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Metabolites changes after pre-bloom gibberellic acid (GA_3) application for inducing seedless grape

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Abstract

Gibberellic acid (GA_3) treatment is a useful method for inducing seedless grape berries in the seeded grape bunch before flowering. In this work, we applied 100 ppm of GA_3 on 'Tamnara' grape flower cluster at 14 days before flowering to find metabolites significantly related to seedlessness. Three bunches of grape flower samples were collected at nine different stages (Day before full bloom; DBF13, 10, 7, 5, 2, flowering (0) and day after full bloom; DAF 2, 5, 9). Metabolites of each collected sample were analyzed using GC-MS with derivatization method (MSTFA). Metabolite contents of GA_3 treatment flower were compared with non-treated controls in all stages and analyzed using Partial least squares discriminate analysis (PLS-DA). As a result, five sampling times (DBF 13, 10, 2, 0, DAF 9) showed significance differences using GA_3 treatments. Total of 13 metabolites were recognized to relate to differences in five specific sampling times and mainly affected the initial stages (DBF 13 and 10). Tartaric acid, D-glucose, phosphoric acid, and D-mannose, inositol were increased by GA_3 treatment at the early-flower developing stage. Dehydroascorbic acid, caffeic acid, citric acid, and gluconic acid were mainly increased at the time of GA_3 treatment but decreased approaching full bloom. All stages of GA_3 treatment, L-glutamine, L-serine, and D-allose was decreased, but fructose increased. In particular, the metabolite contents before GA_3 treatment provides new clues on the role of GA_3 in the early stage of grape berry development.

Keywords: Gibberellic acid (GA), Grape, Metabolite, Seedless.
Abbreviations: FAME_Fatty Acid Methyl Ester; GA_3_Gibberellic acid 3; MSTFA_N-methyl-N-(trimethylsilyl)-trifluoroacetamide; OPLS-DA_orthogonal projections to latent structures discriminant analysis; PCA_principal component analysis.

Introduction

The GA_3 application is a useful agricultural practice in the viticulture worldwide. In grape production, GA_3 is a plant growth regulator that plays a variety of roles such as berry enlargement (Coombe, 1960), reduce shattering, loose compact bunch, and flower thinning (Weaver, 1976). However, GA_3 application induces seedlessness in seeded grape cultivars (Weaver, 1976; Fellman et al., 1991; Lu et al., 1997). In Korean vineyard, GA_3 treatment has been used for producing seedless table grape, but GA_3 treatment time and concentration differ among cultivars. For example, diploid cultivar 'Delaware' needed 100 ppm of GA_3 two times, one 14 days before flowering and the other at full bloom (Motomura and Hori, 1977), while the tetraploid cultivar 'Kyoho' required 25 ppm of GA_3, two times at full bloom and ten days after flowering (Jeong et al., 1998).

Gibberellin is a tetracyclic di-terpenoid which have an essential role in the plant growth regulation (Chhun et al., 2007). In the 1930s, T. Yabuta and T. Hayashi isolated gibberellins from the fungus Gibberella fujikuroi. All gibberellins (Gas) are acidic (Salisbury and Ross, 1992), and GA_3 concentrations in plant tissues are often at levels of $ng.g^{-1}$ fresh weight (Binks et al., 1969). As a plant hormone, endogenous or exogenous GA treatment act to promote growth plant cell (Hedden and Sponsel, 2015), germinate dormant seeds (Bewley, 1997), regulate flowering (Pharis and King, 1985), and develop parthenocarpic fruit (Gustafson, 1936, Salisbury and Ross, 1992). There are many reports on the role of GA_3 to remove seeds. GA causes morphological deformation of flower organs and prevents seed formation. In previous reports, GA induces numerous abnormal flower organ, such as disrupted stamen development (Plackett et al., 2011), block signal to anther and pollen development in rice (Murray et al., 2003), and defect pollen tube elongation in rice (Chhun et al., 2007). GA is a signaling molecule and maintains equilibrium homeostasis in the plant. Recently, GAF1 and DELLA proteins regulate GA homeostasis, which provides the molecular basis for GA regulation and the accumulation in the plant (Fukazawa et al., 2014). In transcriptome research, GA_3 application at pre-bloom alters transcription levels of grape GA metabolic genes at nearly full bloom (Jung et al., 2014).

Metabolic profiling is a useful technique to provide valuable information within the whole metabolite scale (Sumner et al., 2003). GC-MS metabolite profiling can use both well-organized stable protocol from sampling to data analysis and broad coverage of metabolite library (Lisec et al., 2006; Warren, 2013). The multivariate data analysis, principal component analysis (PCA) and orthogonal projections to latent structures discriminate analysis (OPLS-DA) mainly use comparative

analysis methods. PCA is an unsupervised method that reduces the dimension of the variances. OPLS-DA is a supervised method that provides improved models for transparency and interpretability in the comparison between two different treatments (Ericksson et al., 2006). Combination of GC-MS and multivariate data analysis is suitable to find metabolite changes in the whole scale metabolomic studies with statistical significance. The role of GA_3 treatment in seedless grape production is not well-known yet. Also, depending on the grape cultivar, the treatment concentration and timing of GA_3 are different and have not yet clarified. In this study, we tried to find specific metabolites in the seedless processing grape upon different GA_3 treatments during pre- and post-flowering stages in the grape cv. 'Tamnara'. We also tried to identify related metabolites in pre-bloom gibberellic acid (GA_3) application on a grape flower and immature bunches using GC-MS metabolic profiling with derivetization method. We were then attempted to find the correlation between the variability of metabolites and seed development using multivariate statistical analysis. This new methodology provides new insight into the role of GA_3 in grape seed development.

Results

Metabolite differences using PCA method

GA_3 concentration and treatment time are essential factors for producing seedless grape cultivars. The concentration, treatment stage, and times are specific to cultivars and reported various GA_3 treatment conditions (Schwabe and Mills, 1981). In this experiment, seedless berries were successfully induced by GA_3 treatment (100 ppm) at the 14 days before flowering (DBF) in the grape of 'Tamnara'. From GC-MS metabolite profiling, a total of 41 metabolites were detected and annotated using retention indexing in the metabolic Fiehn library (Table 1). The PCA of whole stages showed no grouping patterns in the PCA score and loading plot (data not shown). After bloom stages, metabolites data did not sufficiently explain influence of total variation after GA treatment because there are too many insignificant sampling data. In the previous report, GA_3 treatment is related to embryo abortion in the early stage of grapevine flowering. It reveals that metabolite variation between GA_3 treatment and none-treated control is different from early sampling stages. As a result, metabolite variation between GA_3 treatment and control influenced at the pre-bloom stages as shown in the PCA (Fig. 1A). In the PCA plot, two of the main variation collected using data of pre-bloom stages, principal components (PC), accounted for 27.2% of total variations, PC1 for 17.4% and PC2 for 10.6%. GA treated and none treated control data were located right at bottom side together in the PCA score plot (Fig. 1. A). GA_3 treatment leads to more little metabolite changes than not-treated control. Control sampling data spread from right bottom to left upper side of the score plot that is a more fluctuated metabolite variation than GA_3 treatment. There are two distinct groups between GA_3 treatment and control, in the PCA loading (Fig 1. B) plot. The important metabolites affected such a grouping, displayed the same position in score plot. Metabolites in the upper right side are related to not GA_3 treated control, and on the bottom left side are related to GA_3 treated samples.

Metabolite differences using OPLS-DA method

The supervised analysis method, OPLS-DA, is similar to PLS-DA, but a single component used as a predictor for the model, and the other components describe the variation orthogonal to the first predictive component (Westerhuis et al., 2010). In OPLS analysis, values of R_2 and $Q_2 > 50\%$ are considered satisfactory for metabolic experiments (Azizan, 2012). However, cross-validated analysis of variance (CV-ANOVA) uses a statistical diagnosis method in the PLS and OPLS models (Eriksson et al., 2008). We used the CV-ANOVA of OPLS-DA to identify metabolites with significant differences between GA_3 treatments and, none treated control at each flowering stage (Table 2). As a result, only four of the six comparing models were statistically significant, with CV-ANOVA ($p < 0.05$). It means that four different stages (DBF 13, 10, 2 bloom stages) had important metabolites for affecting the apparent difference between GA_3 treatment and none treated control. GA_3 treatment time in the diploid cultivar of grapevine for making seedless is 14 days before bloom (Kimura et al., 1996 and Okamoto and Miura, 2015). However, other stages have no significance of metabolite contents difference between GA_3 treatment and none-treated control. It means that GA_3 induces metabolite changes quickly, but it does not involve all development stages.

Metabolites contents of the pre-bloom stage in grapevine

In DBF 13, most of the metabolites were related to GA_3 treatment with high correlation values (Table 3, Fig. 1, B). Four carboxylic acids (Caffeic acid, gluconic acid, citric acid, and phosphoric acid) and three carbohydrates (D-glucose, D-mannose, and allo-inositol) were related to GA_3 treatment. However, D-allose was related to none-GA_3 treatment control. In the DBF 10, three carboxylic acids (Tartaric acid, caffeic acid, and citric acid) and one amino acid (L-glutamine) related to GA_3 treatment and only one carbohydrate, D-allose, was related to none- GA_3 treatment. Those results showed that GA_3 treatment induces a very immediate metabolic response to immature flowers of grapes. In contrast, at the stage of bloom (DBF 2), three carboxylic acids (caffeic acid, dehydroascorbic acid, and gluconic acid), two amino acids (L-serine and L-glutamine) and one carbohydrate (D- allose) related to none-GA_3 treatment control, but only one carbohydrate of fructose was related to GA_3 treatment. Likewise, at the bloom (DBF 0), three carboxylic acids (caffeic acid, dehydroascorbic acid, and gluconic acid), and one amino acid (L-glutamine) were related to none of the GA_3 treatment. However, only one carbohydrate of fructose was related to GA_3 treatment. After flowering stage (DAF 9), gluconic acid contents related to positive but fructose content related negative compared with none treated control. The correlation value of each metabolite (Table 3) is a difference in the content of the metabolites between the two treatments. The content of metabolites were changes inversely on DBF 7 and DBF 5. The reason might be the lack of statistical significance on the DBF 7 and DBF 5 between GA_3 treatment and none treated control. As a result of this experiment, the metabolites were classified into four different types (Fig. 2). Type 1 metabolites content was increase in the initial stage (DBF 13, 10) but a decrease in the flowering and after the flowering stage by GA_3 treatment. Type 1 metabolites are allo-inositol, caffeic acid, dehydroascorbic acid, phosphoric acid, and D-mannose. Type metabolites, gluconic acid, and D-

glucose seems like type 1 metabolite but increase after the flowering stage (DAF 9) by GA_3 treatment. Type 3 metabolites, citric acid, fructose, and tartaric acid, contents increase in the pre-flowering stage (DBF 13, 10, 2, 0) by GA_3 treatment. Type 4, which are D-allose, L-glutamine, and L-serin contents, reduce in the GA_3 treatment in all stages. Type 1, 2, three metabolites seem to involve in response to GA_3 treatment in the early stage. These metabolites are responses to GA_3 treatments, but each group changed the content in the different stage immediately. Type 4 metabolites are which are a negative response to GA_3 treatment in flower developing stage in grape tissue.

Discussion

GA_3 sensitive stage in the grape flower development

The GA_3 treatment time and concentration for producing seedless grape depend on the grape cultivar (Fellman et al., 1991; Iwahori et al., 1968; Lu et al., 1997; Weaver, 1976). Also, GA_3 treatment time differs depending on diploid and tetraploid cultivars in table grape production (Jeong et al., 1998). It indicates that the inducing seedless reaction and the GA_3-induced response to GA_3 differ from cultivars in the grapevine. This study showed that there was a significant difference in specific early flower development stages but not in all stages. In this case, we can find that gene variation or metabolites variation may affect to induce the seedlessness of grapes at the specific stages. GA treatment showed that metabolites differences in the producing seedless grapes are in the specific stages. Our result, DBF 13, 10, 2, and full bloom stage, show that metabolite significantly different from GA_3 treatment and control grape flower tissue. GA_3 treatment causes morphological differences in grape at the early stage of fertilization and seed development. It previously reported that pollen germination and pollen tube growth are severely inhibited by GA_3 (Kimura et al., 1996; Okamoto and Miura, 2015). In another case, GA_3-induced seedless is a result of abnormal seed development by an impairment of redox homeostasis in flowers resulting in oxidative damage to the seeds (Cheng et al., 2013).

In this experiment, we applied GA_3 at the DBF 14 in the diploid cultivar 'Tamnara' and DAF 13, and 10, which has a significant metabolite difference, is the early flower development stage. In the previous report, microspore development is different from cultivars between 'Tamnara' and 'Himrod Seedless' during the pre-flowering developmental stage (Yim et al., 2015). In this report, DBF 14 stage in the 'Tamnara' grape is a unicellular microspore stage, which is a pre-meiosis stage. GA_3 treatment does not inhibit pollen mature but inhibits pollen tube growth in the pistil (Okamoto and Miura, 2015). Because it is a tetraploid cultivar, the farmers apply GA_3 at the flowering stage. Because of time, when the morphological difference appears, it coincides with the GA_3 processing time. We need more explanation about upstream gene expression connected to poor fertilization and seed development to understand the different patterns of metabolite at this time.

Many reports demonstrated early-stage gene regulation related to GA_3 treatment. HvGAMYB is a barley transcription factor which has negative control in anther development by GA_3 treatment early flower development stage (Murray et al., 2003). When external GA_3 threat to grape flower, seed development genes have altered expression, decrease in antioxidant enzymes, and increase in cell damage with ROS (Cheng et al., 2013) will occur. Exogenous GA_3 treatment at the pre-bloom stage leads to altering GA_3 oxidase genes (Jung et al., 2014). DELLA protein (transcription factor) controlled GA_3 mediated plant growth, which targeted the protein for proteasome-mediated proteolysis (Hedden and Sponsel, 2015). The effects of GA application in the present study, pollen tube growth inhibition, and alteration in expressions of GA oxidase genes and GA level are in congruent with de Jong et al. (2011). The GA application not only changes the GA levels but other hormones, including auxins (Jung et al., 2014). GA_3 treatment leads to increase in other phytohormones (auxin and cytokinin) in the grape 'Delaware' (Shiozaki et al., 1997). These results suggested that the upstream factors, including metabolites, change morphological characteristics at specific stages. In fact, in the results of previous reports of GA_3 treatment in grapevine, the expression levels of each GA biosynthetic gene differed by GA_3 treatment (Jung et al., 2014). These recent results imply that the induction of seedlessness with GA_3 treatment has a complicated role with the metabolic homeostatic equilibrium.

Metabolite response to GA_3 treatment before blooming

In the previous studies, numerous genes expression rapidly occurs by external GA_3 treatment in grape flowers (Richards et al., 2001; Cheng et al., 2013; Hedden and Sponsel, 2015). Alteration of gene expression of metabolite contents can trigger morphological changes. Our results of DAFs 7 and 5 shows that there is no apparent difference in GA treatment. It means that GA only affects the initial stage (DAF 13 and 10) but disappears at later stages. Significant differences in metabolites after GA_3 treatment have a pattern, mainly increasing at the time of GA treatment but decreasing when approaching full bloom. Many metabolites related to seedless grape induced by GA_3 treatment are reacted rapidly by GA_3. In the initial stage of DAF 13, several sugars play a specific role in response to GA_3 treatment.

Fructose, D-glucose, D-mannose, and allo-inositol include primary metabolic pathway, which relates to a phenolic compound and polyamine pathway. Acid invertase, hydrolyzes sugars (glucose and fructose) from phloem to the grape berries after seed development. GA_3 stimulated sugar concentration and invertase activity as early as 24 and 32h after applications in the grape cv. 'Sultana' (Pérez and Gómez, 2000). GA_3 treatment does not affect photosynthesis, but causes higher levels of sucrose and glucose contents in the GA_3 treatment grape flower (Domingos et al., 2015). Our result agrees with previous observations. The content of inositol and fructose relates to the sucrose because inositol and fructose are downstream metabolites in the glycolysis pathway from the sucrose. Also, alditols (sorbitol and inositol) protect cells against stress caused by metabolic imbalances in grapevine leaves (Pillet et al., 2012). Thus, the high inositol levels in flower may be related to maintaining homeostasis from rapid metabolites fluctuations by GA_3 treatment.

Another sugar family, D-mannose, is a C-2 epimer of D-glucose, which is connected to the glycolysis pathway. GA_3 treatment increases carbon sources in the grape flower-like as glucose, which not only uses an energy source for flower development but plays a durable sink itself. Unlike other metabolites, we found relatively low content of D-allose in GA_3 treatments in the flower tissue till full bloom. D-allose is a rare sugar which

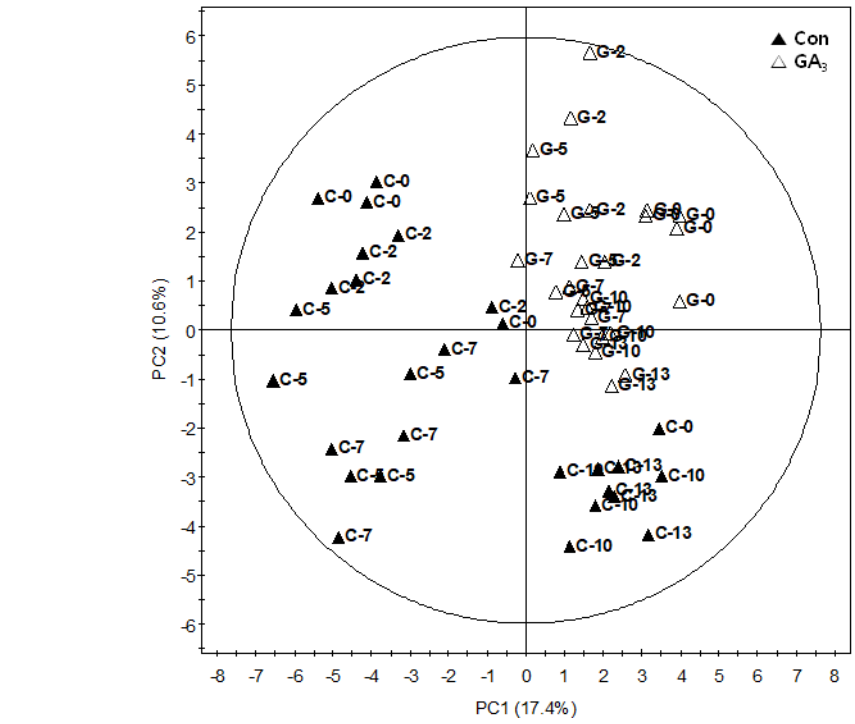
Table 1. Metabolites retention time, model ion, and mass fragmentation of GC/MS in GA_3 treated and none treated control of 'Tamnara' grape flower.

No.	CAS No.	Metabolites	RT	Model ion	Mass fragmentation
1	79-33-4	L-(+) lactic acid	6.9	147 m/z	73, 117, 140, 190, 207, 295
2	56-41-7	L-alanine	7.9	116 m/z	73, 147
3	72-18-4	L-valine	10.9	144 m/z	75, 218
4	7664-38-2	Phosphoric acid	12.5	299 m/z	73, 314
5	147-85-3	L-proline	13.0	142 m/z	73, 147, 245, 299
6	110-16-7	Maleic acid	13.2	147 m/z	73, 245
7	29915-38-6	Succinic acid	13.4	147 m/z	73, 147, 247
8	473-81-4	Glyceric acid	14.0	73 m/z	147, 189, 292
9	17013-01-3	Fumaric acid	14.2	245 m/z	75, 147
10	56-45-1	L-serine	14.8	204 m/z	73, 218
11	72-19-5	L-threonine	15.5	218 m/z	73, 101, 117, 147, 291
12	6263-10-08	Citramalic acid	17.6	247 m/z	73, 147
13	617-48-1	D-malic acid	17.8	73 m/z	147, 233
14	463-00-3	4-guanidinobutyric acid	18.9	174 m/z	73, 84, 147, 230, 304
15	526-99-8	Mucic acid	20.9	292 m/z	73, 117, 147, 219, 246, 305
16	56-86-0	L-glutamic acid	21.1	246 m/z	73
17	133-37-9	Tartaric acid	21.9	279 m/z	73, 147, 189, 219, 292
18	87-99-0	Xylitol	23.1	307 m/z	73, 103, 147, 217, 319
19	10094-62-9	Glucoheptonic acid	24.3	333 m/z	73, 103, 147, 217, 292, 307
20	56-85-9	L-glutamine	24.5	156 m/z	73, 147, 217
21	138-59-0	Shikimic acid	25.4	204 m/z	73, 147
22	5949-29-1	Citric acid	25.6	273 m/z	73, 147, 347, 363, 375
23	490-83-5	Dehydroascorbic acid	26.1	73 m/z	147, 157, 173, 245, 316
24	57-48-7	Fructose	27.0	74 m/z	103, 147, 217, 277, 307
25	87-81-0	Tagatose	27.2	148 m/z	73, 103, 217, 277, 307
26	59-23-4	D-glucose	27.5	73 m/z	147, 160, 205, 217, 319
27	579-36-2	D-allose	27.8	319 m/z	73, 103, 147, 160, 205, 217
28	526-95-4	Gluconic acid	29.7	73 m/z	147, 217, 277, 292, 305, 319, 333
29	87-73-0	D-saccharic acid	30.2	333 m/z	73, 147, 217, 292, 305
30	87-89-8	allo-inositol	31.0	305 m/z	73, 147, 191, 204, 217, 265, 318
31	331-39-5	Caffeic acid	31.3	396 m/z	73, 219
32	56-73-5	D-glucose-6-phosphate	35.1	387 m/z	73, 174, 217, 299, 315, 357
33	90-80-2	Gluconolactone	36.7	217 m/z	73, 105, 147, 204
34	13241-33-3	Neohesperidin	37.8	361 m/z	73, 147, 204, 217
35	57-50-1	Sucrose	40.1	361 m/z	73, 217
36	528-50-7	Cellobiose	41.2	361 m/z	73, 147, 204, 217
37	1109-28-0	Maltotriose	41.5	204 m/z	73, 147, 217, 361
38	35323-91-2	Epicatechin	43.3	368 m/z	73, 217, 310, 355
39	3687-64-7	Galactinol	45.3	204 m/z	73, 147, 191, 204, 361
40	520-18-3	kaemferol	48.1	487 m/z	73, 82, 217, 361, 487
41	96-82-2	lactobionic acid	48.3	204 m/z	73, 147, 204, 217, 361, 444

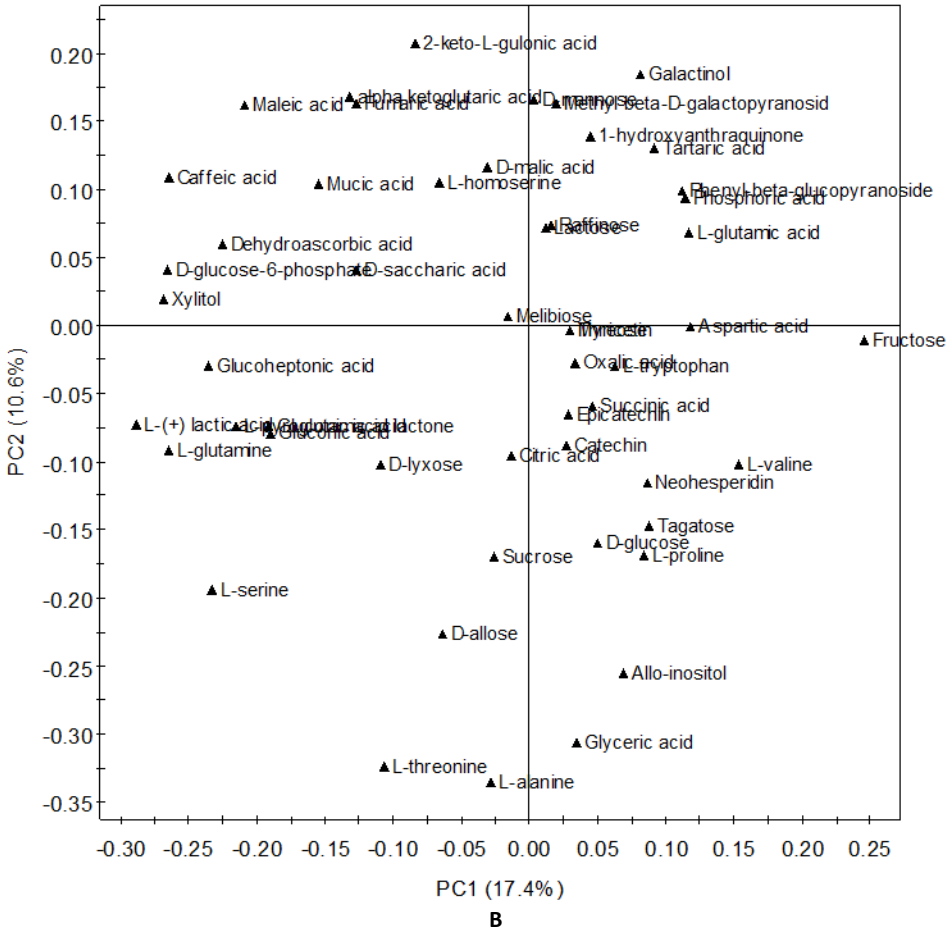
Table 2. Cross-validation of OPLS-DA models in metabolites on grape 'Tamnara' flower after GA_3 treatment at the different flowering development stages.

Flower stage[a]	Fitting values		CV-ANOVA	
	Component[b]	R_2X	$Q_{2(cum)}$	*p*
DBF 13	(1+1)	0.663	0.943	**0.005**
DBF 10	(1+2)	0.651	0.908	**0.033**
DBF 7	(1+1)	0.471	0.461	0.459
DBF 5	(1+1)	0.453	0.735	0.102
DBF 2	(1+1)	0.678	0.875	**0.042**
Flowering (DBF 0)	(1+2)	0.731	0.919	**0.026**
DAF 2	(1+1)	0.717	0.626	0.219
DAF 5	(1+1)	0.659	0.121	0.990
DAF 9	(1+1)	0.704	0.946	**0.002**

[a] DBF(Days Before Flowering), DAF (Days After Flowering); Young flower treated GA_3 at the 14 days before flowering and collected each flower stage.
[b] Component number (predictive + orthogonal) of OPLS-DA.



A



B

Fig 1. Principal component analysis (PCA) plots of metabolite variation at the different flower development stages. A. PCA score plot between GA treatment (G, black triangle) and none treatment control (C; blank triangle). The number is tissue-collecting day (the days before flowering; DBF). B. PCA loading plot showed related metabolites to GA treatment (upper right), and untreated control (lower left).

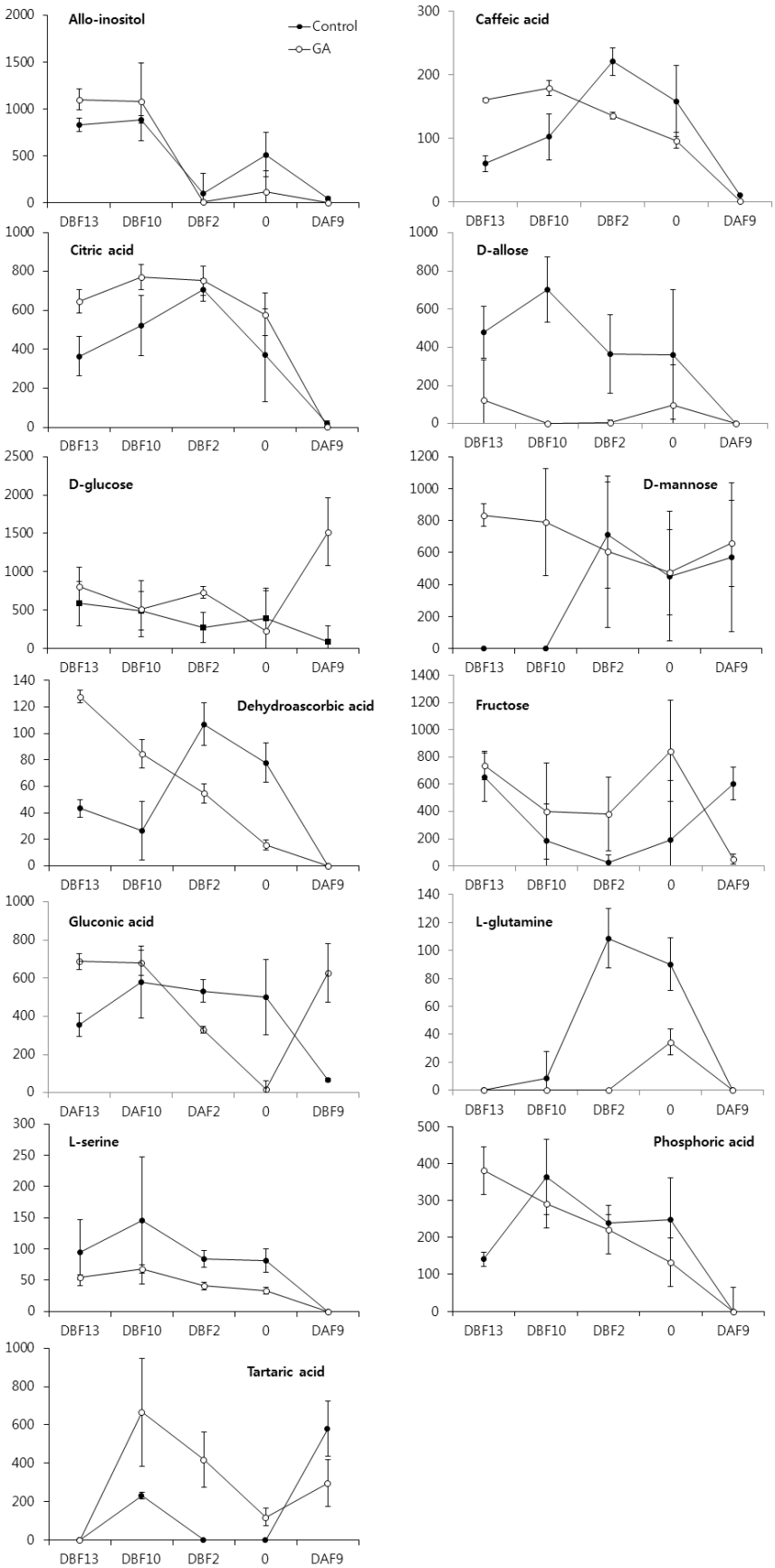


Fig 2. Putative metabolites are significantly related to the GA3 treatment in the grape 'Tamnara' immature flower and berries. (control; dot, GA3 treatment; circle). In the y-axis, minus numbers are days before flowering, and plus numbers are days after flowering.

Table 3. Correlation values in the OPLS-DA between GA3 treatment and none treated control immature flower and berries in grape 'Tamnara'.

Class	CAS No[a]	Metabolites	Sampling time				
			DBF 13[b]	DBF 10	DBF 2	0	DAF 9
Amino acid	56-45-1	L-serine			-0.9257		
	56-85-9	L-glutamine		0.8583	-0.9705	-0.9013	
Carbohydrate	57-48-7	Fructose			0.7738	0.6766	-0.9659
	579-36-2	D-allose	-0.8312	-0.9492	-0.794		
	59-23-4	D-glucose	0.5139				
	87-78-5	D-mannose	0.9983				
	87-89-8	Allo-inositol	0.8886				
Carboxylic acid	331-39-5	Caffeic acid	0.9661[c]	0.8509	-0.9556	-0.6596	
	5949-29-1	Citric acid	0.8655	0.7889			
	490-83-5	Dehydroascorbic acid			-0.9093	-0.7382	
	526-95-4	Gluconic acid	0.9556		-0.9303	-0.8955	0.9374
	7664-38-2	Phosphoric acid	0.9328				
	133-37-9	Tartaric acid		0.6619			

a. CAS registry number. A unique numerical identifier assigned by Chemical Abstracts Service (CAS).
b. DBF is days before flowering; DAF is days after flowering. Only five stages have significance to compare with GA3 treatment and control.
c. Plus values related to control and minus value related to GA3 treatment. Each value cut off by VIP score (>1) in OPLS DA.

suppresses GA_3 signaling in the rice (Fukumoto et al., 2011). D-allose inhibits GA_3 signal transduction through hexokinase-dependent pathway. Our results also support it. In the initial stage of DAF 13, several carboxylic acids play a unique role in response to GA_3 treatment. Dehydroascorbic acid, caffeic acid, citric acid, gluconic acid, and phosphoric acid have the same variation at the different stages. The plant cell wall is elongated after GA_3 treatment. The will lead to cell wall degradation when the expression of genes involved in antioxidation is decreased. Finally, cell wall degradation products such as H_2O_2 cause cell wall degradation (Richards et al., 2001). GA_3 induce seed abortion, which may result from the accumulation of ROS (Cheng et al., 2013). Our result show that, dehydroascorbic acid content increases immediately after GA_3 treatment. This is not related to cell wall degradation. Because of DAF 13, 10 is a unicellular stage in the microspore development in grape (Yim et al., 2015). The dehydroascorbic acid content in the early stage after GA_3 treatment is just a response to excessive exogenous GA_3, acting as stress or a signal molecule following the later stage. Citric acid has protective roles in tall fescue as an antioxidant in response to heat stress. Exogenously applied citric acid might be responsible for maintaining membrane stability, root activity, and antioxidant response activation with HSP genes (Hu et al., 2016). In this study, the dehydroascorbic acid content appeared like citric acid. Caffeic acid reported an increase in the basal parts of elm shoots and influenced endogenous IAA content with the appropriate hormonal balance for root induction (Volpert et al., 1995). Caffeic-acid enhanced polymerization solidified plant cell walls and restricted the growth in the soybean roots like an allelopathy chemical (Bubna et al., 2011). These results suggest that caffeic acid is involved in plant development pathways related to hormone balance. Our result is insufficient to explain the caffeic acid role. However, dehydroascorbic acid, citric acid, and caffeic acid are similar that may be related to responses at the early stage in GA_3 treatment like an antioxidant.

Antioxidant enzyme genes, SOD1, SOD2, SOD3, CAT1, CAT2, and POD, tended to be down-regulated in flowers after treatment with GA_3 (Cheng et al., 2013). The effect of gluconic acid is not fully understood in the flower development. However, in our result, gluconic-acid related both glucose hydrolysis (Ramachandran et al., 2006) and tartaric acid synthesis (Saito and Kasai, 1984). Plant growth promotion bacteria make a specific organic acid, in which one of the primary organic acids is gluconic acid (Vyas and Gulati, 2009). The gluconic acid content in the GA_3 treatment flower at DBF 13 is related to ascorbic acid degradation against stress from GA_3 treatment. Grapes are one of the rare fruits that contain tartaric acid. Tartaric acid is a notable organic acid in grape, which contents increase 50 days after flowering (Saito and Kasai, 1968). The tartaric acid content in the grape flower is not fully reported and understood. In the pollen development, DBF 10 is a unicellular stage; DBF 2 is a bicellular stage (Yim et al., 2015). Tartaric acid and phosphoric acid content in the GA_3 treatment flower expect the reasonable relationship between carboxylic acid and flower tissue development at a specific stage in the pre-bloom.

The contents of L-glutamine and L-serine were decreased in the DBF 13 stage and every other stages. Glutamine is a major amino acid in the N assimilation, and glutamine synthetase and glutamate synthetase pathway is the primary nitrogen cycle. N remobilization and resorption retarded by GA_3 treatment in Paris polyphylla. GA_3 treatment decreases in the C/N ratio, total free amino acid content, and proportions of glutamine and asparagine (Yu et al., 2012). GA_3 treatment related to nitrogen mobilization as a sink, but their role affected by numerous unknown factors. Our result shows that glutamine content in the young flower decrease from DBF 13 to full bloom with GA_3 treatment. It shows the same trend as the previous research reports (Domingos et al., 2015). Another amino acid, L-serine content is a response to exogenous GA_3 treatment. Serine carboxypeptidase (PsCP) transcription in developing fruits and seeds are induced by gibberellins. PsCP is also expressed in developing seedlings but not in cotyledons, suggesting that it not be involved in the mobilization of storage materials (Cercós et al., 2003). L-serine participates in the biosynthesis of sphingolipids for plant cell proliferation (Ros et al., 2014). D-serine has a signaling role that communicates male gametophyte and pistil, like as neuromodulator in the animal (Michard et al., 2011). In our result, GA-treated flowers have low serine content that will be good evidence to support the inhibition of cell wall weakness at the early stage in GA_3 treatment.

Parthenocarpic seedless grapes expected to have lower carbohydrates and endogenous GA_3 levels. Resulting in a higher sensitivity to exogenously applied GAs and a reduction

of fruit set compared with the seeded grape. Sensitivity to exogenously applied GA_3 reported to be inversely related to endogenous gibberellin levels (Boll et al., 2009). GA signaling is necessary for ABA, and ethylene-induced phenolic production, but ABA and ethylene signaling are probably not necessary for GA_3-induced phenolic production (Liang et al., 2013). In many cases of phytohormone research, the main part is gene function study in the signal transduction pathway. That will be important to understand the roles of GA_3 in plant development but can not explain metabolite variation in the flower organ. First, plant metabolites with various structures not easily recognized at once. Second, many of primary and secondary metabolites are related to plant growth, and the metabolic pathways linked to each other. In our work, wev tried to find metabolite differences between GA_3 treatment and no treated control flower tissue. Each metabolite contents are affecting GA_3 treatment in the pre-flowering development. Correlation between metabolites change and inducing seedless grape is not clear in our work. Our result too insufficient to understand whole scale metabolite changes upon GA_3 treatment, but our results suppose new clues for later related studies. That will need more studies for finding other relation controlling in the plant metabolite constancy.

Materials and Methods

Plant materials

GA_3 treatments applied on six-year-old grape cultivar 'Tamnara' in the experiment field of the National Institute of Horticultural and Herbal Science (NIHHS) in Suwon Korea. Grape 'Tamnara' was bred from a cross between 'Campbell Early' (*Vitis labruscana*) and 'Himrod SDS'(*Vitis sp*.) (Park et al., 2004). Grapevine planted at 3 m x 3 m spacing, using a modified-T trellis (Kim et al., 2014) and three vines each, treated with GA3 or none treated control. In GA3 treatment, whole grape flower bunches on grapevine were dipped in the 100 ppm of GA_3, 14 days before flowering (DBF) to produce seedlessness. Three flower samples collected at different stages (DBF 13, 10, 7, 5, 2, 0). Three immature berries collected at the different stages (Day after flowering; DAF 2, 5, 9). Each flower or immature berry was quickly frozen in liquid nitrogen (LN_2). 10 immature flowers or berries tissue was ground in a mortar with LN_2. Each tissue sample prepared five replicate, and each 50 mg of powder transferred into a 1.5 ml tube. Ground samples were kept at the -80℃ deep freezer and analyzed within one week.

Sample extraction

The sample preparation method was modified to Weckwerth (2004) and Kim (2015). Total metabolites were extracted from macerated samples using extraction buffer, which containing degassed methanol, chloroform, water (5:2:2, v/v/v). In the sample tube, 1 ml of extraction butter was added and was shaken for 5 minutes at 4℃. The extracts were centrifuged at 20,000 rpm, and the supernatant was transferred to a new tube and dried using a speed vacuum concentrator (Bioneer, Korea). For derivatization, we added 20 µl of methoxyamine (Sigma 226904, USA), 20 mg^{-1}·ml pyridines (Sigma 270407, USA) to the dried sample tubes and shaken for 90 minutes at 28℃. 180 µl MSTFA (Fluka 68768, Swiss) and 10 µl FAME marker (Supelco C8-C24, USA), were used as standard retention time marker. They were added to each tube and then shaken for 30 minutes at 37℃. Each 100 µl of prepared sample was transferred to auto-sampler vial with insert (Supelco #24722, USA).

GC-MS analyze

GC-MS system consisted of a gas chromatograph (Agilent 6890, USA) and mass spectrometer (Agilent 5985, USA). 1 µl of prepared sample injected into a splitless mode, operating at 230°C with a helium gas flow rate of 1 $ml \cdot min^{-1}$. The column used an HP-5MS, 5% phenyl methyl siloxane. The temperature was 3 min heating at 80°C followed by a $5°C \cdot min^{-1}$ and final 8 min heating at 280°C. Separated ion was detected by MS detector at 250°C and recorded at two scans per sec with an m/z 50-600 scan range. One blank sample and one QC samples injected at the start of a logical sequence. The analysis order was randomly composed of samples. Data files after GC-MS analysis were treated with deconvolution process by AMDIS (Agilent, USA) with standard parameter (component width = 12; model Ion 0, 73, 207, 281; resolution, sensitivity and shape requirements = medium). Retention time variation was adjusted to compare to FAME marker as a retention time standard in a process inside AMDIS program.

Annotation and multivariate analysis

Spectra was cut to 5% base peak abundance and matched to metabolomics Fiehn DB (Agilent G1676AA, USA) entries from most to least abundant spectra using the following matching filters. Equivalent to about ± 2 s retention time, unique ion must be included in apex masses and present at > 3% of base peak abundance. The mass spectrum similarity must fit criteria dependent on peak purity (<1.0) and signal/noise ratios (5:1). Peak annotation results were exported as text files matched metabolite (>80%) in the DB and exported to text files (Kind et al., 2009). For statistical analysis, each data treated rescaling, which divided by the square root of standard deviation (Pareto scaling; Van den Berg et al., 2006) of each sample variance. Statistical analysis of metabolic profiling with principal component analysis (PCA) and orthogonal projections to latent structures (OPLS) analyzed by the SIMCA-P+ software (vol. 12.0, Umetrics, Umea, Sweden; Ericksson et al., 2006).

Conclusion

GA_3 treatment led to change metabolite contents immediately in the grape flower cluster when applied at the day before flowering for making seedlessness. However, those changes have a statistical significance at the specific sampling times (DBF 13, 10, 2, 0, DAF 9). These results indicate that the GA_3 application changes metabolite in the grape flower at the specific developing stage. A total of 13 metabolites were related to seedlessness in the 'Tamnara' grape after GA_3 application at 14 days before flowering. Metabolites were classified into four groups depending on the increase or decrease pattern at the specific sampling times.

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