Functional Identification of 9-*Cis*-Epoxy-carotenoid Dioxygenase Genes in Double Dormant Plant-Herbaceous Peony

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Abstract

Seed dormancy and germination is a complex process, which is affected by external environmental conditions and internal factors independently or mutually. Phytohormones play an important regulatory role in this process. ABA was the main phytohormone affecting herbaceous peony seed dormancy release. However, the mechanism of ABA in the dormancy release of herbaceous peony needs to be further explored. Here, transcriptome data was screened from the perspective of ABA metabolism, and significantly differentially expressed *PINCED1* and *PINCED2* were obtained. We found that their expression trends were positively correlated with ABA content. Among them, *PINCED2* had a stronger regulatory effect on ABA content and was more sensitive to exogenous ABA. Overexpression and silencing of *PINCEs* in callus could affect the expression of *PICYP707As* and the content of endogenous ABA. Through the observation of seed germination of *Arabidopsis thaliana* (*A. thaliana*), we found *PINCED1* and *PINCED2* promoted seed dormancy, and the promotion effect of *PINCED2* was more obvious. In general, *PINCED1* and *PINCED2* participated in the dormancy release of herbaceous peony seeds by regulating the accumulation of endogenous ABA. Our work can reveal the molecular mechanism and related theories of ABA involved in herbaceous peony seed dormancy release.

Key Message

PINCED1 and *PINCED2* played a critical role in seed dormancy by regulating the capacity of the Absciscic acid (ABA) metabolism enzyme in herbaceous peony.

1. Introduction

Seed is the basic guarantee for the continuation of plant species (Bewley, et al. 2013). Seed dormancy is an ecophysiological characteristic of plants that adapt to the environment during long-term growth and development. From a biological perspective, seed dormancy can ensure the survival of species in harsh environments, reduce the competition between individuals in the same species, and prevent seed germination in unsuitable seasons (Bewley 1997). It plays a positive role in plant individuals, survival, evolution, and the protection of plant germplasm resources (Willis, et al. 2014). But in terms of the production of cultivated species, seeds often need to germinate quickly, neatly and grow quickly to obtain high economic yield (Lafta and Mou 2013; Mutlu, et al. 2020). At the same time, an early sprouting phenomenon caused by the lack of seed dormancy has a negative impact on the production of cereal crops (Finkelstein, et al. 2008). Therefore, based on the characteristics of seed dormancy and the actual needs of agricultural and forestry production, scholars have discussed this issue from different scientific perspectives. Seed dormancy release is accompanied by a series of physiological and biochemical reactions, including the repair of metabolic changes, decomposition and utilization of storage substances and energy metabolism (Li and Min 2020; Liu, et al. 2020; Vigliocco, et al. 2020). Seed dormancy is coordinately regulated by external stimulation (such as light, temperature and humidity, etc.) and endogenous factors (such as phytohormones, sugars and nitrogen compounds, etc.) (Chen, et al. 2020a; Chen, et al. 2021; Klupczynska and Pawlowski 2021; Malavert, et al. 2020; Sano and Marion-Poll 2021;

Wala, et al. 2021). Phytohormones are important growth regulators in this process. Ethylene (ETH) can negatively regulate seed dormancy by inhibiting ABA synthesis and signal transduction, or affect seed germination and early seedling growth by interacting with sugar signals (Naing, et al. 2021; Xia, et al. 2018). Brassinosteroid (BR) can reduce the sensitivity of seeds to ABA, thereby stimulating seed germination (Ha, et al. 2018; Kim, et al. 2019). Cytokinin (CTK) promotes seed germination by indirectly antagonizing with ABA (Shen, et al. 2020). Auxin also cannot independently regulate seed dormancy and germination, but positively regulate ABA signal by enhancing the sensitivity of seeds to ABA, thereby affecting seed dormancy and germination (Liu, et al. 2013; Munguia-Rodriguez, et al. 2020).

ABA content was positively correlated with seed dormancy (Tognacca and Botto 2021). The final concentration of endogenous ABA in plants depends on the dynamic balance of ABA synthesis and catabolism (Eggels, et al. 2018; Yan, et al. 2022). ABA biosynthesis pathway mainly includes C15 direct pathway and C40 indirect pathway. In higher plants, ABA is mainly synthesized through an indirect pathway. In the indirect pathway, zeaxanthin is used as the starting point to form violaxanthin catalyzed by zeaxanthin epoxidase (ZEP), and the violaxanthin is converted into 9'-*cis*-Neoxanthin and 9'-*cis*-Violaxanthin. Then these two by-products are oxidized to xanthoxin under the action of 9'-*cis*-epoxycarotenoid dioxygenase. Subsequently, xanthoaldehydes leave the plastid and enter the cytoplasm, which is catalyzed by short-chain dehydrogenase/reductase (SDR) to form ABA aldehyde, and finally oxidized by aldehyde oxidase (AAO) to form ABA (Taylor, et al. 2005). The biological decomposition pathways of ABA mainly include: oxidative binding inactivation and oxidative inactivation. In higher plants, ABA is mainly decomposed through oxidative inactivation. The oxidative inactivation pathway of ABA is divided into 7'-hydroxylation, 8'-hydroxylation and 9'-hydroxylation according to the methyl site, which generate 7'-hydroxy-ABA, 8'-hydroxy-ABA and 9'-hydroxy-ABA, respectively, and causing oxidative inactivation of ABA. Among the three oxidative inactivation modes, 8'-hydroxylation reaction is proved to be the most important metabolic pathway of ABA in higher plants. ABA is catalyzed by 8'-hydroxylase to form 8'-hydroxy-ABA, and its spontaneous isomerization generates phaseic acid (PA). PA eventually generates diammine phaseic acid under the action of PA reductase (PAR) (Okamoto, et al. 2006; Saito, et al. 2004; Weng, et al. 2016). Studies have shown that ABA-deficient mutants of *A. thaliana*, tomato and maize show early dormancy breaking and turning into germination stage, whereas ABA-overexpressing plants show delayed dormancy (Jia, et al. 2021; Martin-Rodriguez, et al. 2016; Qin and Zeevaart 2002; Wu, et al. 2014). It can be verified that the change of endogenous hormone ABA content is significantly positively correlated with the degree of seed dormancy (Wang, et al. 2015).

ABA is a key factor affecting seed dormancy and germination. Previous studies have been confirmed in many plants (Eggels, et al. 2018; Pan, et al. 2018; Shen, et al. 2018). After exogenous application of ABA inhibitors, the germination rate of *A. thaliana* seeds was significantly increased, and the ABA-deficient mutant seeds had no dormancy characteristics (Léon-Kloosterziel, et al. 1996; Lopez-Molina, et al. 2001). *NCED* and *CYP707A* encode key enzymes in ABA synthesis and decomposition pathways, respectively. Different members of family genes have different regulatory roles in plant seed dormancy. How they affect seed dormancy and their regulatory mechanism have been thoroughly studied in model plant - *A. thaliana*. *AtNCED* family members have different expression sites in seeds. *AtNCED6* is only expressed in

endosperm, while *AtNCED9* is expressed in both embryo and endosperm (Lefebvre, et al. 2006). They can not only regulate ABA content in *A. thaliana* embryo, but also promote and maintain embryo dormancy. *AtNCED5* is up-regulated at the late stage of seed maturation in *A. thaliana*, and cooperates with *AtNCED6* and *AtNCED9* to enhance seed dormancy (Frey, et al. 2012; Lefebvre, et al. 2006). *AtCYP707A1* mainly express in seed coat and endosperm at the middle stage of seed maturation, and the seed dormancy of *cyp707a1* mutant is significantly enhanced (Okamoto, et al. 2006; Wu, et al. 2022). *AtCYP707A2* is mainly expressed in the seed coat, embryo and endosperm of *A. thaliana* at the late stage of seed imbibition and maturation (Ikeya, et al. 2020). The ABA content of *A. thaliana cyp707a2* mutant seeds is five times that of wild-type *A. thaliana* seeds, and the seeds show deep dormancy (Chen, et al. 2020b; Saito, et al. 2004).

The effects of *NCED* and *CYP707A* on seed dormancy were also verified in other plants. During the imbibition process of *Zoysia japonica* seeds, *CYP707A* plays a leading role in the decrease of ABA content (Dong, et al. 2021). Overexpression of *Oryza sativa OsNCED3* in wild-type *A. thaliana* can increase ABA content and promote seed dormancy (Liao, et al. 2021). The expression of *PvNCED* in *Phaseolus vulgaris* can increase the seed dormancy (Enomoto, et al. 2017). Overexpression of *LeNCED1* in *Lycopersicon esculentum* delays seed germination (Thompson, et al. 2000). *AhNCED2* plays a positive role in maintaining seed dormancy in *Arachis hypogaea* (Bo, et al. 2010).

Herbaceous peony (*Paeonia lactiflora* Pall.) is the herbaceous perennial flower of *Paeoniaceae*. In the long-term systematic evolution process, the seeds of herbaceous peony form a unique double dormancy characteristic of upper and lower hypocotyls. In the breeding process, the dormancy is often not released or incompletely released, which greatly reduces the germination rate and seriously affects the actual cultivation and production, especially the breeding of new varieties and the process of hybrid breeding (Li 1999). At present, the research on the dormancy release technology of herbaceous peony seeds mainly focuses on mechanical breaking, low temperature and hormone treatment, endogenous inhibitor determination and so on (Ren 2016; Sun, et al. 2012; Zhang 2015). However, there are few studies on the molecular mechanism of genes related to seed dormancy release. The previous study of our laboratory found that the endogenous ABA content in herbaceous peony seeds was significantly negatively correlated with seed germination (Li, et al. 2020). Herbaceous peony seeds endogenous ABA content is mainly regulated by ABA synthesis key gene *PINCEdS* and metabolism key gene *PICYP707As* (Li, et al. 2020). In this study, from the perspective of ABA synthesis, ten family members of *PINCEd* were found from transcriptome data (Supplement Figure 1 and Figure 2). Finally, c53147_g1 (*PINCEd1*) and c69372_g1 (*PINCEd2*) with significant differential expression were selected as research objects. In order to further identify the role of *PINCEd1* and *PINCEd2* in seed dormancy release, we first cloned the full-length cDNA of *PINCEd1* and *PINCEd2* from herbaceous peony seeds, and determined the specific action sites of its encoded protein in plant cells. Finally, the role of *PINCEd1* and *PINCEd2* in ABA metabolism and seed germination was clarified through homologous and heterologous genetic transformation. The results of this study can enrich the related theory of herbaceous peony seed dormancy and provide a scientific basis for finding effective seed dormancy breaking methods of herbaceous peony in the future.

2. Materials And Methods

2.1 Plant material and growth condition

Herbaceous peony hybrid seeds ('Fen Yu Nu' × 'Fen Yu Lou') were harvested in the Shenyang Agricultural University germplasm resources nursery (Shenyang, Liaoning, China) in August 2019. The filled hybrid seeds were used in variable temperature wet sand storage. According to the anatomical structure observation during the herbaceous peony seeds dormancy release process (Fei, et al. 2017), seeds of the six key dormancy release stages were collected (Figure 1) and quick freezing in liquid nitrogen, stored at -80°C as the plant materials for cloning and analyzing gene expression experiments. Using cotyledons obtained from conventional embryo induction methods as explants to induce callus of herbaceous peony (Liu, et al. 2020). Callus induction and proliferation culture medium was MS + 0.5 mg/L 2,4-D + 0.5 mg/L NAA + 0.5 mg/L TDZ + 1 g/L PVP + 30 g/L agar, the culture condition was 25°C, 2000 lx, 14 h/d, with the medium replaced every 30 d. Wild type (WT) *A. thaliana* (Col-0) and mutant seeds were grown following previously reported methods (Fei 2018). The *nced5-2* (GK_328D05), *nced9-1* (SALK_033388), *aba2-1* (CS156) and *aba3-1* (CS157) mutant were obtained from the Arabidopsis Biological Resource Center (ABRC, <http://abrc.osu.edu>). *A. thaliana* seeds and herbaceous peony callus were used for the function analysis experiment.

2.2 Exogenous hormone treatment

Based on the optimal concentrations of ABA and fluridone (FLU) that had significant effects on the herbaceous peony seed germination screened by our research group (Supplemental Table 1) (Song 2020), 30 mg/L ABA and 150 mg/L FLU were used to soak seeds in the dark for 24 h, and then carried out on variable temperature wet sand storage treatment. Six critical stages of herbaceous peony seeds were sampled for detected the key enzyme genes expression in the ABA synthesis pathway.

2.3 RNA extraction, cDNA synthesis and qRT-PCR

In our previous study, we found the key gene-*PINCEdS* in dormancy release process of herbaceous peony seeds by comparing transcriptome data. Next, we explored the expression pattern of *PINCEdS* in the dormancy release of herbaceous peony seeds by qRT-PCR. Total RNAs (S1-S6) were extracted by RNAprep pure Plant Kit (TianGen, China). The cDNAs were synthesized by PrimeScript™ RT Master Mix kit (Perfect Real Time) (Takara, China). The qRT-PCR primers were designed with the Primer Premier 5 software based on the full-length coding sequence (CDS) of *PINCEdS* in transcriptome. *PIACTIN* (GenBank accession no. JN105299.1) was used as the endogenous reference gene (Li, et al. 2020). The primers used for qRT-PCR are listed in Supplemental Table 2. qRT-PCR was performed with StepOne™ 7500 Real Time PCR using the TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, China). The reactions were accomplished according the three-step method, holding stage: 95°C for 30 s; cycling stage: 40 cycles of 95°C for 5 s, 60°C for 30 s; and melt curve stage: 95°C for 15 s, 60°C for 1 min, 95°C for 15 s. Each experiment was performed with three biological and technical replicates. The results were analyzed according to the $2^{-\Delta\Delta C_t}$ methods, and exhibited by GraphPad Prism 8.

2.4 Cloning and sequences analysis

We obtained the CDS of *PINCEdS* through transcriptome database. The amino acid sequences of *PINCEdS* were deduced using ORF Finder (<https://www.ncbi.nlm.nih.gov/orffinder/>). Multiple sequence alignments were performed using MEME software and constructed the phylogenetic tree by MEGA 7.0 software with the neighbor-joining method and bootstrap evaluation was setted 1000 replications, and iTOL v6 (<https://itol.embl.de/>) was used to optimize the trees. The conserved domains were predicted online at (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). The physicochemical properties of *PINCEdS* protein were analyzed using Expasy ProtParam tool (<http://web.expasy.org/protparam/>).

Using total RNA as template, 1st Strand cDNA was synthesized using 3' RACE Adaptor primers. According to the CDS of *PINCEdS*, we designed the gene specific outer and inner primers (Supplemental Table 2) to amplicate the 3' untranslated region (UTR) sequences of *PINCEdS* using 3'-Full RACE Core Set with PrimeScript™ RTase kit (Takara, China). The miRNA binding sites of 3' UTR sequences were predicted by MiRanda software.

Genomic DNA was extracted by Plant Genome DNA Rapid Extraction Kit (Aidlab, China). According to the verified known intronless sequence, three specific primers were designed, namely, SP1, SP2 and SP3 (Supplemental Table 2) to amplify the 5' end sequence of *PINCEdS* containing 5' UTR and promoter regions through Genome Walking Kit (Takara, China). *cis*-acting elements of promoter were analysed by PlantCARE.

2.5 Subcellular localization analysis

A. thaliana leaf protoplasts were extracted by *Arabidopsis* Protoplast Preparation and Transformation Kit (Coolaber, China) for subcellular localization. The CDS of *PINCEdS* were cloned into 16318-hGFP to construct the 16318-hGFP-*PINCEdS* recombinant vectors. The 16318-hGFP empty vector was used for a blank contrast. After 16 h of incubation in darkness, the green fluorescence protein (GFP) expression was captured by ultra-high-resolution laser scanning confocal microscope (Leica TCS SP8 STED).

2.6 Vector construction and plant transformation

The CDS of *PINCEdS* were recombined into pCAMBIA1300-35S-flag to construct pCAMBIA1300-*PINCEdS*-35S-flag for the constitutive overexpression of *PINCEdS*. The obtained recombinant vectors were transformed into *Agrobacterium* strain EHA105, and then infected WT *A. thaliana* and the solid mutant (*nced5-2* and *nced9-1*) inflorescences by the floral-dip method (Lough and Bent 1998), and herbaceous peony callus by the method reported previously of our laboratory (Li 2020).

To silence *PINCEdS* expression in herbaceous peony callus, the fragment of *PINCEdS* (*PINCEd1*: 565 bp; *PINCEd2*: 387 bp) were amplicated by primers with XbaI and BamHI restriction sites and recombined into the linearized pTRV2 empty vector. The positive pTRV2-*PINCEdS* vectors were transformed into EHA105 competent cells. The infection solution containing pTRV1 *Agrobacterium* was mixed with the infection

solution containing pTRV2 and pTRV1-PINCEDs *Agrobacterium* at a volume ratio of 1:1 for callus infection with the method mentioned above.

The seeds germination rate assay was performed in WT *A. thaliana*, solid mutant, functional complementation mutants and transgenic lines (T_3 generation stable genetic lines), which grown at the same time under the equal conditions. Gene expression tendencies were analysed by qRT-PCR (The primer sequences are listed in Supplemental Table 2).

2.7 Measurements of ABA contents

After the herbaceous peony seeds in S1-S6 period were ground with liquid nitrogen, 0.5 g tissue sample was added into 5 mL 80% methanol, and lixiviated overnight in dark at 4°C. 0.1 g polyvinyl pyrrolidone was added on the next day, vortex oscillation was performed for 10 min, and centrifuged at 10000 rpm for 10 min. The supernatant was transferred to a new centrifuge tube, and extracted twice with 2.5 mL 80% methanol. The supernatant was combined and vacuum concentration to dry, then added 3 mL phosphate buffer solution (pH=8.0, 0.1 mol/L). The above solution was frozen in an ultra-low temperature refrigerator at -80°C for 30 min. After thawing at 4°C, it was centrifuged at 10 000 rpm for 15 min to remove impurities, and adjusted pH to 2.5-3.0 with 2 mol/L HCl, then extracted with an equal volume of ethyl acetate, vortex oscillation for 5 min, transferred the supernatant to the new centrifuge tube, and extracted with an equal volume of ethyl acetate for 2 times, combined with the supernatant (ester phase) and vacuum concentration to dry. The residue was eluted with 1 mL 50% methanol and filtered by 0.22 μ m membrane for the measure.

The endogenous ABA content was determined by ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). The liquid phase conditions were as follows: Waters ACQUITY UPLC BEH Shield RP C18 column (2.1 mm×50 mm, 1.7 μ m in diameter), column temperature of 40°C, flow rate of 0.2 mL/min, injection volume of 3 μ L, mobile phase composed of acetonitrile (A) and water (B), gradient elution. Mass spectrometry conditions were as follows: electrospray ionization ESI, negative ion scanning, multiple reaction monitoring mode, capillary voltage 0.8 KV, cone voltage 30 V, desolventizing gas temperature 650°C, desolventizing gas flow rate 1000 L/Hr, and cone gas flow rate 5 L/Hr.

3. Results And Discussion

3.1 Expression of *PINCEDs* were closely related to the process of seed dormancy release

We detected the dynamic changes of ABA by UPLC-MS/MS. Except for a slight increase in S3-S4 period, ABA content showed a downward trend in the process of dormancy release (Figure 1).

The expression level and trend of *PINCED1* and *PINCED2* were not the same during seed dormancy release (Figure 2). During S1 to S2 period, the expression level of *PINCED1* increased sharply. During the dormancy release of hypocotyls from S2 to S3 period, the expression level decreased significantly, which

was almost the same as that in S1 period. Subsequently, with the dormancy release of hypocotyls, the expression level increased gradually. The expression trend of *PINCED2* and *PINCED1* in S1 to S3 period were the same, but the expression level was significantly improved. *PINCED2* showed a decreasing trend from S2 to S6 period, which was similar to the change trend of endogenous ABA content, indicating that *PINCED2* had a greater impact on endogenous ABA synthesis.

PINCED1 and *PINCED2* have different effects on ABA content and dormancy release in herbaceous peony. When the radical break seed coat, the content of *PINCED1* was consumed a lot. With the continuous release of seed dormancy, the expression level of *PINCED1* showed a rising trend. However, since the content was always lower than that in the S2 period, the content of *PINCED1* was still declining as a whole. After germination rate test, we found that *PINCED1* indeed promoted seed dormancy (Figure 9). Different from *PINCED1*, the expression of *PINCED2* had been positively correlated with endogenous ABA content, and was more sensitive to ABA. Compared with *PINCED1*, *PINCED2* may have a stronger function of inhibiting seed germination. Therefore, we infer that although both of them have a promoting effect on seed dormancy, maybe there is no functional redundancy between *PINCED1* and *PINCED2* due to the differences in the intensity and mechanism involved in the function of genes.

3.2 Effects of exogenous ABA and FLU on key enzymes of endogenous ABA synthesis in herbaceous peony

Our previous studies showed that 30 mg/L ABA had a significant inhibitory effect on seed germination of herbaceous peony, which delayed 10 days germination compared with the control group (clean water treatment) and 150 mg/L FLU had an obvious promoting effect on seed germination of herbaceous peony, which germinated 5 days earlier than the control group (clean water treatment). Based on this phenomenon, we measured the expression of *PINCED1* and *PINCED2* in herbaceous peony seeds treated with exogenous hormones at effective concentrations.

As can be seen from Figure 2 and Figure 3, the expression trend of *PINCED1* and *PINCED2* in peony seeds treated with exogenous ABA/FLU showed no significant difference compared with the control group. However, the expression of key enzyme genes of ABA synthesis increased and decreased during S1-S2 due to exogenous ABA/FLU. The change of *PINCED2* expression was significantly greater than that of the control group, indicating that *PINCED2* was more sensitive to exogenous ABA/FLU treatment.

3.3 *PINCEDs* pertained to the ABA synthesizes rate-limiting enzymes

Sequence analysis showed that the gene *PINCED1* and *PINCED2* corresponded to a full-length cDNA (Figure 4) with an open reading frame of 1515 and 1323 bp that encoded a protein consisting of 505 and 441 amino acids, with a calculated molecular weight of 56.03 and 49.01 kDa and a theoretical *pI* of 6.0 and 5.7, respectively. On the basis of above mentioned CDS sequence, *PINCED1* and *PINCED2* obtained 142 and 300 bp 3' UTR, 732 and 1855 bp 5' end sequence (containing 5' UTR and promoter regions),

respectively. The sequence of this fragment was presented in GenBank (GenBank accession number *PINCED1*: OL744236; *PINCED2*: OL744237).

The CD-search section of NCBI website was used to analyze the conserved domain types of *PINCED1* and *PINCED2* proteins, and it was found that *PINCED1* and *PINCED2* proteins all had the typical RPE65 conserved domain of NCED family (Supplement Figure 2), which was related to the degradation of carotenoids in plants. NCED proteins from different plants were shown in (Supplement Figure 3). The *PINCED1* and *PINCED2* protein had the highest identity to the PoNCED protein (74.70%) and the JrNCED (62.43%), respectively (Table 1). This similarity demonstrated that *PINCEDs* are relatively conserved with respect to diverse plants. According to the low consistency with high similarity sequences used in the multi-sequence alignment of *PINCED1* and *PINCED2*, the genetic relationships between *PINCED1* and *PINCED2* were analyzed, respectively. The genetic relationship analysis results indicated that *PINCED1* and *PINCED2* had closest relationship with the homologues from *Paeonia ostii* and *Vitis vinifera*, respectively (Figure 5).

Through online software prediction, it was found that the 3'UTR regions of *PINCED1* and *PINCED2* were regulated by different miRNAs (Supplemental Table 3). These miRNAs have their own functions, such as mdm-miR319c regulating seed development, osa-miR169f.2 affecting seed root growth, osa-miR5149 regulating heat shock modules under high temperature stress, osa-miR2863a responding to *Rhizoctonia solani*, zma-miR160c-3p mitigating drought stress and ptc-miR7817a regulating cold response genes (Chopperla, et al. 2020; Gelaw and Sanan-Mishra 2021; Kushawaha, et al. 2021; Ma, et al. 2013; Parreira, et al. 2021; Zhou, et al. 2019). Therefore, we predicted that *PINCED1* and *PINCED2* may play a certain role in seed development, biological and abiotic stresses, which is consistent with the role of ABA in plant growth.

In addition, the promoter core elements and *cis*-acting elements of *PINCED1* and *PINCED2* genes were analyzed (Supplemental Table 4). It was found that in addition to the promoter core elements TATA-box and CAAT-box, there were also response elements (TCA-element, ABRE, AuxRR-core, TGA-box, TGACG-motif and CGTCA-motif) involved in jasmonic acid, abscisic acid, auxin and jasmonic acid. It is predicted that *PINCED1* and *PINCED2* may be regulated by transcription factors in different phytohormones pathways. Continued excavation of this regulatory pathway, different pathways between ABA and other phytohormones may be elucidated, and provide new ideas for exploring seed dormancy.

3.4 The *PINCEDs* capacited as a structural gene located in the nucleus or cytoplasm

To determine the subcellular localization pattern of *PINCEDs*, GFP fluorescence signals were detected by confocal fluorescence microscope in *A. thaliana* protoplasts. In the positive control, GFP signal intensity was distributed in the protoplast cell membrane, nucleus and cytoplasm (Figure 6), the 16318-hGFP-*PINCED1/2* fusion proteins were only observed in nucleus and cytoplasm, respectively (Figure 6).

At present, most NCED protein are located in chloroplasts (Jia, et al. 2018; Lee, et al. 2018), but the result of this paper is that PINCED1 is located in the nucleus, while PINCED2 is located in the cytoplasm. Since DAPI was not used for co-localization test of nucleus or chloroplast in this experiment, we used Plant-PLoc online software to predict the localization of PINCED2 protein (Supplemental Figure 5B). We found that PINCED2 was expressed in chloroplast, which not only verified the fact that PINCED2 was localized in cytoplasm, but also further predicted that PINCED2 was localized in chloroplast that is contained in cytoplasm. After analyzing the nuclear localization signal of PINCED1 by PredictNLS software (Supplemental Figure 5A), it was found that PINCED1 protein was localized in the nucleus, which was consistent with the results of this experiment.

3.5 Effect of *PINCEDs* on expression of ABA metabolism genes in herbaceous peony

At present, some achievements have been made in the establishment of stable genetic transformation system of herbaceous peony. But due to the difficulty of inducing adventitious buds through indirect pathway and the proliferation is slow. The number of adventitious buds induced by the direct pathway is small and the adventitious buds regenerate difficultly (Shen, et al. 2015). These problems hinder the establishment of efficient and stable regeneration system. The callus of herbaceous peony is relatively easy to obtain, and it is also a suitable genetic transformation receptor material. Therefore, we analyze the function of *PINCED* in endogenous ABA metabolism of herbaceous peony by using transgenic callus. The results showed that, compared with the control, the expression of *PINCED1* and *PINCED2* in transgenic herbaceous peony callus were significantly changed. The expression levels of *PINCED1* and *PINCED2* in over-expressed callus were about 14 times that in normal callus, and the expression levels in silent callus were about 0.4 times that in normal callus, indicating that transgenic callus of *PINCED1* and *PINCED2* were successfully obtained (Figure 7).

As shown in figure 8, compared with the control group, the expression level of *PINCED2* in the over-expressed *PINCED1* callus increased by about 5 times, and the expression level of *PINCED1* in the over-expressed *PINCED2* callus increased by about 6 times. In addition, the expression level of *PICYP707A* gene encoding ABA decomposition enzyme of herbaceous peony was detected, and it was found that the three members of the gene family increased in varying degrees compared with the control group. By detecting the expression levels of related genes in the silenced herbaceous peony callus, it was found that with the silencing of *PINCED1* gene, *PINCED2* increased slightly compared with the control group. After silencing *PINCED2*, *PINCED1* was almost unchanged compared with the control group. Subsequently, the expression levels of *PICYP707A* family members in silenced callus was detected, and it was found that the three members of the gene family also increased to varying degrees compared with the control group, but the *CYP707A* members with the most increased expression level in silent callus were not consistent with those in the over-expression callus.

It was found that the change trend of *PINCED2* expression level was consistent with the dynamic change trend of endogenous ABA content in the process of dormancy release of herbaceous peony seeds (Figure

1 and Figure 2). In the callus with overexpression of *PINCED2*, the expression level of *PICYP707As* increased accordingly, and the increase of *PICYP707A3* expression level was the highest. In the callus with silenced *PINCED2*, the expression level of *PICYP707As* also increased, and the increase of *PICYP707A2* expression level was the most. Through previous studies on the relationship between *PICYP707As* and endogenous ABA content (Supplemental Figure 4), it was found that the effect of *PICYP707A* family members on endogenous ABA content was not the same (Li 2020). *PICYP707A2* and *PICYP707A3* had the greatest impact on endogenous ABA content. When the expression level of *PICYP707A2* increased, the endogenous ABA content showed a downward trend, while when the expression level of *PICYP707A3* increased, the endogenous ABA content showed an upward trend. Based on the above results, we speculated that *PINCED2* positively regulated endogenous ABA content.

3.6 PINCEDs inhibited seed dormancy release

A. thaliana is a typical model plant for gene research, which is widely used in gene transformation experiments. Since there is no genetic transformation system for herbaceous peony, stable genetic transgenic offspring cannot be obtained. Meanwhile, it takes four to five years for herbaceous peony from sowing to flowering, and the observation period is long. Therefore, in this study, the genetic transformation system of *A. thaliana* was used to explore the role of *PINCED* in dormancy release.

In order to further identify the function of *PINCEDs* in seed dormancy release process, the seed germination time of wild type *A. thaliana*, ABA-deletion and *NCED*-deletion *A. thaliana* mutants, overexpression lines of *PINCED1* and *PINCED2* and functional complementation *A. thaliana* constructed by our laboratory were observed in this experiment.

Figure 9A showed that the seeds of mutants and wild type germinated at 48 h, but the seeds of overexpression lines did not germinate. At 68 h, the seeds of *PINCED1* overexpression line began to germinate, while the seeds of *PINCED2* overexpression line began to germinate at 78 h. Figure 9B and figure 9C revealed that the seed germination of *PINCED1* complementation line was earlier than *PINCED2* complementation line, whether at *Atnced9-1* or *Atnced5-2* was used in the functional complementation tests.

5. Conclusion

This study found that *PINCED1* and *PINCED2* were a positive regulator of seed dormancy. Overexpression of *PINCED1* and *PINCED2* promoted seed dormancy in transgenic *A. thaliana*. Gene overexpressing and silencing of *PINCED1* and *PINCED2* effect ABA content in herbaceous peony. Therefore, *PINCED1* and *PINCED2* played a critical role in seed dormancy by regulating the capacity of the ABA metabolism enzyme. This study not only understand the specific functions of ABA metabolism genes and dormancy germination of herbaceous peony seeds, but also provide scientific basis for obtaining effective dormancy breaking methods of herbaceous peony seeds by molecular means in the future.

Declarations

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Author contribution statement

Author Contributions Xiaomei Sun and Riwen Fei conceived and designed research. Riwen Fei, Jiayuan Ge and Tianyi Sun conducted the experiments. Riwen Fei, Siyang Duan analyzed the data. Riwen Fei wrote the manuscript. Xiaomei Sun revised the manuscript. All authors read and approved the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Tables

Table 1 is not available with this version

Figures

Figure 1

ABA content changes during the dormancy release of herbaceous peony seeds. S1: dry seed; S2: imbibition seed; S3: the radical break seed coat; S4: the length of seed root is 3-4 cm; S5: the basal part of seed root turns red; S6: the seed germ breakout.

Figure 2

Relative expression level of *PINCEDs* during seed dormancy release process.

Figure 3

Relative expression level of *PINCEDs* under ABA and FLU treatments.

Figure 4

Full-length cDNA sequence of *PINCEd*s.

Figure 6

Subcellular localization of PINCEDs in *A. thaliana* protoplast. Scale bars: 10 μ m.

Figure 7

***PINCEDs* expression level in different transgenic herbaceous peony callus.** WT, normal callus; 35S::flag, empty overexpressed vector; pTRV2, empty silent vector; 35S::PINCED1-flag, *PINCED1* was overexpressed; pTRV2-PINCED1, *PINCED1* was silenced; 35S::PINCED2-flag, *PINCED2* was overexpressed; pTRV2-PINCED2, *PINCED2* was silenced.

Figure 8

Relative expression level of *PINCEDs* and *PICYP707As* expression under different transgenic treatment.

(A) *PINCED1* was overexpressed. (B) *PINCED2* was overexpressed. (C) *PINCED1* was silenced. (D) *PINCED2* was silenced.

Figure 9

Detection of seed germination rate of different transgenic *A. thaliana*. (A) WT, Col-0; mutant (*aba2-1*, *aba3-1*, *nced9-1*, *nced5-2*); 35S::PINCED1, *PINCED1* was overexpressed under Col-0 background; 35S::PINCED2, *PINCED2* was overexpressed under Col-0 background. (B) 35S::PINCED1/*nced9-1*, *PINCED1* was overexpressed under *nced9-1* background; 35S::PINCED2/*nced9-1*, *PINCED2* was overexpressed under *nced9-1* background; (C) 35S::PINCED1/*nced5-2*, *PINCED1* was overexpressed under *nced5-2* background; 35S::PINCED2/*nced5-2*, *PINCED2* was overexpressed under *nced5-2* background.

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