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Full Length Article

Boric Acid Mediates Self-Incompatibility Response by Affecting Sugar Metabolism in Radish

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Abstract

Physiological changes and gene expression related to self-incompatibility (SI) in radish were studied when treated with boric acid (BA). The results showed that at 24 and 48 h after the 0.4% BA treatment, the soluble sugar was 14.93 and 37.04% lower than the control, while the content of soluble protein increased by 7.07 and 6.78% compared with the control, respectively. Nonetheless, BA had a little effect on the free proline content. BA treatment also caused deferential expression of genes in radish pistils. Totally, 2021 differentially expressed genes (DEGs) were identified, of which 1024 were up-regulated and 997 were down-regulated. Gene ontology (GO) analysis revealed that the DEGs were grouped into three categories viz., biological process, cell component and molecular function. The binding and catalytic activity of molecular function and cellular process, metabolic process and single-organism process in biological process played major roles. Furthermore, Kyoto encyclopedia of genes and genomes (KEGG) analysis enriched 20 main pathways. The pathways of pentose and glucuronate interconversions, glucosinolate biosynthesis, starch and sucrose metabolism were the most possible pathways affected by BA. The genes affected by BA in the pathways of starch and sucrose metabolism, and pentose and glucuronate interconversions were 39 and 32, respectively. Two DEGs related to β-1, 3-glucanase were identified, both of which were down-regulated. Moreover, four DEGs related to SI protein S1 were found, all of which were down regulated. The quantitative real-time PCR (qRT-PCR) analysis showed that the transcript levels of four SI related genes and two β-1, 3-glucanase genes were consistent with the trends of transcriptome data. These results suggested that exogenous BA remarkably affected the expression of genes which may be related to callose deposition and SI reaction in radish. © 2020 Friends Science Publishers

Keywords: Radish; RNA sequence; Gene expression; Callose; Self-incompatibility

Introduction

The radish (*Raphanus sativus* L.; 2n=2x=18) belongs to the Brassicaceae family, wherein the self-incompatibility (SI) phenomenon exists widely. SI plays an important role in maintaining species diversity, adaptation to environmental changes, and avoidance to inbreeding depression (Fujii *et al*. 2016). SI also provides a favorable approach for the utilization of heterosis in plants, one of the main breeding methods of radish to produce F_1 hybrids. SI in Brassicaceae is controlled by multiple alleles of S-locus (Watanabe *et al*. 2012). There are three closely linked genes at the S-locus including S-locus receptor kinase (SRK), S-locus cystine rich protein (SCR) and S-locus protein 11(SP11) or S-locus glycoprotein (SLG). SRK and SLG are expressed in stigma, and SRK is the only determinant of stigma SI, nonetheless, the SLG and SRK gene sequences were highly homologous (Takayama *et al*. 2001). SLG was once thought to be the determiner of SI, but later it turned out to be not, although it promoted the SI reaction.

In self-cross-pollination, SLG and SCR form a complex. The signal peptides at the SLG-C terminal guide the SCR-SLG complex through the intercellular space to the cell wall of the stigma epidermal cells. The conformation of the complex changes after signal dissociation, and the SCR ligand specifically binds to the extracellular domain of the stigma homologous SRK, then it activates the intracellular kinase (Tsuchimatsu *et al*. 2010; Biicherl *et al*. 2013). The SRK conformation is affected by SLG, whereas a stable SRK conformation can enable it to form dimers in inactive state (Tsuchimatsu *et al*. 2010; Biicherl *et al*. 2013). Notably, the SCR and SP11 are expressed in anthers. While the SCR is the key determinant of male SI, it also occurs with the SLG and SRK in stigma to trigger the SI reaction. The protein encoded by SCR gene is the ligand carried by pollen. After meiosis, hydrophilic polypeptides bind to the coat protein of pollen, which is carried by pollen to the mastoid cell wall of stigma and bound to the extracellular domain of SRK to activate the SRK kinase and thereby activating the transmission of SI signal (Kachroo *et al*. 2001; Shiba *et al*. 2001). ARC1 (arm-repeat containing 1), MLPK (M-locus protein kinase), EX070A1 (a member of Exocyst 70 family) and THL1 (thioredoxin-H-like-1) can interact with SRK kinase domain and then affect the SI reaction.

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The ARCl gene possesses the activity of E3 ubiquitinated ligase, which can increase the ubiquitinated protein on the stigma and trigger SI reaction (Goring *et al*. 2014; Indriolo *et al*. 2014; June *et al*. 2014). The Exo70A1 may be a downstream signaling receptor for ARC1 (Samuel *et al*. 2009). When the level of Exo70A1 ubiquitination is high, the ubiquitin binding enzyme E2 break off from the (E2/ARC1/Exo70A1) protein complex and ARC1 directs the transfer of Exo70A1 to the proteasome, induces the self-crosslinking affinity protein Exo70A1 to degrade on the proteasome, and finally inhibits the germination of "self-flowering" pollen and the elongation of pollen tubes (Drdova *et al*. 2013). Haffani *et al*. (2004) argued that when its own pollen scattered on the stigma surface, S protein structure domain of SCR and SRK was specifically recognized under the supporting role of SLG and the SRK configuration changed to made THL1/THL2 free, which originally remained combined with SRK and suppressed the phosphorylation. So the SRK phosphorylation and combination with ARC1, MLPK and SRK inducing a series of signaling cascade resulting in SI reaction. The MLPK belongs to serine-threonine protein kinase family and it can be phosphorylated by SRK to form an SRK-MLPK complex. In SI response, the phosphorylated SRK and MLPK complex activate the ARC1 by phosphorylating its arm-repeat domain (Kakita *et al*. 2007).

Boron is needed for pollen germination in most plants. Boron deficiency results in abnormal cell structure of floral organs, destruction of anther sporogenous layer tissues, deformed pollen tubes and abnormal pollen development (Iwai *et al*. 2006). Boron transporter OsBOR4 is involved in rice pollen germination (Tanaka *et al*. 2013; 2014), whereas the RTE and TLS1 (ZmNIP3;1) participate in the development of maize inflorescence and ear (Chatterjee *et al*. 2014; Leonard *et al*. 2014). Boron deficiency inhibits apple pollen germination and prevents pollen tube elongation (Fang *et al*. 2019). Callose is an important polysaccharide in pollen tube wall and a key factor affecting plant SI. The synthesis and distribution of callose are regulated by boron in culture medium (Wang *et al*. 2003). Our previous study found that exogenous boric acid (BA, foliar spray) improved the SI of radish (Wang *et al*. 2018). We speculated that BA might cause some physiological, metabolic and transcriptional changes to affect SI in radish. Therefore, in the present study, we determined the changes in the pistil at the transcript levels after BA treatment to further elucidate the mechanisms of SI as influenced by BA in radish.

Materials and Methods

Plant material

The plant material used in the present research was a self-incompatible radish genotype (*Raphanus sativus* L. cv. LY1101). Radish fleshy roots in similar sizes and shapes were selected and sown in the experimental field on February 21, 2018 for normal management. At the stage of pollen development, 0.4% BA (boric acid) solution was sprayed on flowers, and the pollen was collected for artificial supplementary pollination 30 min after spraying. The pistils were collected at 24 and 48 h after pollination to determine the contents of soluble sugar, soluble protein and free proline (Pro). The pistils were sampled 24 h after pollination for transcriptome analysis. The control was radish, which was treated only with water. All experiments were carried out in a randomized complete block design with three replications.

Measurement of biochemical and physiological parameters

Soluble sugars were determined using the phenol-sulfuric acid method (Dubois *et al*. 1956), where 0.5 g (fresh pistils) of sample was homogenized with deionized water, the extract was filtered and treated with 5% phenol and 98% sulfuric acid, and the mixture was allowed to settle for 1 h. The absorbance at 485 nm was determined using a spectrophotometer (UV-5200 spectrophotometer, Shanghai Metash Instruments Co. Ltd, Shanghai, China).

The free Pro content was determined using the method described by Bates *et al.* (1973), where 0.5 g (fresh weight) of sample was homogenized in 10 mL of 3% aqueous sulfosalicylic acid. 2 mL of the filtrate was reacted with 2 mL of acidic-ninhydrin and 2 mL of glacial acetic acid for one hour at 100ºC. The reaction mixture was then extracted with 4 mL toluene. The chromophore-containing toluene was aspirated from aqueous phase and the absorbance at 520 nm was measured using toluene as a blank. Soluble protein content was determined spectrophotometrically at 595 nm as described by Bradford (1976) using bovine serum albumin (BSA) as the standard.

Analysis and screening of differentially expressed genes (DEGs)

Three biological repeats were taken, each consisting of a mixture of 40 pistils. RNA was extracted and cDNA library was constructed. Illumina HiSeq2500TM system was used for sequencing. RNA sequencing was finished by Biomarker Technologies (Beijing, China). A reference transcriptome analysis was performed at http://www.biocloud.net/ and blasted with databases such as non-redundant protein sequence database (NR), Swiss-prot, gene ontology (GO), clusters of orthologous groups (COG), clusters of eukaryotic orthologous groups (KOG) and Kyoto encyclopedia of genes and genomes (KEGG) and Hammer software with Pfam database. DESeq was used for differential expression analysis and false discovery rate (FDR) <0.01 and fold change (FC) 2 were used as the screening criteria to obtain the differentially expressed gene sets between the two samples. GO functional enrichment and KEGG metabolic pathway information analysis were performed on differential genes.

Real-time quantitative RT-PCR (qRT-PCR) verification

The qRT-PCR was used to verify the expression of DEGs. Material processing was the same as 1.1. Pistils were collected, RNA was extracted. The cDNA was synthesized using primeScript^{TM} RT-PCR kit with MsActin as internal genes. The procedure was followed: 95℃ for 30 s; 95℃, 55℃ for 5 s, 30 s and 72℃ for 30 s, 45 cycles. Finally, $2^{-\Delta\Delta Ct}$ was used to calculate relative expression of genes (Livak and Schmittgen, 2001).

Statistical analysis

Data were subject to analysis of variance (ANOVA) and analyzed using SPSS19.0 Statistical Software. Significant differences between the means were separated by Duncan's Multiple Range Test (P<0.05).

Results

Effects of BA on biochemical and physiological metabolism in the pistils of radish

Compared with the control group, BA treatment had a significant effect on the content of soluble sugar, soluble protein, but it had a little effect on free Pro content in the pistils of radish (Table 1).

BA treatment reduced the content of soluble sugar, which was 14.93% lower than the control at 24 h after treatment, while 37.04% lower at 48 h. This indicated that the longer the treatment time, the more significant was the effect of BA on the content of soluble sugar. At the same time, BA treatment increased the content of protein. The content of soluble protein in BA treatment was higher than that of the control group at 24 and 48 h, which increased by 7.07% and 6.78%, respectively. The changes in free Pro content seemed similar to that of protein, however, the significance analysis showed that the difference between BA treatment and control did not reach a significant level at 24 and 48 h, respectively. Furthermore, among the three indices, BA had the largest influence on soluble sugars and the least influence on the free Pro content.

Screening of DEGs induced by BA

Through analysis, 2021 DEGs were obtained, among which 1024 were up-regulated and 997 were down-regulated. A total of 1987 genes were annotated through functional annotation of the database (Table 2).

GO function analysis of DEGs

The DEGs were divided into three categories through GO function analysis (Fig. 1) including biological process, cellular component and molecular function. These DEGs mainly described the possible molecular functions of gene products, their cellular environment and biological processes, which can further be divided into 22 subcategories, 16 subcategories and 15 subcategories. Binding and catalytic activity in molecular function, as well as cell process, metabolic process and single-organism process in the biological process were dominant in these DEGs.

Enrichment analysis of KEGG pathway of DEGs

KEGG pathway analysis enriched 20 major pathways which involved pentose and glucuronate interconversions, glucosinolate biosynthesis, starch and sucrose metabolism, alpha-linolenic acid metabolism, plant pathogen interaction, endocytosis, 2-oxycarboxylic acid metabolism, etc. (Fig. 2). The reference values of the three pathways of pentose-glucuronate interconversions, glucosinolate biosynthesis, starch and sucrose metabolism were the largest among them. The number of affected genes in the starch and sucrose metabolism and pentose-glucuronate interconversions pathway were more, which were 39 and 32, respectively.

Effects of BA on the expression of SI related genes

At present, it is believed that the SI reaction of plants is closely related to callose. Callose catabolism enzyme, also called β-1, 3-glucanase, is the main enzyme to degrade callose. In the present study, four genes annotated with β-1, 3-glucanase were identified by transcriptome sequencing (Table 3). No significant changes in the expression of two β-1, 3-glucanase genes (Rsa1.0_03670.1_g00002, Rsa1.0_17781.1_g00001) were found under the treatment of BA, but the other two (Rsa1.0_01564.1_g00003 and Rsa1.0_02320.1_g00009) were down-regulated. Furthermore, the expression of Rsa1.0_02320.1_g00009 was completely inhibited by BA. The above results suggested that BA treatment potentially affected the activity of callose enzyme. At the same time, 50 genes were annotated to be related to the SI protein S1, among which four were differentially expressed (down-regulated) due to BA treatment (Table 3). The results indicated that BA treatment also affected the SI reaction of radish.

qRT-PCR analysis of some DEGs

qRT-PCR was used to verify the expression of two β-1, 3-glucanase and four SI protein S1 genes that were differentially expressed, and the results were shown in Fig. 3. The gene expression of SI protein S1 was significantly inhibited under BA treatment compared with the control group (Fig. 3A), which was consistent with the transcriptome data (Table 3). These data indicated that BA did affect the expression of SI protein S1 genes. It can be seen from Fig. 3B, that the transcript levels of two β-1, 3-glucanase genes were also inhibited under BA treatment, in which Rsa1.0_02320.1_g00009 was completely

Table 1: Effects of BA treatment on biochemical and physiological indices of pistils

Treatments	soluble sugar content (g/100 g)	soluble protein content (mg/g)	free Pro content (μg/g)
0.4%BA treatment (24 h)	5.47b	18.77b	125.67c
0.4%BA treatment (48 h)	4.30c	19.83a	137.33a
Control (24 h)	6.43a	17.53c	120.42c
Control (48h)	6.83a	18.57b	133.85ab

Table 2: Annotation of DEGs induced by BA

Total DEGs	COG	GO	KEGG	KOG	NR	Pfam	Swiss-Prot	eggNOG
1987	759	1842	720	1074	1983	1570	1602	1944

Table 3: SI related genes of DEGs in radish

Genes	FDR	regulation	function annotation
Rsa1.0_01564.1_g00003	0.00331	down	1,3-beta-glucanase [Brassica rapa subsp. chinensis]
Rsa1.0_02320.1_g00009	7.145E-28	down	1,3-beta-glucanase [Brassica rapa subsp. chinensis]
Rsa1.0_03670.1_g00002	-	-	1,3-beta-glucanase [Brassica rapa subsp. chinensis]
Rsa1.0_17781.1_g00001	-	-	1,3-beta-glucanase homologue [Brassica napus]
Rsa1.0_00081.1_g00018	0.0000233	down	Plant self-incompatibility protein S1
Rsa1.0_00081.1_g00019	0.000380	down	Plant self-incompatibility protein S1
Rsa1.0_00044.1_g00015	2.144E-08	down	Plant self-incompatibility protein S1
Rsa1.0_00253.1_g00029	9.28E-09	down	Plant self-incompatibility protein S1

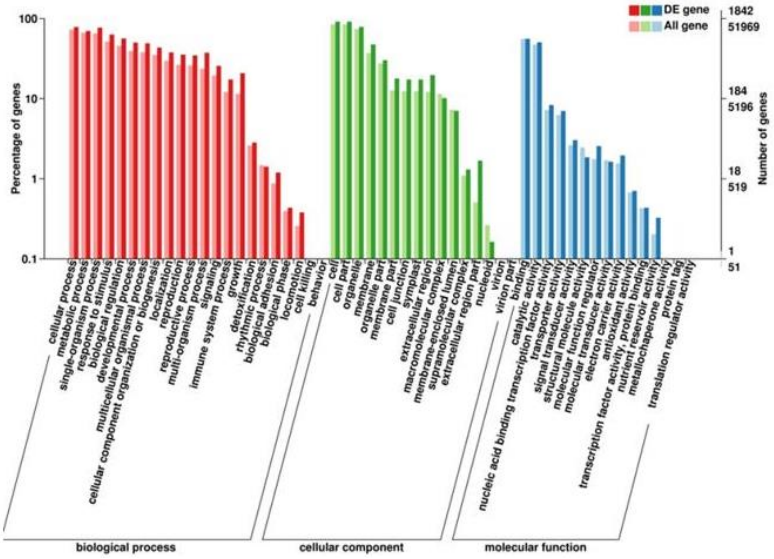


Fig. 1: GO function analysis of DEGs

inhibited. These results were basically consistent with the data in Table 3. So, it is concluded that BA treatment affected not only the callose degradation, but also the SI protein S1 and these two components were closely related to SI reaction in radish.

Discussion

It is believed that callose deposition at the top of pollen tube hinders the growth of pollen tube (Dumas and Knox 1983; Julius *et al*. 2018). Meanwhile, the callose deposits between the cell wall and the plasma membrane of the stigma mastoid cell also prevent the pollen tube from passing through the stigma, resulting in ultimate failure of pollination (Chen *et al*. 2017; Xiong *et al.* 2019). The principal component of callose is β-1, 3- glucan, which is synthesized from uracil diphosphate glucose (UDPG) by the catalysis of callose synthase (GSL) and is decomposed by β-1, 3-glucanase. In this study, it was found that BA down-regulated the expression of two β-1, 3-glucanase genes. Because β-1, 3-glucanase is the enzyme that breaks down callose, the amount of callose deposition after BA treatment should be more than that of the control in theory, but our previous results showed that the callose deposition under BA treatment was less than that of the control (Wang *et al*. 2018). The most probable reason was that BA treatment also inhibited callose synthesis resulting in decreased callose deposition. It is possible that the enzymes catalyzing

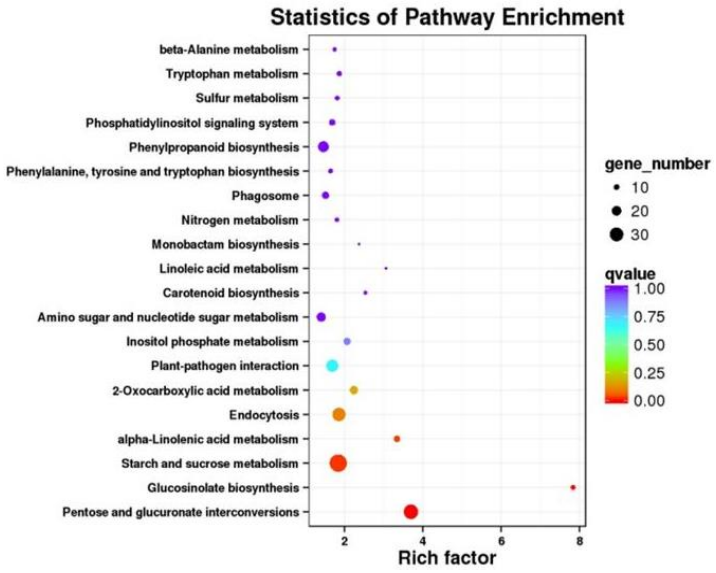
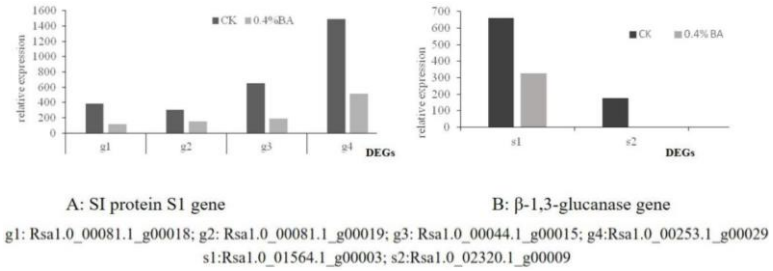


Fig. 2: Pathway Enrichment analysis of DEGs



A: SI protein S1 gene B: β-1,3-glucanase gene

g1: Rsa1.0_00081.1_g00018; g2: Rsa1.0_00081.1_g00019; g3: Rsa1.0_00044.1_g00015; g4:Rsa1.0_00253.1_g00029
s1:Rsa1.0_01564.1_g00003; s2:Rsa1.0_02320.1_g00009

Fig. 3: qRT-PCR analysis of some DEGs

callose synthesis (for example GSL) was also inhibited. However, no GSL genes were found to be inhibited by BA in this study. Moreover, KEGG pathway enrichment proved that BA affected the starch and sucrose metabolism in radish. UDPG was a precursor of sucrose synthesis. Therefore, it was most likely that the raw material (UDPG) for callose synthesis decreased. The determination of soluble sugar content also showed that the content of soluble sugar under BA treatment was significantly lower than that of control (Table 1). Sugar is mainly transported as sucrose in plants, while UDPG is the precursor of sucrose synthesis. The synthesis of UDPG requires boron, so boron is crucial for the synthesis and transportation of sucrose (Niu and Zhang 2003). It is plausible that BA down-regulated UDPG content resulting in reduced callose synthesis. Bellaloui *et al*. (1999) and Brown *et al.* (1999) showed that the absorption, transport and utilization of boron were closely related to sugar, boron and sugar can form complex and boron-containing polysaccharides. Furthermore, the role of boron in the synthesis and transport of sugars has been confirmed by many studies. For instance, foliar applications of boron significantly increased the contents of total sugar, reducing and non-reducing sugars in pineapple (*Ananas comosus* L.) and guava (*Punica granatum* L.) fruit (Kar *et al*. 2002; Awasthi and Lal 2009). Boron also stimulated sucrose accumulation in watermelon (Li *et al*. 2010). A foliar application of boron increased the fruit sugar content significantly (Nagy *et al*. 2010). Hegazi *et al.* (2018) also proved that the total soluble sugars significantly increased as the boron application rate increased in olive leaves. Conversely, no significant differences were recorded concerning the total levels of translocated sugars (sucrose + sorbitol + mannitol) in *E. japonica* (Papadakis *et al*. 2018). Li *et al.* (2010) showed that boron promoted the synthesis of watermelon sucrose by regulating sucrose metabolism enzymes, such as acid invertase (AI), neutral invertase (NI), sucrose synthase (SS) and sucrose phosphorylase (SPS).

From what has been discussed above, we conclude that although the effects of boron on sugars in plants vary from organ to organ and from developmental stage to developmental stage with differential fluctuations, it is certain that boron is closely related to glucose metabolism in plants. Studies have shown that boron affects the transport of sugar mainly because it affects the deposition of callose on sieve tubes (Riaz *et al*. 2019). Callose deposits are known to block cell to cell movement of solutes, hormones, and proteins, therefore, the control of callose deposition is a crucial mechanism of phloem sap transport, resource

allocation and plant development (Chen and Kim 2009; Cui and Lee 2016; Julius *et al*. 2018). Wang *et al.* (2003) conferred that boron affected the synthesis of callose, changed the properties of cell membrane, and eventually caused changes in the synthesis and distribution of callose. Combined with previous results that BA improved SI of radish (Wang *et al*. 2018), we speculated that the main reason for BA to improve the affinity of radish might be that it affected the synthesis and transportation of UDPG (the raw material for callose synthesis).

Pollens are rich in Pro, and pollination adds exogenous Pro to stigma (Britikov *et al*. 1970). Pro acts as a potent reserve (as a source of energy and nitrogen) and is utilized during germination of pollens. This is one of the reasons for the reduction of free Pro content along with the pollen germination. Moreover, Pro directly incorporates to proteins (structural proteins and biologically active proteins). There are many Pro-rich proteins and peptides that may affect SI in plants (Linskens and Tupý 1966; Ebert *et al*. 1989; Wang *et al*. 1993). In the current study, free Pro was measured in pistils and the results showed that BA had a little effect on the free Pro content. These results suggested that BA affected radish SI not through Pro metabolism. In a word, BA may affect radish SI mainly by affecting the metabolism of sugar, particularly the synthesis and transport of UDPG. Of course, how does it affect the synthesis and transport of UDPG remains to be elucidated.

Conclusion

BA caused changes in soluble sugar and protein content in the pistil of radish and induced differential expression of related genes. KEGG analysis showed that the pathways of pentose-glucuronate interconversions, glucosinolate biosynthesis, starch and sucrose metabolism were the most possible ways affected by BA. While the number of affected genes in the starch and sucrose metabolism and pentose-glucuronate interconversions pathway were more, which were 39 and 32, respectively. The qRT-PCR verified that two β-1, 3-glucanase and four SI protein-related genes were differentially expressed. This study deepens our understanding that BA may regulate radish SI by affecting sugar metabolism in radish pistils.

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