INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
21–0044/2021/26–2–251–256
DOI: 10.17957/IJAB/15.1831
http://www.fspublishers.org

Full Length Article

Expression Changes of Genes Related to Germination Based on EST Database under Priming Treatment by Gibberellic Acid in *Perilla frutescens* (Korean Perilla)

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Received 12 January 2021; Accepted 30 April 2021; Published 10 July 2021

Abstract

It is very important to establish an optimal seed priming process in order to increase the vitality of the seeds and promote the metabolism for the germination of the seeds. The optimum concentrations and species of priming agents to improve seed germination of both medicinal plants were also estimated. To improve the germination rate of *Perilla frutescens* (Korean perilla) seeds, various seed priming agents were used to analyze seed germination rates in the Saeyeopsil, Okdong and 141 collection Korean perilla cultivars. The agents used for seed priming were $CaCl_2$, $Ca(NO_3)_2$, NaCl, K_3PO_4, polyethylene glycol, and gibberellic acid (GA_3). When 0.1 m*M* GA_3 was used for seed priming, germination rates of Okdong, and the 141 collection showed a greater than 70% increase compared to the controls. Nine genes were selected for expression analysis by searching for genes related to seed germination and plant development in the EST (Expressed Sequence Tag) database of the Korean perilla cDNA library. GA_3 priming treatment for 1 d induced higher transcriptional levels of genes related to germination and plant development than controls treated with water only. These genes were identified as protochlorophyllide reductase-like, magnesium-chelatase subunit ChlI, heme-binding protein 2-like, glyceraldehyde 3-phosphate dehydrogenase A, Chlorophyll a-b binding protein 6, B2 protein, 2-Cys peroxiredoxin BAS1, and 21 kDa protein. From these results, we suggest that when priming Korean perilla seeds with GA_3, a large number of genes involved in plant development at early stages of seed germination play a role in improving the seed germination rate. Also, these induced genes are ideal candidate biomarkers for seed priming of Korean perilla. Specially, protochlorophyllide reductase-like is thought to be a potential gene for future molecular marker. © 2021 Friends Science Publishers

Keywords: EST database; GA_3; Germination rate; *Perilla frutescens*; Seed priming

Introduction

Perilla frutescens is a plant native to regions of Southeast Asia and has various uses such as an ingredient in natural products and food, and as a medicinal pigment (Seong *et al.* 2009). This plant has long been utilized as a raw material for oil extraction and is commonly known as "Dlggae" in Korea. Recently, consumption of perilla has increased significantly in Korea; more than 60% of the total unsaturated fatty acids (FAs) in perilla seeds comprises α-linolenic acid (Ichikawa 2006), an essential FA required for human growth and development, in addition to its known major role in preventing and treating blood vessel diseases (Shahidi and Miraliakbari 2005). Many flavonoids, sterols, terpenoids and phenolic acids have been extracted from seeds of Korean perilla and studied, with several studies reporting on the importance of flavonoids and phenolic compounds in relation to biological activity (Ozturk *et al.* 2010; Kim *et al.* 2019).

Seed priming technology using $Ca(NO_3)_2$, KNO_3, $MgSO_4$, $NaNO_3$, KCl, K_3PO_4, NH_4NO_3 and PEG 6000PEG (polyethylene glycol) involves pretreatment of seeds with different agents with varying concentration, duration, or temperature conditions, with the goal of improving seed production under given environmental conditions (Park *et al.* 2013). The success of priming is strongly involved in the hydration of the metabolism and process by which the seed absorbs a limited amount of water (Rahimi 2013).

To cite this paper: Seong ES, BJ Kang, JH Yoo, JG Lee, NY Kim, CY Yu (2021). Expression changes of genes related to germination based on EST database under priming treatment by gibberellic acid in *Perilla frutescens* (Korean perilla). *Intl J Agric Biol* 26:251–256

The complex network involved in seed metabolism is dependent on the agent used, duration, and temperature of the priming treatment, as well as vigor, dehydration, and storage conditions of primed seeds (Dezfuli *et al.* 2008). Seeds priming to enhance seed quality show increase pattern of germination rate which result in high levels of abiotic stress resistance. All these characteristics directly correlate to seed vigour, plant genotype and physiology controlled by multiple genetic and environmental factors (Jisha *et al.* 2013). Priming method is generally used to treat vegetables seeds such as carrot, celery, lettuce, pepper and tomato (Paparella *et al.* 2015). However, the establishment of seed priming techniques for medicinal crops is extremely limited. Therefore, it is necessary to improve the germination rate, shorten the number of days it takes to germinate, and establish optimal priming conditions for uniform seedling production in medicinal crops.

The effect of priming has been proven to improve seed germination and seedling growth using numerous chemical factors in various crops such as wheat, beans, sunflower, corn, and brassica (Cho *et al.* 2011a). For instance, the germination characteristics of corn seeds were improved after gibberellic acid (GA_3) or hydropriming treatment (Subedi and Ma 2005).

Gibberellic acid (GA_3) is essential for seed germination and flower development; for example, Arabidopsis exhibiting a deficiency in GA_3 content showed defects in seed germination and organ formation (Kim *et al.* 2014). In addition, loss-of-function studies have identified many genes involved in GA_3-induced seed germination (Cao *et al.* 2006). Genetic markers responding to GA_3 may be used to assess the specificity, which *AtGA3ox1*(GA4) was downregulated by GA_3 activity (Silverstone *et al.* 2001). Gene expression regulated by GA_3 during the germination process has also been studied, helping to explain the GA_3 response mechanism (Cao *et al.* 2006).

The purpose of this study was to establish an optimal germination system for Korean perilla through priming treatment, using various agents such as $CaCl_2$, $Ca(NO_3)_2$, NaCl, K_3PO_4, polyethylene glycol (PEG), and GA_3. In this report, we investigated the germination ratios resulting from the application of all the agents used in the seed priming treatments. Furthermore, this study aimed to reveal the genetic relationship between seed germination and GA_3 response, using gene expression data from the *Perilla frutescens* EST database generated in our previous study (Seong *et al.* 2015).

Materials and Methods

Priming treatments for Korean perilla seeds using various agents

All seeds used in this study were stored at 4°C and the priming conditions were tested on three sources of seed: Saeyeopsil, Okdong, and the 141 line. The agents used for priming treatment were $CaCl_2$, $Ca(NO_3)_2$, NaCl, K_3PO_4, PEG 6000 and GA_3. The concentrations of $CaCl_2$, $Ca(NO_3)_2$, NaCl and K_3PO_4 used were 100, 300 and 500 m*M*, respectively, and 0.6 and -0.9 MPa for PEG 6000. GA_3 was used at concentrations of 50, 100, 300 and 500 μM. Among priming techniques, osmotic priming and biopriming are the most widely used. Chemicals related to osmotic priming include $CaCl_2$, $Ca(NO_3)_2$, NaCl, K_3PO_4, and PEG 6000, and GA_3, a metabolite related to biopriming, was also selected and applied to the experiment. Treatment for concentrations of priming agents and seeds were preceded at 20°C for 3 days in a dark condition (Park *et al.* 2013).

Germination of primed Korean perilla seeds

Korean perilla seeds were sterilized with 70% ethanol for 5 min and 1% hydrogen peroxide for 5 min, and then dried naturally for 1 hour to achieve moisture balance of seeds. Next, 100 mL of priming solution and 5 g of sterilized perilla were placed in an Erlenmeyer flask. Priming treatment with $CaCl_2$, $Ca(NO_3)_2$, NaCl, K_3PO_4 and PEG 6000 was carried out for 3 days, at 20°C in the dark on a shaking incubator. Priming treatment with GA_3 was performed under dark condition at 20°C for 1 day.

Gene selection and primer design from the EST databases of Korean perilla

In our previous study, we analyzed and reported the metabolic classification for genes from the EST database contained in the Korean perilla cDNA library (Seong *et al.* 2015). As a result of Seong *et al*. (2015), nine genes related to seed germination were selected for analysis and are shown in Table 1, with numbers and the annotation of the EST database (Seong *et al.* 2015). To analyze the expression patterns of the 9 selected genes, RT-PCR were performed with 20-mer primers designed using the PICK primer program on the Bioneer homepage (https://www.bioneer.co.kr/index.php/).

RNA extraction from Korean perilla treated with GA_3

The prepared samples were placed in a pre-frozen pestle bowl with liquid nitrogen and ground to a fine powder using a stick. The ground sample was placed in a tube with TRIzol® Reagent (Thermo Fisher Scientific, USA), allowed to stand at room temperature for 5 min, with shaking, for thorough mixing. The samples were separated using a centrifuge at 13000 rpm and the supernatant transferred to a new tube, chloroform was added and left for 10 min, with shaking. The sample was again centrifuged at 13000 rpm and the supernatant transferred to a new tube. The supernatant was slowly mixed with 2–3 times volume of isopropanol and stored overnight at -20°C. The following day, samples were thawed, centrifuged at 13000 rpm, and the supernatant discarded. The resulting pellets were washed

with DEPC-treated 70% alcohol and dried. The total RNA was dissolved in DEPC-treated sterilized water and quantified on an agarose gel.

RT-PCR analysis

After cDNA synthesis from the quantified total RNA samples, RT-PCR was performed using primers (forward and reverse) for the Korean perilla actin gene. After confirming the expression level of the actin gene, the RT-PCR analysis was performed using primers for genes related to germination (Table 2). PCR conditions were as follows: initial denaturation at 94°C for 5 min; 28 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and 1 min extension at 72°C, followed by an additional 10 min extension time at 72°C. Aliquots of 12 μL of the reacted samples were loaded and separated by electrophoresis on a 1% agarose gel. The reaction was done in triplicate for clarity of results. The band detected on the agarose gel was cloned into a pGEM T-easy vector, followed by sequencing, and homology was confirmed by aligning with sequences of the original genes.

Statistical analysis of germination rates

After treatment with the priming reagent, a germination test was carried out by in triplicate with 50 seeds for each treatment at 25°C for 10 days. To investigate the germination characteristics resulting from priming treatments, the average number of germinating seeds was determined after 15 days and was performed in triplicate. Statistical significance was analyzed using Duncan's Multiple Range Test (DMRT) using the IBM SPSS Statistics software (SPSS v. 23, International Business Machines Corp., Armonk, NY, USA). Statistical significance was determined at the 5% level.

Results

Improvement in germination rates by priming of Korean perilla seeds

In this study, the germination rates of Korean perilla were analyzed after treatment with six priming agents viz. $CaCl_2$, $Ca(NO_3)_2$, NaCl, K_3PO_4, PEG and GA_3. When seeds were primed with $CaCl_2$ at the concentrations of 100, 300 and 500 mM, the germination rates were 50.00 ± 1.63% for Saeyeopsil and 62.00 ± 1.63% for line 141 with 100 m*M*, and 68.66 ± 8.99% for Okdong with 300 m*M*. For priming with $Ca(NO_3)_2$, the germination rate was 56.00 ± 2.82% at 100 m*M* for Saeyeopsil and 72.66 ± 8.37 and 61.33 ± 6.79% at 300 m*M* for Okdong and line 141, respectively. The germination rate for all Korean perilla seeds primed with 100 m*M* NaCl ranged from 44.00 ± 3.26 to 53.33 ± 8.21%. However, NaCl treatment resulted in a lower germination rate compared to the control without priming treatment. The germination rate for the priming treatment with -0.96 MPa PEG was 58.00 ± 4.32% for Saeyeopsil and 64.00 ± 4.32% for Okdong, respectively. For the 141 collection, was higher value as 55.33 ± 2.49% in that of -0.6 MPa PEG. Priming with 0.1 m*M* GA_3 showed the best values among the priming treatment agents for all the Korean perilla seeds, presenting values ranging from 62.66 ± 1.88 to 70.66 ± 4.10%. However, no germination was observed with treatment at any concentration of K_3PO_4. Among various priming agents, 'Saeyeopsil' and 'Okdong' showed a high germination rate of 60~70% or more under GA_3 treatment, and '141 collection' showed a high germination rate of 70% or more under treatment with 100 m*M* $CaCl_2$ or 0.1 m*M* GA_3 (Table 3).

Gene expression by GA_3-priming treatment in Korean perilla

As GA_3 proved to be the most effective at increasing germination rates in Korean perilla among all the priming agents used, it was selected as the priming agent for the analysis of the expression patterns of nine genes related to plant development. Gene expression patterns were compared between Korean perilla seeds treated or untreated with 0.1 mM GA_3 for 1–5 d. We found no significant difference in the transcriptional levels of genes between GA_3 treated and untreated controls in Saeyeopsil. However, gene expression levels were higher in Okdong seeds treated for 1 d with GA_3 than in the water-only controls. The genes showing the greatest induction after GA_3 treatment for 1 d, were: protochlorophyllide reductase-like, magnesium chelatase subunit ChlI, heme-binding protein 2-like, glyceraldehyde 3-phosphate dehydrogenase A (GAPDH), Chlorophyll a-b binding protein 6 (LHCP), B2 protein, 2-Cys peroxiredoxin BAS1, and 21 kDa protein (Fig. 1).

Higher transcriptional levels were also observed for 141 collection seeds with GA_3 treatment for 1 d compared to controls. The highest expression levels were recorded for protochlorophyllide reductase-like, magnesium chelatase subunit ChlI, heme-binding protein 2-like, GAPDH, 2-Cys peroxiredoxin BAS1 and 21 kDa protein. Gene expression in Korean perilla seeds treated with GA_3 for 5 d showed a similar expression pattern compared to water treatment alone (Fig. 1). These results show that various genes are involved in seed germination metabolism during the early stages in Korean perilla seeds primed with GA_3.

Discussion

In general, priming agents should be free of toxicity and kept under constant water conditions for effective plant growth. Priming treatment agents inhibit cellular osmotic regulation, and high concentrations of ions can inhibit germination by destroying enzymes and membrane (Seo *et al.* 2009). The ion concentrations of the priming solution can affect germination and seedling appearance, as the

Table 1: Genes related to germination analyzed from the EST data of Korean perilla cDNA library

EST NO.	Annotations by blast results of EST
Perilla-1-1a_pTriplEx2-seq_E22	PREDICTED: 1-aminocyclopropane-1-carboxylate oxidase [*Sesamum indicum*]
Perilla-1-4a_pTriplEx2-seq_J14	PREDICTED: 21 kDa protein [*Sesamum indicum*]
Perilla-1-2a_pTriplEx2-seq_M18	PREDICTED: 2-Cys peroxiredoxin BAS1, chloroplastic-like [*Sesamum indicum*]
Perilla-2-1a_pTriplEx2-seq_I15	PREDICTED: B2 protein [*Sesamum indicum*]
Perilla-1-1a_pTriplEx2-seq_C22	PREDICTED: chlorophyll a-b binding protein 6, chloroplastic [*Sesamum indicum*]
Perilla-1-1a_pTriplEx2-seq_A24	PREDICTED: glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic [*Sesamum indicum*]
Perilla-3-2a_pTriplEx2-seq_G10	PREDICTED: heme-binding protein 2-like [*Sesamum indicum*]
Perilla-1-1a_pTriplEx2-seq_K12	PREDICTED: magnesium-chelatase subunit ChlI, chloroplastic-like [*Sesamum indicum*]
Perilla-2-2a_pTriplEx2-seq_C12	PREDICTED: protochlorophyllide reductase-like [*Sesamum indicum*]

Table 2: The primers designed to gene expression of EST selected from Korean perilla cDNA library

Actin gene and EST No.	Forward	Reverse
Pfactin	ACAGAGGCACCTCTCAACCC	ATCACGACCAGCAAGATCCA
Perilla-1-1a_pTriplEx2-seq_E22	GCGAAAACTGGGGTTTCTTC	AGGAAGAAGGTGCTCTCCCA
Perilla-1-4a_pTriplEx2-seq_J14	TGGAGGAGCTGTCTGACTCG	CGCCACATTCACAATCTTCC
Perilla-2-2a_pTriplEx2-seq_M18	CTAGTGACCGAGTGCCGAGA	GCTTGCAAGTGCTTCGTTTC
Perilla-2-1a_pTriplEx2-seq_I15	GTGCATGGCAACCTACAAGG	GATGCACGTAAGCACCCATC
Perilla-1-1a_pTriplEx2-seq_C22	CCGTCCTCTCTTCCTCCAAG	GTGGGTCGAATCCGAAATCT
Perilla-1-1a_pTriplEx2-seq_A24	TTGTGATCGAGGGAACTGGA	AGGAAGCGTTGCTGATGATG
Perilla-3-2a_pTriplEx2-seq_G10	TGATTTGGAGGATATCGGCA	CCTCTCTTTGTGAAAGGGGC
Perilla-1-1a_pTriplEx2-seq_K12	GAGCCAGAGGCCAGTTTACC	TCTCCCTCACTTCAGGACCC
Perilla-2-2a_pTriplEx2-seq_C12	CCCCTCTAACAAGGGAGCAG	GTTCGGGTACACTGACACGC

agents penetrate into the seeds and may have toxic effects. Additionally, increases in ion accumulation of a priming solution can reduce the priming effect by interfering with metabolism (Seo *et al.* 2009).

In a previous report, the germination rate of *Hippophae rhamnoides* seeds was shown to 52.6% of 300 m*M* and 50.9% of 400 m*M* under $CaCl_2$ priming treatment, respectively (Choi 2012). On the contrary, in a priming study of *Sorbus alnifolia* seed, $CaCl_2$ treatment resulted in a reduced germination rate compared to the control (Park *et al.* 2013). $Ca(NO_3)_2$ priming treatments for Saeyeopsil and Okdong produced higher germination rates than with $CaCl_2$. $Ca(NO_3)_2$ priming treatment was effective in tomato, but application resulted in a decrease when compared to the control in sesame seeds (Cho *et al.* 2011b). This indicates that the effect of priming treatment is crop-dependent. In this study, the germination rates with the NaCl priming treatment were lower compared to the non-treated controls, while K_3PO_4-treatment completely inhibited germination. Inorganic salts such as NaCl and K_3PO_4 are often used when salt priming is applied. Nitrogen-containing salts are more effective at improving germination rates than salts containing phosphoric acid (Bose *et al.* 2018). However, in this report, germination rates of Korean perilla seeds did not show any improvement with NaCl priming treatment.

PEG is known to play a role in regulating osmotic equilibrium (Ismail *et al.* 2005). The germination rates of Korean perilla seeds under PEG priming treatment increased compared to the controls, as was previously reported for germination rates and germinative power of *Alnus* sibirica (Park *et al.* 2013). In the case of *Zanthoxylum piperitum* seeds, GA_3 has been reported to increase the germination rate with increasing immersion time and concentration (Lim *et al.* 2015). The germination rate was significantly improved with GA_3 levels of 25 ppm, and the germination rate tended to increase with increasing GA_3 concentrations in *Lithospermum erythrorhizon* seed (Kim *et al.* 2014). Among all the priming agents tested, the results indicate that GA_3 had the greatest effect, showing an increase of over 70% in the germination rates of Okdong and the 141 collection cultivars of Korean perilla.

In the past, many studies on seed priming with GA_3 and related genes in various plants such as vegetables or *Arabidopsis* have been reported, but these results are very limited in medicinal plants (Ogawa *et al.* 2003). Therefore, in our results, optimal germination conditions of Korean perilla were established during GA_3 priming, so we studied to analyze the correlation with genetic changes at the cellular level. DNA repair and antioxidant mechanisms are involved in minimization of growth inhibition for seeds during seedling development. The effects of the priming agent on DNA repair mechanisms are essential to optimize priming methods (Balestrazzi *et al.* 2015). Therefore, the induced genes according to the establishment of priming optimization during seed germination of Korean perilla were identified. It is expected that these genes can be used as biomarkers to create a cultivation environment that increase the germination rate of Korean perilla by investigating genes induced during seed germination using GA_3.

In peas, protochlorophyllide reductase has been shown to play a post-transcriptional regulatory role in protein elongation and conversion. Protein expression patterns differ between monocots and dicots, but protochlorophyllide reductase is present in higher plants (Cahoon and Timko 2000). Magnesium-chelatase subunit ChII is known to be active in plant-cell interactions, chelating magnesium on protoporphyrin IX and mediating plastid-to nucleus retrograde signaling (Papenbrock *et al.* 2000; Nott *et al.*

Table 3: Germination rate of three different cultivars depending on priming treatments in *Perilla frutescens*

Seed Treatment		*Perilla frutescens*		
		Saeyeopsil	Okdong	141 collection
Priming Agents	Concentrations	Germination rate (%)		
Control		46.67 ± 12.85^{bcdef}	58.00 ± 5.29^{de}	54.67 ± 1.15^{cdefg}
$CaCl_2$	100 m*M*	50.00 ± 2.00^{abcef}	62.00 ± 3.46^{cd}	75.33 ± 3.06^{a}
	300 m*M*	32.67 ± 5.77^{g}	68.66 ± 11.02^{abc}	49.33 ± 3.06^{fg}
	500 m*M*	42.00 ± 2.00^{efg}	56.67 ± 4.62^{de}	45.33 ± 6.43^{g}
Ca $(Na_3)_2$	100 m*M*	56.00 ± 3.46^{abcd}	69.33 ± 8.33^{abc}	59.33 ± 7.02^{cdef}
	300 m*M*	54.00 ± 6.93^{abce}	72.67 ± 10.26^{ab}	61.33 ± 8.32^{bcde}
	500 m*M*	43.33 ± 12.22^{defg}	60.67 ± 3.06^{cd}	48.67 ± 2.31^{g}
NaCl	100 m*M*	44.00 ± 4.00^{cdefg}	55.33 ± 5.03^{de}	53.33 ± 10.07^{defg}
	300 m*M*	40.00 ± 4.00^{fg}	50.00 ± 6.00^{e}	48.67 ± 7.02^{g}
	500 m*M*	34.00 ± 3.46^{g}	50.00 ± 2.00^{e}	33.33 ± 6.10^{h}
K_3PO_4	100 m*M*	ND	ND	ND
	300 m*M*	ND	ND	ND
	500 m*M*	ND	ND	ND
PEG	-0.6 Mpa	48.67 ± 16.65^{bcdef}	62.00 ± 10.39^{cd}	55.33 ± 3.06^{cdefg}
	-0.9 Mpa	58.00 ± 5.29^{ab}	64.00 ± 5.29^{bcd}	51.33 ± 4.16^{efg}
GA_3	0.05 m*M*	62.00 ± 2.00^{a}	74.67 ± 2.31^{a}	59.33 ± 9.02^{cdef}
	0.1 m*M*	62.67 ± 2.31^{a}	78.67 ± 4.16^{a}	70.67 ± 5.03^{ab}
	0.3 m*M*	54.00 ± 7.21^{abce}	74.00 ± 2.00^{ab}	63.33 ± 5.03^{bcd}
	0.5 m*M*	56.66 ± 5.03^{abc}	75.33 ± 1.15^{a}	64.66 ± 4.16^{bc}

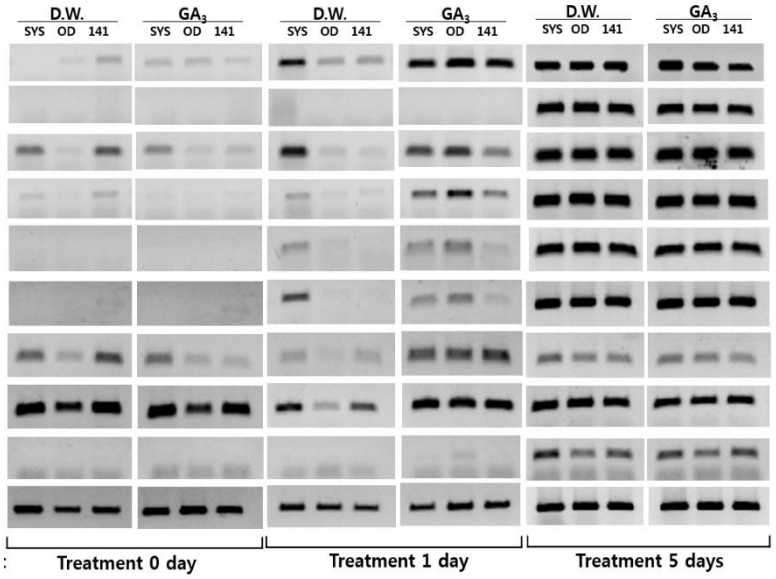

Fig. 1: Expression patterns of genes related to germination from EST analysis data of Korean perilla after seed priming with water and GA_3

2006). HBP is induced by oxidative stress and is involved in various functions of the protein (Lee *et al.* 2012). GAPDH catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate and has two isoforms, GAPCp1 and GAPCp2, both of which are important for the plastidial glycolytic pathway in plant primary metabolism (Munoz-Bertomeu *et al.* 2009). The LHCP gene shows an expression pattern specific to chloroplast-containing tissue, and mRNA expression can be determined by its associated factors (Wang and Grimm 2021). The 2-Cys peroxiredoxin BAS1 gene has antioxidant properties that regulate cellular redox states and is associated with the soluble chloroplast fraction function of mesophyll protoplasts in higher plants (Cerveau *et al.* 2016).

Conclusion

In this study, genes from the Korean perilla selected from the EST database that were induced by GA_3 treatment are related to oxidative stress, plastidial metabolism, tissue specificity, redox reactions, and chloroplast function in plant cells. It was found that the method to increase the germination rate of Korean perilla is the optimal concentration treatment of GA_3. Under this optimal condition, these marker genes such as protochlorophyllide reductase-like, magnesium-chelatase subunit ChlI, heme-binding protein 2-like, glyceraldehyde 3-phosphate dehydrogenase A, and Chlorophyll Since ab binding protein 6, B2 protein, 2-Cys peroxiredoxin BAS1, and 21 kDa protein genes are induced and this pattern is

thought to be involved in GA_3 priming. We suggest that these genes induce substances related to the initial stages of germination metabolism of Korean perilla seeds under GA_3 priming, thus improving the germination rate. In the future, we propose studying the functional relationship between these genes and the germination of Korean perilla seeds.

Acknowledgements

This study was supported by the Bioherb Research Institute, Kangwon National University, Republic of Korea.

Author Contributions

ES Seong and BJ Kang performed experiment design and writing of manuscript. CYY supervised the experi ment. JH Yoo, JG Lee and NY Kim performed editing of manuscript.

Conflicts of Interest

The authors declare that they have no confict of interest.

Data Availability

Data presented in this study are available with the authors.

Ethics Approval

There are no researches conducted on animals or human.

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