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Article

BcSRC2 interacts with BcAPX4 to increase ascorbic acid content for responding ABA signaling and drought stress in pak choi

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Abstract

As a reducing substance, ascorbic acid functioned well in abiotic and biotic stress. However, the regulatory mechanism of drought resistance is rarely known in pak choi. Here we found a gene *BcSRC2* containing a C2 domain that responds to ABA signal and drought regulation in pak choi. Silencing of *BcSRC2* reduces ascorbic acid content and drought resistance of pak choi. In *Arabidopsis*, BcSRC2 overexpression promotes ascorbic acid accumulation and increases drought tolerance. Meanwhile, transcriptome analysis between WT and *BcSRC2*-overexpressing pak choi suggests that ascorbic acid-related genes are regulated. BcSRC2 interacts with BcAPX4 and inhibit APX activity *in vitro* and *in vivo*, increasing the ascorbic acid content. We also found that drought stress increases ABA content, which reduces the expression of BcMYB30. BcMYB30 bound to the promoter of *BcSRC2* and reduced its expression. Overall, our results suggest that a regulatory module, BcMYB30-BcSRC2-BcAPX4, plays a central role in increasing ascorbic acid content for responding ABA-mediated drought regulation in pak choi.

Introduction

Pak choi [*Brassica campestris* (syn. *Brassica rapa*) ssp. *chinensis*] comes from China and is popular around the world. Pak choi can provide a balanced diet rich in fiber, soluble sugars and vitamins [1, 2]. However, salt, drought, extreme temperature, abiotic and biotic stresses influence the quality and productivity of pak choi [3, 4]. Drought stress causes water loss and affects plant growth and quality [5]. When water is scant, plants accumulate higher levels of reactive oxygen species (ROS), further impairing cell development and enzyme activity and leading to plant death [6]. Greater ROS productions cause enzyme inactivation, protein degradation and membrane lipid peroxidation [7].

Drought tolerance involves complex biological processes involving different response pathways. Drought stress accumulates abscisic acid (ABA). ABA affects stomatal closure to keep water in the plants. In plants, ABA transduces drought signals to ABA receptors, like PYRABACTIN RESISTANCE/PYR1-Like /Regulatory Components of ABA Receptor proteins. These ABA receptors interact with clade A protein phosphatases type 2C to form stable subsequences. The interaction complexes activate sucrose nonfermenting 1-related protein kinase 2, which further phosphorylates regulated genes [8–11]. The PYR/PYL/RCAR-PP2Cs-SnRK2s signaling pathway has been confirmed in several studies and is involved in MYB transcription factor genes [12, 13]. In *Arabidopsis*, MYB2 bind to the promoter of *drought-responsive 22* to participate in drought regulation [14]. AtMYB30, AtMYB60 and AtMYB96 act as an ABA signaling cascade to regulate drought stress by participating in redox oxidation [15–17].

ROS contain H_2O_2, OH^-, and O_2^- and are produced by unfavorable growth conditions [18]. Plants have evolved many methods to scavenge ROS, including nonenzymatic antioxidants (ascorbate, glutathione, and vitamin E) and enzymatic antioxidants (APX, GPX, CAT, SOD, and POD) [19]. Ascorbate protects cells from oxidative stress [20]. AsA has been confirmed to react directly with OH^- and O_2^- to produce H_2O and H_2O_2 [21]. AsA also acts as a reducing agent to produce e^-, which is used by APX to eliminate H_2O_2 [22, 23]. In *Arabidopsis*, low AsA content increased sensitivity to environmental stresses [24]. The regenerative metabolism of the ascorbic acid cycle has been well studied; how it responds to ABA signaling and drought stress is not yet clear. For pak choi, it is crucial to explore mechanisms of ascorbic acid regulation by ABA and drought signaling.

In this study, a previously identified gene, *BcSRC2*, containing a C2 domain was characterized. Silencing of *BcSRC2* significantly reduced AsA content and increased APX activity compared to control plants during drought treatment in pak choi. Transcriptome analysis between *BcSRC2*-overexpressed plants and wild type plants revealed an ascorbic acid metabolism-related gene, *BcAPX4*. BcSRC2 was found to interact with BcAPX4 and regulated its APX enzyme activity. These results showed that *BcSRC2* upregulated ascorbic acid content by inhibiting APX activity to combat drought. Here, we also found that *BcSRC2* responded to the ABA signal and reduced the sensitivity to ABA. In addition, a

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samples were analyzed with anti-Flag (Sigma-Aldrich) and anti-His (Abcam, America) antibodies.

Yeast one-hybrid assay and β-galactosidase activity

For yeast one-hybrid (Y1H) assay, the ORF of *BcMYB30* were cloned into pJG vector. The promoter of *BcSRC2* was fused into pLacZi vector (primers are listed in Table S1, see online supplementary material). The pIG-BcMYB30 and pLacZi-proBcSRC2 were co-transformed into EGY48 strain. The transformed cells were cultured on the SD-Trp/-Ura medium for 3 d at 28°C and then placed on SD/−Trp/-Ura/Gal/Raf/X-Gal (20 μg/mL) medium for 3–5 d. The combinations, pLacZi and pIG-BcMYB30, pLacZi and pJG, pLacZi-proBcSRC2 and pJG, were negative controls. The β-galactosidase Assay Kit (Beyotime, Beijing, China) was used to measure the β-galactosidase activity in different groups.

Transient dual-luciferase assays

For luciferase (LUC)/Renilla (REN) assays, the promoter of *BcSRC2* was inserted into pGreenII 0800-LUC vector as reporter, the ORF of BcMYB30 was constructed into overexpression vector as effectors (primers are listed in Table S1, see online supplementary material). Then the recombined vectors were transformed into *Agrobacterium* and co-expressed in 4-week-old tobacco leaves. The luciferase signal was observed using a charge-coupled device (CCD) camera after spraying with 100 mM D-luciferin. The LUC and REN activity were measured following the Dural-luciferase Reporter Assay System (Yeasen, Shanghai, China).

Electrophoretic mobility shift assay

The ORF sequence of BcMYB30 was fused into pET-28a vector and then transformed into *BL21* strain to extract BcMYB30-His proteins using HisSeq Ni-NTA Agarose gel (Beyotime, Beijing, China). Two 24-bp probes of *BcSRC2* promoter labelled with or without biotin label were designed and synthesized from the company (Sangon, Shanghai, China). The core *cis*-element, ACGTG, was turned to AAAAA as a mutant probe. The fusion protein and probes were incubated at 24°C for 30 min and then separated using a chemiluminescent EMSA kit (Thermo Fisher, California, USA).

Chromatin immunoprecipitation assay

The ChIP assay was described following the previous method [54]. In brief, 2 g *BcMYB30*-overexpressed pak choi leaves were treated with 0.1% formaldehyde for cross-linking the protein-DNA complexes. After isolation and ectraction, the supernatants were immunoincubated with Flag-antibody (Sigma-Aldrich). Then the protein-DNA complex was reversed and further DNA purification according to the EpiQuikTM Plant ChIP Kit (Base Catalog # P-2014-24, Epigentek, New York, USA). The purified DNA was used as templates for qPCR. Primers are listed in Table S1 (see online supplementary material).

Determination of ascorbic acid, abscisic acid content, and AsA-GSH pathway enzyme activities

The ascorbic acid content, DHA content, and total AsA content were extracted and measured using the HPLC system following the previous protocol [55]. POD and SOD activity were measured using the kit (Sangon, Shanghai, China).

After drought treatment, the leaves of control and silent lines were collected and extracted with 80% methanol overnight. Abscisic acid content was measured as described previously using a Liquid Dhromatography Coupled to Triple Quadrupole Mass Spectrometryto (Triple Quad 6500+, AB Siex) [56].

The enzyme activities in AsA-GSH pathway, GSH, GSSG, AAO, and DHAR were measure following the kits' protocols (Solarbio, Beijing, China). The ascorbate peroxidase activity (APX) consumed ascorbic acid to remove hydrogen peroxide using SpectraMax Paradigm. According to the decreasing value of OD_{290} per min, the APX activity was calculated. The solution was mixed with 0.1 mM EDTA-Na_2, 5 mM AsA, 20 mM H_2O_2, and 40 μL samples and then immediately determined the change of OD_{290} value in 1 min at 20°C.

DAB, NBT staining and malondialdehyde (MDA), H_2O_2 content and relative water contents (RWCs) measurements

The rosette leaves of WT and overexpression plants after 3 days ABA treatment were used for DAB and NBT staining. Briefly, leaves were cut into bottles and soaked in DAB buffer (1 mg/ml) or NBT buffer (0.5 mg/ml) in the dark at 100 rpm for 12 h. Then leaves were transferred into 95% ethanol and boiled at 95°C for 10 min. Finally, the color changes of leaves were observed. The MDA content and H_2O_2 content were measured as per the kits (Solarbio, Beijing, China). RWC was calculated following the previous study [57].

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Author contributions

Z.Y. designed the research and wrote the manuscript. X.C. analysed data. Z.C., H.W., S.H.A.H., T.L., and X.H. revised the manuscript. Y.L. advised on the design, results, and edited the manuscript. All authors read and approved the final manuscript.

Data availability

The RNA-seq data of WT and BcSRC2-overexpressed pak choi can be found in the NCBI database with the accession code PRJNA1063936. All data can be found in the main text.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

Supplementary data

Supplementary data is available at *Horticulture Research* online.

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