antioxidant responses of the perennial *xerophyte Capparis* ovata Desf. to drought. *Environ Exp Bot*, **66**(3): 487-492.

Priya R, Siva R. 2015. Analysis of phylogenetic and functional diverge in plant nine-*cis* epoxycarotenoid dioxygenase gene family. *J Plant Res*, **128**(4): 519-534.

Probst Y. 2015. A review of the nutrient composition of selected *Rubus* berries. *Nutr Food Sci*, **45**(2): 242-254.

Qin X, Zeevaart J A D. 2002. Overexpression of a 9-*cis*-epoxycarotenoid dioxygenase gene in *Nicotiana plumbaginifolia* increases abscisic acid and phaseic acid levels and enhances drought tolerance. *Plant Physiol*, **128**(2): 544-551.

Raghavendra A S, Gonugunta V K, Christmann A, *et al*. 2010. ABA perception and signalling. *Trends Plant Sci*, **15**(7): 395-401.

Ranieri A, Petacco F, Castagna A, *et al*. 2000. Redox state and peroxidase system in sunflower plants exposed to ozone. *Plant Sci*, **159**(1): 159-167.

Sun L, Yuan B, Zhang M, *et al*. 2012. Fruit-specific RNAi-mediated suppression of *SlNCED1* increases both lycopene and *β*-carotene contents in tomato fruit. *J Exp Bot*, **63**(8): 3097-3108.

Tung S A, Smeeton R, White C A, *et al*. 2008. Over-expression of *LeNCED*1 in tomato (*Solanum lycopersicum* L.) with the *rbcS*3*C* promoter allows recovery of lines that accumulate very high levels of abscisic acid and exhibit severe phenotypes. *Plant Cell Environ*, **31**(7): 968-981.

Verslues P E. 2016. ABA and cytokinins: challenge and opportunity for plant stress research. *Plant Mol Biol*, **91**: 629-640.

Vishwakarma K, Upadhyay N, Kumar N, *et al*. 2017. Abscisic acid signaling and abiotic stress tolerance in plants: a review on current knowledge and future prospects. *Front Plant Sci*, **8**: 161.

Xu P, Cai W. 2017. Functional characterization of the *BnNCED*3 gene in *Brassica napus*. *Plant Sci*, **256**: 16-24.

Yang G H, Chen Y X, Yu H, *et al*. 2021. Raspberry (*Rubus idaeus* L.) *NCED1* gene enhances high salinity and cold tolerance in *Arabidopsis*. *In Vitro Cell Dev-Pl*, **57**(5): 811-819.

Yoshida T, Mogami J, Yamaguchi-Shinozaki K. 2014. ABA-dependent and ABA-independent signaling in response to osmotic stress in plants. *Curr Opin Plant Biol*, **21**: 133-139.

Zhang L, Xi D M, Luo L, *et al*. 2011. Cotton *GhMPK*2 is involved in multiple signaling pathways and mediates defense responses to pathogen infection and oxidative stress. *FEBS J*, **278**(8): 1367-1378.

Zhang W, Yang H, You S, *et al*. 2015. *MhNCED*3, a gene encoding 9-*cis*-epoxycarotenoid dioxygenase in *Malus hupehensis* Rehd., enhances plant tolerance to Cl^- stress by reducing Cl^- accumulation. *Plant Physiol Bioch*, **89**: 85-91.