Characterization of Tissue-specific Transcriptomic Responses to Seedlessness Induction by Gibberellin in Table Grape

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Gibberellins are widely used to induce seedlessness in table grape production, but are not effective for every cultivar or developmental stage. In order to characterize the potential physiological mechanism behind gibberellin-induced seedlessness, we conducted tissue-specific transcriptome analysis of ovules and ovaries from cultivars with different levels of receptiveness to seedlessness induction. GA₃ treatment at full bloom highly induced gibberellin signaling and various phytohormone signaling pathway genes in ovules at two days after treatment, but had less effect in ovaries, which suggests that GA₃ directly affects seed development. During the development to six days after GA₃ application, cell cycle-related genes in the ovules were markedly downregulated, indicating that this may be related to ovule abortion induction. The marked transcriptomic responses were largely absent in a cultivar with low receptiveness to gibberellin-induced seedlessness, suggesting a crucial role for the observed tissue-specific responses in the successful induction of seedlessness by GA₃ application. Collectively, our transcriptome analysis highlights distinct tissue-specific reactions to GA₃ during early berry development, providing important insights for the successful production of seedless berries by gibberellin application.

Key Words: gene expression, phytohormone, RNA-seq, seed development.

Introduction

Phytohormones are signaling molecules in plants that control various physiological processes. Phytohormones are generally effective at low concentrations and have been intensively applied in agricultural practices (Jiang and Asami, 2018; Šimura et al., 2018). One of the most successful examples of the practical application of phytohormone is gibberellins (GAs) for seedless table grape (*Vitis vinifera*, *V. labrusca*, and their hybrids) production. GA application on developing flowers induces seed abortion and results in seedless berries (Ledbetter and Ramming, 1989). Also, GA application is routinely used to increase berry size and cluster thinning in the production of genetically

seedless (stenospermocarpic) cultivars (Ledbetter and Ramming, 1989; Acheampong et al., 2017). Currently, in Japan, seedlessness is a commercial standard for table grapes (Yamada and Sato, 2016). In many commercial cultivars, seedlessness is practically achieved by the application of GA₃ at full bloom to three days after full bloom. For example, GA₃ application at flowering is known to be effective to induce seedlessness for the tetraploid hybrids 'Kyoho' and 'Pione', and the diploid hybrid 'Shine Muscat' (Kishi and Tazaki, 1960; Fukunaga and Kurooka, 1988; Kondo and Kawai, 1998; Yamada et al., 2008).

GA-induced seedlessness for table grape was achieved firstly for 'Delaware' (*V. labruscana*) in the 1960s. Seedlessness in 'Delaware' is known to be achieved by applying GA₃ at 14 days before anthesis, but if GA₃ is applied around the flowering stage, seedlessness is not induced perfectly and the cluster consists of many seeded berries. Some studies suggested that the seedlessness of the 'Delaware' grape is attributed to the production of sterile pollen and delayed embryo development caused by GA₃ application at 14 days before anthesis (Sugiura and Inaba, 1966; Sugiura, 1969). As

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mentioned above, it is also known that inducing seedlessness by applying GA₃ for the cultivars bearing large-sized berries, such as ‘Kyoho’ and ‘Pione’, should be at full bloom to three days after full bloom, whereas it should be at 14 days before anthesis for small-sized berry cultivars such as ‘Delaware’. Details on the seedlessness induction mechanisms for the large-sized berry remain unclear.

We previously showed that some cultivars with large-sized berries can be divided into two types according to the process in GA-induced seedlessness (Yoshimura et al., 2021); one type is naturally difficult to fertilize due to abnormality of embryo development, and the other type can develop normal embryo. The former normally causes a large amount of berry drop around anthesis, but GA₃ application from full bloom to three days after full bloom promotes fruit set and development, and produces seedless berries. ‘Kyoho’ and ‘Pione’ belong to this type. The latter type fertilizes and produces seeded berries in the cluster at a quite high rate in natural conditions; induced seedlessness by GA₃ application as in the former type is also possible. ‘Shine Muscat’ and ‘Honey Venus’ belong to this type, but have different sensitivities to GA-induced seedlessness. Yoshimura et al. (2021) reported that a higher rate of seedless berries could be obtained in ‘Shine Muscat’ than in ‘Honey Venus’ in a GA₃ treatment experiment. This difference in response to GA was suggested to be a heritable trait (Nagata and Kurihara, 1982). Due to the variation in responsiveness to GA, not all genetic resources are currently available for seedless berry production. Understanding the mechanisms of the GA response may have significant implications for large-sized berry grape production and breeding.

GA is a phytohormone that is essential for many developmental processes in plants, including seed germination, stem elongation, leaf expansion, pollen maturation, and the induction of flowering (Achard and Genschik, 2009; Davière and Achard, 2013). GA signal transduction in Arabidopsis seeds positively regulates seed germination (Vishal and Kumar, 2018). In Arabidopsis, the molecular regulatory mechanisms of GA signaling for ovule initiation, integument development, and late embryogenesis control were uncovered (Gomez et al., 2016, 2018; Hu et al., 2018); these mechanisms may also be associated with the success of seedlessness induction by GA treatment in grapevines, but have not been examined in detail. GA signaling is often regulated by the amount of GA, and the levels of bioactive GAs in plants are maintained via feedback regulation of GA metabolism (Yamaguchi, 2008). GA2ox, which catabolizes active GAs, is often one of the major regulators of GA levels and is highly responsive to changes in GA signaling to ensure GA homeostasis (Rieu et al., 2008; Galinha et al., 2009; Sun, 2011). In contrast, expression of the genes encoding the GA biosynthesis enzymes GA20ox and GA3ox is high-

ly elevated in a GA-deficient background, whereas these genes are downregulated after application of bioactive GAs in Arabidopsis (Phillips et al., 1995; Matsushita et al., 2007; Yamaguchi, 2008). It was suggested that this feedback regulation is conserved in grapevines, at least in developing berries (He et al., 2019).

Furthermore, previous reports suggested the potential molecular mechanisms underlying seedlessness induction by pre-bloom GA application. Cheng et al. (2015) reported changes in the expression of genes for the biosynthesis and signaling of phytohormones, including GA, jasmonic acid, and salicylic acid, various transcription factors, and secondary metabolites by GA₃ treatment at 12 days before flowering in ‘Kyoho’. In addition, Cheng et al. (2013) reported changes in redox homeostasis by GA₃ treatment at 6–12 days after treatment in ‘Kyoho’, ‘Red Globe’, and ‘Thompson Seedless’ berries treated 16–18 days before flowering. The above studies showed general features of physiological responses upon GA₃ application; however, the following points are still not clear: (1) the key process responsible for GA-induced seedlessness, (2) the tissue-specificity of the responses caused by GA, and (3) the mechanisms underlying the differential response among large-sized berry grape cultivars, e.g., between ‘Shine Muscat’ and ‘Honey Venus’.

In this study, the details of the response to GA₃ treatment were analyzed with tissue-specific transcriptome analysis of large-sized berry grape cultivars, ‘Shine Muscat’ and ‘Honey Venus’, that shows different levels of receptiveness to seedlessness induction. In particular, tissue-specific transcriptome profiles and GA signaling-related genes were analyzed to discuss the variation in the GA₃ response during the flowering period.

Materials and Methods

Plant materials and GA treatment

This study was conducted in the 2019 harvest season. Four-year-old ‘Shine Muscat’ (SM) plants, grafted on ‘Kober 5 BB’ grown at the Maki experimental farm of Ryukoku University (Otsu, Japan), were used in this study. Five SM plants grown in 30-L pots were trained and cultivated using the short cane pruning cultivation system. The plants were cultivated with a conventional cultivation method (a similar method was presented in Suehiro et al., 2019). A few days before flowering, all flower buds in a cluster except for those bearing up to 3.5–4 cm from the tip were thinned, leaving approximately 45 flowers in each cluster. The cluster was dipped in a 25 ppm GA₃ solution, which is a standard concentration for the production of cultivars bearing large-sized berry in Japan, on the day all the flowers in the cluster had opened. The no-GA₃ treatment clusters, which had the same bloom day as the treated clusters, were used as control.

We also analyzed six-year-old ‘Honey Venus’ (HV)

and SM plants grafted on ‘Kober 5 BB’ in the orchard at the Grape and Persimmon Research Division, NARO Institute of Fruit Tree and Tea Science (Higashi-Hiroshima, Japan). One vine was used for each field-grown cultivar and trained using the long cane pruning cultivation system. GA₃ application was conducted as mentioned above. Pedigrees of SM and HV are available in references (Yamada et al., 2003, 2008).

To investigate seedlessness in the GA₃-treated and control clusters, clusters were collected in the harvest season (4th September) for the plants at Otsu, and just after véraison (3rd July) for the plants at Higashi-Hiroshima. The berries were cut with a razor blade to visually determine the presence or absence of seeds. The number of seeds per berry was counted and the percentage of seeded berries per cluster was calculated for each cluster.

For the analysis of transcriptome, ovule and ovary tissues were separately collected at 2 and 6 days after treatment (DAT) from the berries of SM (Otsu) and HV (Higashi-Hiroshima) (Supplementary Fig. S1). The samples were immediately frozen in liquid nitrogen and stored at -80°C until use. We selected the 2 and 6 DAT stages because there was no obvious difference in ovule size between treatment and control at 2 DAT, whereas the difference began to be visible at 6 DAT. Five berries were used as one sample, and four or five biological replicates were provided for RNA-Seq analysis. It should be noted that at least six days after flowering, there was no visible difference in the size and morphology of ovules and ovary tissues between the two cultivars in the control samples.

RNA extraction, library preparation, and sequencing

Total RNA was isolated by the hot borate method (Wan and Wilkins, 1994) from the ovule and ovary samples and quantified using Qubit 4 Fluorometer (Invitrogen, Carlsbad, CA, USA). Five hundred nanograms of total RNA were used to prepare mRNA-seq libraries. Double-stranded cDNA was prepared and fragmented according to a previous report (Burkart-Waco et al., 2013). The fragmented cDNA was subsequently processed with the GenNext[®] NGS Library Prep Kit (TOYOBO, Osaka, Japan) and the TruSeq DNA CD Indexes (Illumina, San Diego, CA, USA) for library preparation. Seventy-nine libraries in total were multiplexed and sequenced with three lanes of the Illumina HiSeqX system (PE150) at Macrogen (Daejeon, Korea). All RNA samples used for the RNA-seq analysis are listed in Supplementary Table S1. All sequence data are available in the DDBJ Sequenced Read Archive (accession number DRA011277).

RNA-seq data analysis

All sequences were pre-processed using fastp (Chen et al., 2018) with a minimum read length threshold of 35 bp. The reads from each library were aligned to the

coding sequence of the 12X *Vitis vinifera* reference genome with version 2.1 gene annotation (Jaillon et al., 2007), which was available at Phytozome v13 <https://phytozome-next.jgi.doe.gov/info/Vvinifera_v2_1>, using BWA-MEM with default parameters (Li and Durbin, 2009). Low-quality alignments were filtered out by using SAMtools view with -q 20 option (Li et al., 2009). All libraries were checked to ensure they had mapped read counts greater than one million. The number of reads mapped to each contig was counted from the SAM files with a custom python script: when one read was mapped to multiple genes, it was counted for each gene, i.e., there could be multiple counts for one read (Nishiyama et al., 2018). Gene expression level was represented as reads per kilobase per million reads (RPKM).

The DESeq2 package (Love et al., 2014) was used to define differentially expressed genes (DEGs) between GA-treated and control samples with a threshold of FDR < 0.05. The DEGs were hierarchically clustered by Morpheus software <<https://software.broadinstitute.org/morpheus>> with the average-linkage method, using a matrix of mean RPKM values of the biological replicates.

In addition to the functional annotations from the v2.1 database, the amino acid sequences of the annotated genes were searched against the representative protein sequences of the Araport11 Arabidopsis database with blastp. The best hit with an e-value threshold < e^{-20} was retrieved for each gene. A unique list of the GO terms from the v2.1 annotations and the GO terms assigned to the best-hit Arabidopsis locus was prepared and used for GO enrichment analysis. GO enrichment analysis was conducted using the topGO (version 2.24.0; <<https://bioconductor.org/packages/release/bioc/html/topGO.html>>) R package with the weight01 algorithm and the option for Fisher's exact test. GO terms with *P*-values < 0.05 and with gene counts > 2 were considered enriched GO terms.

Results

Effect of GA application on seed development

The rates of seeded berries per cluster (Fig. 1A), as well as the number of seeds per berry (Fig. 1B), were significantly lower in the GA₃ treatments than the controls in both SM (from Otsu and Higashi-Hiroshima) and HV (from Higashi-Hiroshima). Moreover, GA₃ treatment induced more seedless berries in SM than in HV.

Tissue-specific transcriptome profile

Principal component analysis showed that biological replicates were plotted close together without exception (Supplementary Fig. S2). Along with the first principal component, samples were separated at 2 and 6 DAT in both ovules and ovaries of SM, whereas no cluster separation was associated with GA₃ treatment. Although

SM (loc1, Otsu) and HV (Higashi-Hiroshima) had different genetic backgrounds, sampling locations, and management, the plots of the principal component analysis almost overlapped and there was no clear separation of cultivars.

In the differential expression analysis, 3,739 and 1,798 unique DEGs were identified in SM and HV, respectively, between the GA₃-treated and control samples (Table 1). The number of DEGs increased in the samples at 6 DAT compared to the samples at 2 DAT, except for the HV ovary samples. Several DEGs overlapped between SM and HV ovules at 6 DAT, whereas the other samples showed very limited shared DEGs. In the following, the results obtained from SM, with high-level receptiveness to GA₃, are mainly described.

Exploration of key systems involved in GA-induced seedlessness

The 3,739 DEGs that responded to GA₃ treatment in SM were divided into four clusters by hierarchical clustering (Fig. 2). Cluster 1 included genes that were highly expressed in control ovule samples. This cluster contained genes with basic functions such as “translation” (Supplementary Table S2). Cluster 2 showed a significant expression pattern in the ovule samples at 2 DAT and appeared to be an important cluster of genes associated with the very early response to GA₃ treatment and the difference in GA₃ responsiveness between SM and HV. The genes assigned to cluster 2 were

enriched with functions related to various plant hormones, including ethylene, ABA, cytokinin, and salicylic acid, in addition to GA metabolism and signaling (Supplementary Table S3). Also, the expression of genes annotated with GO terms related to GA, found in cluster 2, was remarkably affected by GA₃ treatment in the SM ovule sample, whereas most of the genes were not affected in the SM ovary (Fig. 3). Cluster 3 contained genes that were markedly differentially expressed in ovule samples at 6 DAT. Cluster 3 was characterized by a high number of genes involved in cell division and development (Supplementary Table S4) and may be associated with the key processes for abortion in GA₃ response. Cluster 4 contained genes that were predominantly expressed in the ovary samples. This cluster contained genes involved in cell differentiation and stress responses (Supplementary Table S5).

We also characterized gene expression related to GA metabolic and signal transduction pathways. The expression of *GA2ox*, encoding an enzyme that catabolizes active GA, and of *GA20ox* and *GA3ox*, major GA biosynthesis genes, were investigated (Fig. 4). The expression levels of *GA2ox* and *GA3ox* were considerably higher than *GA20ox*. Particularly, markedly high expression of *GA2ox* was observed in the SM ovules and approximately doubled on average by GA₃ treatment at 2 and 6 DAT. Variations in the expression of *GA2ox* in the SM control samples were large, and the

Fig. 1. Effect of GA₃ treatment on seed development of ‘Shine Muscat’ (SM) and ‘Honey Venus’ (HV). GA₃ treatment was performed at flowering. A: Ratio of seeded berries per cluster. Student’s *t*-test was conducted on the arcsine transformed value. B: Number of seeds per berry. loc1 represents samples harvested at Otsu on 4th Sep. loc2 and HV samples were harvested at Higashi-Hiroshima on 3rd Jul. Error bars indicate standard error. The numbers in parentheses indicate the number of clusters (A) and berries (B) tested.

Table 1. The number of differentially expressed genes (DEGs) between GA₃-treated and control samples.

Sample	Ovary			Ovule			Unique counts of total DEGs
	2d	6d	Shared between 2d and 6d	2d	6d	Shared between 2d and 6d	
Shine Muscat (SM)	651	1087	68	664	2057	64	3739
Honey Venus (HV)	328	181	15	355	1218	96	1798
Shared between SM and HV	19	47	0	13	351	1	703

Fig. 2. Overview of expression patterns of the differentially expressed genes between the GA₃-treated and control groups of the SM samples. Gene expression levels were represented as a heatmap based on the average RPKM value of each group, and the color scheme was normalized for each gene. Blue indicates low expression and red indicates high expression. Full lists of the enriched GO terms are shown in Supplementary Tables S2–S5.

effect of GA₃ was statistically not significant. Notably, this response was not observed in the ovary tissue. In addition, expression of the GA biosynthesis genes, *GA20ox* and *GA3ox*, was significantly changed by GA₃ treatment in the SM ovary, but not in the SM ovules. Collectively, the GA catabolism gene was generally upregulated by GA₃ application in the SM ovule, but the GA biosynthesis genes were not affected. In the ovary tissue, the presumed GA feedback system was less active than in the ovule tissues.

To identify the potential key genes responsible for seedlessness, the DEGs detected in the SM ovule samples were ranked by fold change and the top genes are shown in Table 2. The highly upregulated genes included several ABA-related genes, such as *MYB15*, *ABR1*, *CBF4*, and *HVA22A*, as well as genes related to somatic embryogenesis, such as *EP3* and *PSK4* (Table 2). Conversely, *OPF16*, a member of the *OVATE* family, and *SPX2*, which is involved in phosphate signaling, were strongly downregulated by GA₃ treatment (Table 3).

Comparison of GA responses between HV and SM

To validate the results obtained above, we referred to the GA₃ response in the low-level receptiveness cultivar HV. The feedback reaction of GA metabolism, which was typical in the SM ovules, was also observed in the HV ovules and ovary samples, but the early strong reaction of *GA20ox* at 2 DAT in the SM ovules was completely absent in the HV ovules (Fig. 4). In the HV samples, unlike SM, expression induction of GA-related genes by GA₃ treatment was largely absent in the ovules and ovaries, except for *GA20ox* at 6 DAT (Fig. 4). The cell cycle-related DEGs detected in the SM ovules, which are thought to be important for abortion in response to GA₃ (Fig. 2), were not differentially expressed in the HV ovules (Fig. 5).

Discussion

Tissue-specific transcriptome reactions to GA in grape flowers

The effect of GA₃ application on seed development was clear and highly significant. GA₃ application was effective in inducing seedlessness in both SM and HV,

Fig. 3. Expression profiles of GA-related DEGs. DEGs with the GO terms “gibberellin biosynthetic process” (GO:0009686), “gibberellin catabolic process” (GO:0045487), “regulation of gibberellic acid mediated signaling pathway” (GO:0009937), and “gibberellic acid homeostasis” (GO:0010336) are shown. Gene expression levels were represented as a heatmap based on the average RPKM value of each group, and the color scheme was normalized for each gene. Blue indicates low expression and red indicates high expression. The GO terms annotated for each gene are listed in Supplementary Table S6.

although SM was more receptive and was almost entirely seedless when treated with GA₃ (Fig. 1). This result was consistent with previous reports (Yamada et al., 2008; Satoyoshi et al., 2015; Yoshimura et al., 2021). GA-induced seedlessness by the same method as in this study has been widely applied in conventional cultivation of table grapes in Japan (Yamada and Sato, 2016), and it is empirically known that despite some cases where seeds are accidentally developed in some conditions, even with GA₃ application in the GA₃-receptive cultivars, the effect of GA-induced seedlessness is highly stable across years, even in with a different environment and juvenility. We observed this robustness in the SM experiment conducted at two locations (Fig. 1). Therefore, although we collected RNA from berry samples from two locations (SM from Otsu, and HV from Higashi-Hiroshima), we could make a fair comparison on the GA₃ response between the two samples. It should be noted that since *Vitis* generally has two ovules per carpel and two carpels in a flower, there could be four seeds in a berry; whereas it was observed that even in the control a berry contained only approximately 1.4 seeds on average (Fig. 1). Houel et al. (2013) reported that the average seed number per berry over hundreds of cultivars was 1.9, with great variation by cultivar. The lower seed formation rate in natural conditions suggests that GA-induced seedlessness is a mixture of the natural abortion process and GA responsive process. The latter was focused on in this study and

was assumed to be measurable by comparing cultivars with the same level of seed formation ability, which was observed in the control samples (Fig. 1).

The result showed that the reaction to GA₃ treatment is distinct and most likely independent between ovaries and ovules. In the SM samples with high sensitivity to GA₃, dramatic expression changes in phytohormone-related genes were observed in the ovules at 2 DAT (Figs. 2 and 3). The strong response to GA₃ at this early stage is consistent with previous studies investigating the whole flower transcriptome of genetically seeded cultivars (Cheng et al., 2015). Conversely, this response was largely absent in the ovaries (Figs. 2 and 3). A similar pattern was also observed in the GA biosynthesis and catabolism genes (Fig. 4). In the SM ovule samples, *GA2ox*, which encodes an enzyme that catabolizes active GAs, was highly expressed and was upregulated at 2 DAT in response to GA₃ application. Collectively, the expression of the GA metabolism genes was regulated towards balancing the GA level in tissues. The response of the *GA2ox* gene expression to GA₃ was distinct in the SM ovule at 2 DAT, but it was not obvious in the SM ovaries. Taken together, these results showed that the responses to GA₃ differed between ovary and ovule tissues, with the ovules being more responsive.

The present study showed that the dynamic functional changes in response to GA₃ in early developing berries were mainly ovule (or seed trace)-specific reactions (Fig. 2; Supplementary Table S3), and this is a key

Fig. 4. Expression pattern of genes annotated as *GA3ox* (A, B), *GA20ox* (C, D), and *GA2ox* (E, F) in developing ovules and ovaries. The gene ID is based on the 12X *Vitis vinifera* reference genome with version 2.1 gene annotation (Jaillon et al., 2007), and the functional annotation of GA oxidases by Wang et al. (2020) was applied. A, C, E: $\log_2(1 + RPKM)$ scores of each GA oxidase gene. B, D, F: Sum of RPKM values of GA oxidase paralogs. Error bars represented standard error. Asterisks indicated the statistical significance ($P < 0.05$) based on Student's *t*-test for sum of homologous genes.

finding for understanding seed and berry development. The importance of exogenous and endogenous GAs in early berry growth has been repeatedly highlighted in genetically seedless grape production (Cheng et al., 2013; Ravest et al., 2017). Differential expression of various phytohormone-related genes in response to GA₃ application (Fig. 2; Supplementary Table S3) was also observed in studies on GA₃ treatment of stenospermocarpic cultivars based on a whole-berry analysis, and was previously considered a cause of berry enlargement induced by GA₃ application (Chai et al., 2014;

Upadhyay et al., 2018). The functional components responsive to GA₃ application reported by Chai et al. (2014), Upadhyay et al. (2018), and Cheng et al. (2015), based on whole-berry analysis fairly matched with those observed in the present study, suggesting that the responses observed in the previous studies, although at different stages, might track signals mainly from the ovule tissue. Collectively, for the above reasons, the hypothesis of the importance of the differential expression of phytohormone signaling genes in the GA-induced berry enlargement needs to be reformulat-

Table 2. Higher-ranked up-regulated genes by GA_3 treatment in the SM ovule samples. Top ten genes based on the log2 fold change between GA_3 -treated and control of the SM samples detected at two days after treatment are shown.

Contig	Gene expression (RPKM)						treated/control in SM at 2DAT		Best hit Arabidopsis gene model	Description		
	HV-control		HV-treated		SM-control		SM-treated	log2 fold change			P-value	
	2d	6d	2d	6d	2d	6d						2d
VIT_212s0134g00490.1	0.28	0.61	0.11	1.66	0.15	0.03	5.80	0.02	5.078	4.3E-05	AT3G23250.1	ATMYB15, ATY19, MYB15
VIT_207s0031g01980.1	0.94	0.81	0.18	1.35	0.86	0.12	12.88	0.23	3.970	2.8E-11	AT5G64750.1	ABR1
VIT_201s0011g02890.1	0.49	0.90	0.00	3.43	0.32	0.00	4.74	0.06	3.893	6.5E-04	AT1G49000.1	
VIT_201s0010g02450.1	0.41	5.66	0.15	2.88	0.65	0.03	8.61	0.16	3.840	8.8E-05	AT3G48140.1	B12D
VIT_203s0038g01650.1	0.13	0.16	0.33	0.41	0.97	2.56	13.77	2.21	3.796	4.5E-14	AT1G74520.1	ATHVA22A, HVA22A
VIT_218s0001g08760.1	40.17	23.66	24.98	46.13	6.26	0.67	82.01	2.24	3.791	7.3E-10	AT3G49780.1	ATPSK4
VIT_217s0000g07375.1	1.03	2.23	1.53	4.13	0.34	0.94	4.79	0.48	3.762	2.9E-13	AT3G48090.1	ATEDS1, EDS1
VIT_205s0094g00360.1	0.90	1.15	0.62	2.80	8.54	3.68	116.31	4.49	3.714	1.7E-11	AT3G54420.1	ATCHITIV, ATEP3, CHIV, EP3
VIT_206s0061g01390.1	0.04	0.04	0.00	0.39	0.36	0.00	5.23	0.04	3.692	8.9E-03	AT5G51990.1	CBF4, DREB1D
VIT_212s0057g01430.1	7.13	26.71	7.29	52.53	8.20	8.79	98.01	7.62	3.591	5.1E-18	AT3G07600.1	Heavy metal transport/detoxification superfamily protein

Table 3. Higher-ranked down-regulated genes by GA_3 treatment in the SM ovule samples. Top ten genes based on the log2 fold change between GA_3 -treated and control of the SM samples detected at two days after treatment are shown.

Contig	Gene expression (RPKM)						treated/control in SM at 2DAT		Best hit Arabidopsis gene model	Description		
	HV-control		HV-treated		SM-control		SM-treated	log2 fold change			P-value	
	2d	6d	2d	6d	2d	6d						2d
VIT_212s0057g00660.1	49.25	65.37	30.36	61.65	13.19	33.74	0.62	22.99	-4.366	7.7.E-05	AT4G16447.1	
VIT_206s0004g04210.1	7.13	8.08	4.42	5.89	45.08	2.38	2.84	3.69	-4.103	9.0.E-03		
VIT_219s0014g03390.1	0.84	0.31	0.90	0.61	4.76	0.05	0.29	0.04	-3.869	2.4.E-03	AT1G52855.1	
VIT_208s0007g04500.1	16.74	8.91	3.58	13.21	53.36	9.08	4.04	21.81	-3.812	1.9.E-04		
VIT_205s0020g03520.1	43.92	101.75	23.91	47.68	13.51	54.35	1.03	33.10	-3.800	1.8.E-03	AT2G32100.1	ATOPF16, OFP16
VIT_218s0072g00150.1	70.02	101.17	24.27	76.61	67.27	28.03	4.99	19.87	-3.768	1.2.E-09	AT4G09890.1	Mediator of RNA polymerase II transcription subunit, putative (DUF3511)
VIT_211s0016g05330.2	11.63	9.35	5.19	18.55	128.46	34.65	11.97	91.86	-3.461	1.5.E-07	AT2G26660.1	ATSPX2, SPX2
VIT_206s0004g02860.1	7.33	6.81	2.65	5.93	11.38	3.50	1.18	2.61	-3.438	6.2.E-02	AT2G28410.1	
VIT_214s0060g00490.1	570.51	298.00	961.19	306.99	334.68	69.99	39.33	51.40	-3.227	1.0.E-03		
VIT_205s0020g00420.1	31.83	192.16	32.66	145.11	63.12	77.69	7.52	91.71	-3.190	3.5.E-03	AT1G48100.1	Pectin lyase-like superfamily protein

Fig. 5. Expression profile of the cell cycle-related DEGs detected in the comparisons of ovule samples for SM and HV. DEGs with the GO terms “cell population proliferation” (GO:0008283), “regulation of cyclin-dependent protein serine/threonine kinase activity” (GO:0000079), and “DNA replication initiation” (GO:0006270) are shown. Gene expression levels were represented as a heatmap based on the average RPKM value of each group, and the color scheme was normalized for each gene. Blue indicates low expression and red indicates high expression. The GO terms annotated for each gene were listed in Supplementary Table S7.

ed. Correa et al. (2015) reported a correlation between seed number and berry size in an F_1 population, whereas this correlation was not significant under cultivation with GA_3 application. Correa et al. (2015) interpreted that the correlation between seed number and berry size is caused by GA from mature seeds, and that exogenous GA_3 application masked the GA variation in berry among the F_1 individuals. This is partially supported by our results, demonstrating markedly lower GA metabolism in developing ovaries than ovules in early developing berries (Fig. 4). Unfortunately, we could not test the detailed effects of GA_3 application on berry development in the present study; however, a similar sampling scheme as in the present study should result in a deeper understanding of berry size modulation by GA_3 application, as well as seed development.

Various phytohormones and cell cycle control as possible key systems in GA-induced seedlessness

According to the results, a key process of the GA_3 response in ovules was suggested to be the signaling of various phytohormones. In ovule tissue, the GA-related response occurred at 2 DAT, followed by a strong change in the expression of cell cycle-related genes at 6 DAT, suggesting that a key molecular response driving

seed abortion functioned within 6 DAT (Figs. 3 and 5). Interestingly, the responses of various phytohormone-related genes in the ovule tissue were observed at 2 DAT, including ethylene, ABA, cytokinin, and salicylic acid, as well as GA (Fig. 2; Supplementary Table S3). In particular, the salicylic acid-dependent defense mechanism was suggested to be involved in genetic seedlessness and may be the key process for seedlessness (Royo et al., 2018). As mentioned above, the response of phytohormones was reported for GA_3 -treated stenospermocarpic cultivars based on a whole-berry analysis (Chai et al., 2014; Upadhyay et al., 2018). Additionally, seed dry weight of stenospermocarpic individuals was reduced by GA_3 application (Correa et al., 2015), suggesting that the same system observed in this study may function in diverse grapes, including genetically seedless plants. Collectively, the presented results and all the literature support the key role of the phytohormone signaling changes in GA-induced seedlessness.

Extensive expression changes in genes related to embryo development and seed dormancy or germination were also detected in GA_3 -treated ovule samples (Fig. 2; Tables 2 and 3). *OFPI6*, a member of OVATE family proteins (OFPs), was markedly downregulated

by GA₃ treatment (Table 3). OFP is a regulator of various aspects of plant growth and development, and some OFP members are known to have a role in embryo development in Arabidopsis (Pagnussat et al., 2007; Wang et al., 2011, 2019). Furthermore, *MYB15*, *CBF*, and *ABR1*, all of which are associated with ABA signaling, were strongly induced by GA₃ treatment (Table 2). *MYB15* and *CBF* function in cold acclimation, and *MYB15* in Arabidopsis act as a transcriptional repressor of *CBF*, depending on the phosphorylation status of *MYB15* (Agarwal et al., 2006; Kim et al., 2017). It is known that the *CBF* signaling pathway interacts with ABA signaling (Knight et al., 2004; Roychoudhury et al., 2013). Also, *ABR1* in Arabidopsis is known to act as a transcriptional repressor of ABA response and has a role in seed germination (Pandey et al., 2005; Li et al., 2016). In addition, *EP3* and *PSK* are known to affect somatic embryogenesis processes (De Jong et al., 1992; Igasaki et al., 2003). Taken together, these results suggest GA₃ treatment may initially cause dynamic reprogramming of the developmental process in ovule tissue, followed by the alteration of cell division and subsequent seed abortion.

Potential mechanism of low sensitivity to GA-induced seedlessness in 'Honey Venus'

Many potential processes affect seedlessness by GA, from GA recognition to abortion, but the key process responsible for the variation in the GA response in seeds has never been identified. The process needs to be systemic enough to suppress the entire GA response reaction upon GA application, as observed in GA signal transduction (Fig. 3) and GA biosynthesis/catabolism (Fig. 4). The HV ovules largely lacked the dynamic GA₃-responsive reactions observed in the SM ovules (Fig. 3), suggesting that this genetic variation may be conferred by a process before GA signal transduction in the ovules, rather than in downstream processes. Further work on the GA₃ response with a wider range of variation may be beneficial for the breeding of cultivars suitable for seedlessness induction.

Conclusion

This study showed that GA₃ application induces not only GA signaling, but also various phytohormone signaling genes in developing grape flowers. This response was predominantly observed in ovules, but not in ovaries; therefore, it may be inferred that the key system for seedlessness is ovule-specific. Cell cycle-related genes in the ovules were markedly affected in the high gibberellin-sensitivity cultivar SM and may be an important process for ovule abortion. A marked response was observed in the processes of phytohormones and cell cycle genes, and these may be the initial targets for future genetic studies. Although this study showed valid results for only one GA₃-responsive genotype, based on the literature, the results may be general-

izable, and it is potentially meaningful to validate the results with a wider range of genotypes in the future. The results presented in this study provided a basic knowledge of the mechanism behind seedlessness induction, which is crucial for improving seedless table grape production.

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