Identification of ABSCISIC ACID (ABA) signaling related genes in *Panax ginseng*

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Abstract Korean ginseng (*Panax ginseng*) has long been cultivated as an important economic medicinal plant. Owing to the seasonal and long-term agricultural cultivation methods of Korean ginseng, they are always vulnerable to various environmental stress conditions. ABSCISIC ACID (ABA) is an essential plant hormone associated with seed development and diverse abiotic stress responses including drought, cold and salinity stress. By modulating ABA responses, plants can regulate their immune responses and growth patterns to increase their ability to tolerate stress. With recent advances in genome sequencing technology, we first reported the functional features of genes related to canonical ABA signaling pathway in *P. ginseng* genome. Based on the protein sequences and functional genomic analysis of *Arabidopsis thaliana*, the ABA related genes were successfully identified. Our functional genomic characterizations clearly showed that the ABA signaling related genes consisting the ABA receptor proteins (*PgPYLs*), kinase family (*PgSnRKs*) and transcription factors (*PgABFs*, *PgABI3s* and *PgABI5s*) were evolutionary conserved in the *P. ginseng* genome. We confirmed that overexpressing ABA related genes of *P. ginseng* completely restored the ABA responses and stress tolerance in ABA defective *Arabidopsis* mutants. Finally, tissue and age specific spatio-temporal expression patterns of the identified ABA-related genes in *P. ginseng* tissues were also classified using various available RNA sequencing data. This study provides ABA signal transduction related genes and their functional genomic information related to the growth and development of Korean ginseng. Additionally, the results of this study could be useful in the breeding or artificial selection of ginseng which is resistant to various stresses.

Keywords *Panax ginseng*, ABA, Drought stress, Signal transduction, Gene expression

Introduction

P. ginseng C. A. Meyer is an important perennial herb plant belonging to *Araliaceae*. It is a worldwide medicinal plant used for over thousand years in Asia, especially in Korea, Japan and China (Hu 1976). Many studies and clinical trials have demonstrated the efficacy of ginseng, including cancer prevention, HIV virus replication inhibition, and aging inhibition (Shin et al. 2000, Choi et al. 2008). The cultivation of ginseng is a very slow growth rate and requires 4 to 6 years of cultivation period to obtain sufficient active ingredients (Chung et al. 2016). It is susceptible to environmental stresses such as growth environment, salt in soil, pests, drought and high temperature because it has to grow for a long time (Liu et al. 2014). In addition, the yield of ginseng has been greatly reduced due to recent global warming with cold weather, drought, and high temperature. Since the frequency of occurrence of an abnormal climate is increasing, the necessity of varieties cultivating highly adaptable environment is required.

Plants are sessile living organisms that have evolved a variety of signaling mechanisms to respond to and cope with ever-changing environments. Representative reactions are through plant hormones such as brassinosteroid, jasmonic acid, ethylene, abscisic acid (ABA) (Kim et al. 2016, Verma et al. 2016). Plants respond to a variety of stresses through these plant hormones, and also tolerate to environmental stresses such as cold weather or dryness through ABA signaling pathways (Nakashima and Yamaguchi-Shinozaki 2013, Saddhe et al. 2017). The ABA response is mediated by SnRK, which acts as a kinase, with transcription factors including ABA-INSENSITIVE 3, 4 and 5 to modulate the expression of downstream target genes (Nakashima and Yamaguchi-Shinozaki 2013). This response is not inhibited by negative regulators,

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ABA-INSENSITIVE 1 and 2 (ABI1, 2), which is type 2c protein phosphatases (PP2C), unless stress conditions are present. However, when plants are under stress condition, the biosynthesis of ABA is rapidly increased and the canonical ABA signaling is activated by the receptors, PYRABACTIN RESISTANCE 1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) (Nakashima and Yamaguchi-Shinozaki 2013). The combined PYR1 / PYL / RCAR and ABA complexes directly bind with ABA-INSENSITIVE 1, 2 (ABI1, 2). When the phosphatase function of ABI1 and ABI2 is inhibited, the ABA reaction occurs because the inhibition of SNF1-RELATED PROTEIN KINANS (SnRKs) is released (Umezawa et al. 2009). In many studies, these ABA signaling related gene has been used to generate stress resistant plants (Sah et al. 2016). Therefore, molecular breeding using ginseng's ABA signal transduction gene will be able to cultivate environmentally resistant varieties. However, much research has been done on the genome of ginseng, but ginseng is a allotetraploid plant ($2n = 4x = 48$) and has a larger genome size (3.2 Gbp) (Hong et al. 2004, Choi et al. 2014, Jang et al. 2017). So the research on ginseng was focused on pharmacological research and tissue culture (Yang and Yang 2000, Kim et al. 2009). Recent advances in genome sequencing technology have led to the detoxification of genomes and transcripts in *P. ginseng*, which could be used for functional genomic approaches (Jiang et al. 2017, Jo et al. 2017, Waminal et al. 2018).

In this study, ABA signaling related genes in *P. ginseng* were identified from recently reported draft genome and transcript data (Jiang et al. 2017, Jo et al. 2017, Waminal et al. 2018). The expression patterns of the identified ABA signaling related genes in *P. ginseng* were examined in the different age and tissues. We also found that the expression was regulated by exogenous ABA, suggesting that they are involved in ABA signal transduction. It was also confirmed that the ABA responsiveness was restored by complementation of the ginseng ABA related genes in ABA signaling defective *Arabidopsis* mutants.

Materials and Methods

Phylogenetic tree construction, classification expression profiles analysis of ABA related genes from *P. ginseng*

The protein sequences of ABA-related genes were selected from previous studies (Jiang et al. 2017; Jo et al. 2017; Waminal et al. 2018). A phylogenetic tree based on protein sequence alignment was generated using MEGA version 7.0 software

by the neighbor-joining method with a bootstrap value of 1000 (Hall et al. 2013, Kumar et al. 2016). An online program, iTOL (<http://itol.embl.de/>), was applied to beautify the phylogenetic tree. Base on the phylogenetic tree constructed by ABA related genes from *P. ginseng* and *Arabidopsis*, these genes were divided into different groups and subgroups. The expression values were calculated by log₂ (FPKM) and were presented in heat map using XLSTAT software.

ABA treatments and Real Time qRT-PCR

One-year-old ginseng roots were incubated with water containing 100 μ M ABA (Sigma) for 3, 6 h. After treatment, the samples were immediately frozen in liquid nitrogen. Total RNAs were extracted from seedlings using a Total RNA extraction kit (Taesin Bioscience, Korea) according to the manufacturer's instructions. Total RNA concentration and quality were measured using a K5600 Micro-spectrophotometer (Shanghai Biotechnol Co., China). A first-strand synthesis kit (Enzynomics, Korea) with oligo (dT) primers was used for cDNA synthesis from 1 ng of total RNA. The cDNA was then used for real-time quantitative PCR with a Quant Studio 3 (Applied Biosystems, USA) instrument using SYBR Green Real-time PCR Master Mix (Applied Biosystems). Primer lists are followed : *PgTUB1* : 5'-GGCGAAATCTTCGAGA ATGC-3' and 5'-TCGAAACCTAACAAAAGAAAAGG-3'; *PgABI1a* : 5'-GGTATGTGATATGGCTCGAAAGC-3' and 5'-TCCTTGCTGCCCTTTTGAAG-3'; *PgABI2a* : 5'-CCAA TGAGGAGGTATGTGACACA-3' and 5'-TTCCCTTTTGA AGAGCTCGATT-3'; *PgABF1* : 5'-ACGAGCTCGGAAAC AGGCTTA-3' and 5'-CTTTTCCAAGGCATTTTCATCTTC-3'; *PgPYL1* : 5'-GTCATTGAAGGAAGACCTGGTACTC-3' and 5'-TGCAAGCCGCTCTGAAACTT-3'; *PgPYL2* : 5'-GAGC ATATTCTTAGCGTCAGGATTG-3' and 5'-GGTGTTCCTTCTGGCACAT-3'; *PgPYL3* : 5'-TCATGGATGGGAGAC CAGGTA-3' and 5'-CAGCCAAACGCTCAGAGACA-3'; *PgPYL4* : 5'-AGTCATTACTGTCCATCCCGAAGT-3' and 5'-GCCA GTGATTTGAGGTTGCA-3'; *PgPYL5* : 5'-TCAGTCATTA CTGTCCATCCTGAAG-3' and 5'-CAGTGATTTGAGGTT GCACTTGA-3'; *PgSnRK1* : 5'-GCCAATCTGATAAACCC ACACA-3' and 5'-CACTCTCTAGGTCGTCTCCATGT-3'; *PgSnRK2* : 5'-TGATCAGCAGCCAGTTTGAAGA-3' and 5'-TCTCAGGATCGGAGTCCAAGTC-3'; *PgSnRK3* : 5'-GAG CAACCCATGCAGAGCAT-3' and 5'-CCTCTCCACTGCT GTCAATATCAA-3'; Threshold cycle (Ct) values were used to calculate $2^{-\Delta\Delta C_t}$ for expression analysis, where $\Delta\Delta C_t$ for treated plants was determined as follows: (Ct target gene - Ct actin gene) - control plant (Ct target gene - Ct actin gene) (Livak and Schmittgen 2001).

Protoplast transient expression assay

The full-length cDNAs of *PgPYL4*, *PgPYL5*, *PgSnRK1*, *PgSnRK2*, *PgSnRK3*, *PgABF1*, *ARR2* were cloned into plant expression vectors containing *HA*, *FLAG* or *GFP* DNA sequence tags in the C terminus driven by the 35S:*C4PPDK* promoter as previously described (Ryu et al. 2007). For protoplasts transient expression assays, about 4×10^4 protoplasts were transfected with 20 µg of plasmid DNA and then incubated under constant light condition at 20°C for 6 h. For the subcellular localization, GFP-tagged constructs were transfected into protoplasts. *ARR2*-RFP was used as a nuclear marker. GFP and RFP fluorescence were observed with a fluorescence microscope (Nikon).

Plant materials and growth conditions

Arabidopsis thaliana ecotype Col-0 was used as wild-type controls, and *rcar*, *snrk2.2/2.3* and *snrk2.6* knock out mutants were used as the genetic backgrounds of transgenic lines. *Arabidopsis* seeds were germinated on solid media (pH 5.7–5.8) containing 1/2 Gamborg B5 salts (Duchefa, Netherlands), 1% sucrose and 0.8% plant-agar and all plants were grown in a greenhouse under long-day conditions (16-h light/8-h dark cycles) at 22°C.

Transgenic plants and immunoblotting assay

To generate transgenic plants overexpressing HA-tagged *PgPYL3*, 4 and *PgSnRK1*, 3 in the *rcar*, *snrk2.2/2.3*, *snrk2.6* mutant background, respectively, the gene fragment was cloned into *pCB302ES* or *pCAMBIA1303* containing the 35S promoter and double HA tag sequences as described previously (Ryu et al., 2014). Electroporation was used to introduce the *PgPYL3-pCAMBIA1303*, *PgPYL4-pCAMBIA1303* and *PgSnRK1-pCB302ES*, *PgSnRK3-pCB302ES* vector into *Agrobacterium* strain GV3101. All transgenes were integrated into the *Arabidopsis* genome by *Agrobacterium*-mediated floral dipping methods. Transgene expression was verified by immunoblotting. Total proteins from 5-days-old seedlings were extracted with protein extraction buffer (50 mM Tris-HCl (pH 7.5), 75 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 1× protease inhibitor cocktail (Roche), and 1% Triton X-100). Total protein was subjected to SDS-PAGE (10% polyacrylamide), transferred to a PVDF membrane and immunodetected with 1/2,000 dilution of peroxidase-conjugated high-affinity anti-HA (Roche) antibodies.

Physiological analysis of germination and drought stress responses

For seed germination assays, surface-sterilized homozygous seeds were kept at 4°C for 3 days and plated on 1/2 Gamborg B5 medium containing 1% sucrose supplemented with or without the ABA. The seed germination (radicle emergence and green cotyledon) rates were scored. For leaf water loss assays, leaves were detached from 16 days after germination *Arabidopsis* plants and placed under the greenhouse conditions. Leaf fresh weight was measured immediately after separation, then leaves were placed in Petri dishes and weight was measured over time.

Results and Discussion

Identification of ABA signaling related components in *P. ginseng* genome

To identify the genes involved in ABA signal transduction in the *P. ginseng* genome, we analyzed two recently reported full-length draft genome sequence information (Choi et al. 2014, Jiang et al. 2017 Waminal et al. 2018) and transcript data using SMRT PacBio sequencing technique (Jo et al. 2017). The genes predicted to play a role for ABA signaling in ginseng by blast method using the protein sequence information of genes involved in ABA signaling confirmed by functional genomic studies in *Arabidopsis* and several model systems. We identified total putative 67 protein sequences which could be ABA signaling components including 29 PYR1 / PYL / RCARs as ABA receptor proteins (*PgPYL1* to *PgPYL29*), the negative regulatory 7 phosphatase (*PP2C*) *ABI1* and 2 of ABA signaling (*PgABI1a* to *PgABI1c*, *PgABI2a* to *PgABI2d*), 14 phosphorylated enzyme *SnRKs* (*PgSnRK1* ~ *PgSnRK14*), 5 transcription factor *ABI3* (*PgABI3a* to *PgABI3e*), 4 *ABI4* (*PgABI4a* to *PgABI4d*), 12 *ABFs* (*PgABF1* to *PgABF12*) and 6 *ABI5* (*PgABI5a* to *PgABI5f*), respectively (Table 1). The gene sequence information revealed that much number of ABA signal transduction related genes are presented in the *P. ginseng* genome compared to that of other plants. It is possible that this feature of *P. ginseng* is an evolutionary product to maintain the characteristics of heterozygous dielectrics and the ability of ginseng to cope with various external stresses with whole lifetime of more than 100 years. For the further functional genomic study of these predicted genes, the evolutionary similarity among the genes was confirmed based on the protein sequence information of ABA signal transduction genes of *Arabidopsis* (Fig. 1). As shown in Figure 1, the protein

Table 1 Identified ABA-related genes in the genome of *Panax ginseng*

Gene name	[TAIR]Description	Total
ABI1	ABA INSENSITIVE 1	7
ABI2	ABA INSENSITIVE 2	
ABI3	ABA INSENSITIVE 3	
ABI4	ABA INSENSITIVE 4	
SnRKs	SNF1-RELATED PROTEIN KINASE family	14
PYR/PYL/RCAR	REGULATORY COMPONENT OF ABA RECEPTOR family	29
ABFs	ABSCISIC ACID RESPONSIVE ELEMENT-BINDING FACTOR family	12
ABI5	ABA INSENSITIVE 5	6

Fig. 1 Phylogenetic tree analysis of ABA related genes from *P. ginseng* and *A. thaliana* genome. The Neighbor-joining tree was constructed with ABA related genes from *P. ginseng* and *A. thaliana* using MEGA7.0 with a bootstrap of 1000

structure of genes identified from ginseng genomic information is related to those of the ABA signaling system in *Arabidopsis* model plants, which are consisted with the functional receptors, transcription factors (TF), and phosphorylation related functions (Kinase, PP2C). These results strongly support the possibility that the ABA signaling system to cope with diverse external environmental stresses is well preserved evolutionarily in the

P. ginseng genome.

Spatio-temporal expression patterns of ABA signaling related genes

We next confirmed that the ABA signaling related genes were expressed in the different ginseng tissues by reanalyzing

Fig. 2 Heat map of spatio-temporal expression patterns of ABA related genes in different tissues of *P. ginseng*. Heat map of the expression levels of ABA-related genes from *P. ginseng* in 2, 3, 4, 5 and 6 years old roots (A) and different tissues (B). (C) The relative expression levels of *PgABF1-4*, *PgABI1a*, *PgABI2a*, *PgPYL1-5* and *PgSnRK1-3* in *P. ginseng* root treated with ABA 100μM for 0, 3, and 6 hours. Green, high expression; red, low expression; black, intermediate

available RNA-seq data in NCBI (Liu et al. 2017) and our unpublished data (Fig. 2). Relative levels of gene expression were determined by ginseng growth period (2, 3, 4, 5 and 6 year old roots, Fig. 2A) and different ginseng tissues including root developmental stages, stem, leaf tissues, fruits and seeds (Fig. 2B). In case of *PgSnRKs*, most genes were highly expressed during early stage of root developments, but *PgSnRK13* and *14* were gradually increased in the late 4–6 years old roots (Fig. 2A). ABA receptors (*PgPYLs*) and transcription factors (*PgABFs* and *ABIs*) were specifically expressed during early

and late root developmental stages. It was also found that the genes expressed in the upper part and the lower part of ginseng plants were differently regulated in the ginseng tissues. For example, the expressions of *PgSnRKs* were ubiquitously detected in most tissues, but *PgABIs* were mostly expressed in the root tissues (Fig. 2B). However, *PgABI1* and *PgABI2* were rarely expressed in 2 and 3 year olds, but not in late stage of root developments. *PgABI3s* showed lower expression patterns in 2-year-old ginseng roots. Interestingly, the expression patterns of *PgABI5s* in the early stages of development were observed,

but it was decreased in the late stages of development (Fig. 2A). These age and tissue specific expression patterns were similarly observed in the other ABA signaling related genes. These results confirmed that the expression of ABA signaling related genes are correlated with almost coincided with that of seed germination and stress responses. To confirm the ABA responsive gene expression, the expression level of each gene in ginseng treated with ABA for 0, 3, 6 hours was analyzed by real-time quantitative PCR (qRT-PCR) (Fig. 2C). *ABFs* are well known as an ABA responsive gene, and *PgABFs* were upregulated by exogenous ABA. *PYL/PYR/RCAR* showed a decrease in expression when the activity of ABA was increased (Fujii et al. 2009, Ma et al. 2009). Similarly to this previous report, the expression of ABA receptors was down-regulated by ABA. The expression pattern of PP2Cs (*PgABI1a* and *2a*) and SnRK was also decreased by ABA treatments (Fig. 2C). These results suggest that the ABA signal transduction genes are tissue specifically expressed in ginseng and their expression is regulated by ABA, suggesting that these genes are indeed involved in ABA signal transduction pathways in *P. ginseng*.

Subcellular localization of *P. ginseng* ABA signaling components

To confirm the protein localization in the cells, where they are similar to the protein localization of ABA signaling genes of *Arabidopsis*, we next determined the subcellular localization of GFP tagged genes in the protoplasts (Fig. 3). It has been well characterized that PYLs and SnRKs are localized in both cytoplasm and the nucleus in plant cells (Fujita et al. 2009, Ma et al. 2009). We successfully transfected the *PgPYL4*-GFP, *PgPYL5*-GFP, *PgSnRK1*-GFP, *PgSnRK2*-GFP and *PgSnRK3*-GFP into *Arabidopsis* protoplasts (Fig. 3A). When GFP expression was confirmed, all PYLs and SnRKs of *P. ginseng* were clearly detected in the nuclear and cytoplasm similar to *Arabidopsis*. The transcription factor, ABFs in *Arabidopsis* have been reported to be expressed in the nucleus since they are transcription factors (Yoshida et al. 2010, Yoshida et al. 2015). In order to confirm that *PgABF1*-GFP of ginseng is actually expressed in the nucleus, it was confirmed by expression with *AtARR2*-RFP (Lohrmann et al. 2001) (Fig. 3B). It was confirmed that *ARR2*-RFP and *PgABF1*-GFP were co-localized in the nucleus according to the coincidence of intracellular expression positions. These results suggest that ABA signal transduction genes identified in ginseng may play the same roles as previously reported in *Arabidopsis* research.

Functional analysis of *P. ginseng* ABA signaling genes

To determine the functional roles of the identified genes in

ABA signal transduction, the germination rate and resistance to drought stress were investigated with Col-0 and overexpressing lines in the *rcar*, *snrk2.2/2.3* and *snrk2.6 Arabidopsis* genetic mutant backgrounds in the presence of ABA (Fig. 4). We initially confirmed the transgene expression level by a western blotting and germination rates of all wild type control and transgenic lines in the absence of ABA (Fig. 4A, 4C and 4E). All tested seeds were completely germinated within 4 days after sowing (DAS), indicating the rare inhibition effects of ABA related genes in the transgenic plants. However, the germination rate of Col-0 was almost 20% or less, but that of *rcar* and *snrk2.2 / 2.3* mutants was over 90% at 5DAS in the presence of ABA (Fig. 4B, 4D). Nonetheless, the complementation lines which were overexpressed with *PgPYL3*, 4 (*rcar* 35S:*PgPYL3*-HA and *rcar* 35S:*PgPYL4*-HA) and *PgSnRK3* (*snrk2.2/2.3* 35S:*PgSnRK3*-HA # 4 and 6) were completely restored the ABA responses as much as a wild type Col-0 control (Fig. 4B, 4D). These results suggest that *PgPYL3*, 4 and *PgSnRK3* are involved in ABA signal transduction. Surprisingly, the germination rate was not recovered by only overexpression of *PgSnRK1*-HA in the *snrk2.2/2.3* mutant background in the medium containing ABA (Fig. 4D). The *PgSnRK1* is similar to *AtSnRK2.6* (Fig. 1), being a protein known to play a major role in drought stress due to involvement in stomata aperture rather than seed germination controls (Yoshida et al. 2002, Fujii et al. 2007). To test this possibility, we next evaluated if the drought stress susceptibility of the *snrk2.6 (ost1)* plants was recovered by complementation of *AtSnRK2.6* function by overexpression of *PgSnRK1*. The resistance to the drought stress was determined by measuring water loss rate of leaf with the *snrk2.6* 35S: *PgSnRK1*-HA # 1 and 5 plants (Fig. 4E). The water loss of detached leaves from 16 old plants was weighed for 2 hours. After 2 hours, the water loss of the Col-0 plant was reduced by 44% compared with that of the immediately after cutting, but the *snrk2.6* plants decreased by 58%. However, the water loss of two independent *snrk2.6* 35S: *PgSnRK1*-HA transgenic plants significantly was recovered by 49% and 50% at 2 hours, respectively (Fig. 4E). Taken together, ABA signal transduction genes identified from *P. ginseng* in this study have functional similarity with the ABA related genes in *Arabidopsis*. Recent advances in genome sequencing technology have enabled functional genomic analysis of many different plant species (Choi et al. 2014, Jiang et al. 2017, Jo et al. 2017, Waminal et al. 2018). In this study, we analyzed genes involved in the canonical ABA signal transduction pathway in *P. ginseng*, which play essential roles in broad range of environmental stress tolerances. Much research has been conducted on multi environmental stress tolerant plants

Fig. 3 Subcellular localization of *P. ginseng* ABA related genes in plant cells. Subcellular localization of *PgPYL4*, *5*, *PgSnRK1-3* (A) and *PgABF1* (B) were observed in *Arabidopsis* mesophyll protoplasts. GFP tagged ABA related genes from *P. ginseng* were transfected into the protoplasts to confirm the subcellular localization. ARR2-RFP was used as a nuclear marker. Scale bar = 50 μ m

Fig. 4 Overexpression of the *P. ginseng* ABA signaling related genes was completely complemented in the ABA insensitive phenotypes of ABA defective *Arabidopsis* mutants. The ABA-mediated inhibition of seed germination was measured using green cotyledon emergence. Col-0, *rcar*, *rcar* PgPYL and *snrk2.2/3* PgSnRK transgenic seeds were germinated on 1/2 B5 agar plates containing 0 (A, C) or 1μM of ABA (B, D). Western blot showing HA-tagged PgPYL3, 4 (A), PgSnRK1, 3 (C), PgSnRK1 (E) proteins from independent transgenic lines. Col-0, negative control; CBB staining indicates loading control. Error bars indicate S.E.M. ($P < 0.05$; one-way ANOVA). (E) Relative water loss rates were measured by weight of leaves from 16 days-old Col-0, *ost1* (*snrk2.2/3*) and two *ost1* PgSnRK1 transgenic lines grown under LD ($n = 4$). Error bars indicate S.E.M. ($*P < 0.05$, Student's t-test)

through molecular breeding due to the extreme climate changes caused by global warming. It is expected that the identified ABA signaling related genes of *P. ginseng* will be able to use in breed ginseng program for increasing resistant to environmental stress.

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