

Early response to GA3-treatment in the pedicel of table grape genotypes with different susceptibility to berry drop reveals responses elicited in cell wall yield and modification of lignin content

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Abstract

Background Gibberellins (GA₃) are the most sprayed growth regulator for table grape production worldwide, increasing berry size of seedless varieties through pericarp cell expansion. However, these treatments also exacerbate berry drop, which has a detrimental effect on the postharvest quality of commercialized clusters. Several studies have suggested that pedicel stiffening caused by GA₃ would have a role in this disorder. Nevertheless, transcriptional and phenotypic information regarding pedicel responses to GA₃ is minimal. Results Characterization of responses to GA₃ treatments using the lines L23 and Thompson Seedless showed that the former was up to six times more susceptible to berry drop than the latter. GA₃ also increased the diameter and dry matter percentage of the pedicel on both genotypes. Induction of lignin biosynthesis-related genes by GA₃ has been reported, so the quantity of this polymer was measured. The acetyl bromide method detected a decreased concentration of lignin seven days after GA₃ treatment, due to a higher cell wall yield of the isolated fractions of GA₃-treated pedicel samples which caused a dilution effect. Thus, an initial enrichment of primary cell wall components in response to GA₃ was suggested, particularly in the L23 background. A transcriptomic profiling was performed to identify which genes were associated with these phenotypic changes. This analysis identified 1,281 and 1,787 genes differentially upregulated by GA₃ in L23 and cv. Thompson Seedless, respectively. Concomitantly, 1,202 and 1,317 downregulated genes were detected in L23 and cv. Thompson Seedless (FDR ≤ 0.05). Gene ontology analysis of upregulated genes showed enrichment in pathways including phenylpropanoids, cell wall metabolism, xylem development, photosynthesis and the cell cycle at seven days post GA₃ application. Twelve genes were characterized by qPCR and striking differences were observed between genotypes, mainly in genes related to cell wall synthesis. Conclusions High levels of berry drop are related to an early strong response of primary cell wall synthesis in the pedicel promoted by GA₃ treatment. Genetic backgrounds can produce similar phenotypic responses to GA₃, although there is considerable variation in the regulation of genes in terms of which are expressed, and the extent of transcript levels achieved within the same time frame.

Background

The exogenous application of gibberellic acid-3 (GA₃) during key phases of fruit development is critical for seedless grape varieties, since it promotes berry enlargement through elongation of pericarp cells [1, 2]. Although these treatments are beneficial in terms of meeting the market standards at expected fruit caliber, they also exacerbate berry drop (also known as shattering) during postharvest [3]. This disorder affects negatively the general quality of the fruit, generating relevant economic losses. Its extent can also increase over time depending on postharvest storage conditions [4].

Preventing decay through the application of systematic measures during the postharvest handling of table grape is critical to achieve the commercialization of healthy clusters [5]. Hence it is important that marketable clusters present a non-senescent aspect with green stems and pedicels attached to the berry. The pedicel has been proposed as a main factor involved in berry drop [6] and several varieties have been described to be more prone to this postharvest issue under GA₃ treatment conditions; for instance,

Thompson Seedless was characterized as a cultivar sensitive to berry drop several decades ago [7]. Nevertheless, its extent can be kept under control with proper postharvest management practices [4]. Berry drop in cv. Kyoho is related to an increase in rachis hardness caused by expansion and lignification of this structure as a consequence of GA₃ treatments [8]. Other commercial cultivars have been reported as susceptible, such as Flame Seedless [5] and Ruby Seedless [3]. Thus, genetic background and the growth regulators applied to certain cultivars, particularly GA₃, seem to play an important role in postharvest berry drop.

Reports have indicated several morphological changes that take place in the rachis and pedicel following GA₃ treatment [9, 10]. It has been found that GA₃ induces changes in the pedicel by increasing the area of xylem and pith structures [9]. One study proposed that the loss of pedicel flexibility could be one of the main factors underlying berry drop in cv. Thompson Seedless [6]. Recently, analysis of transcripts related to lignin synthesis on genetic backgrounds with contrasting susceptibility to berry drop was reported, suggesting an enhancement of lignification in response to GA₃ treatments [11]. In other woody species an increase in the concentration of bioactive gibberellins (GA) enhanced growth and biomass production [12, 13]. In fact, transgenic lines have been engineered to increase bioactive GA and repress lignin biosynthesis in xylem tissue, resulting in enriched biomass destined to liquid fuel production [14, 15]. Therefore, it is suggested that the expected effects of GA₃ treatments in vascular tissue could be on cell wall-related processes, which in turn impact on the flexibility of this structure.

Transcriptomic approaches have been very useful to characterize the underlying biology of berry development [16–18] and its responses to gibberellin treatments [19, 20]. These studies have been widely conducted on samples obtained from fruit tissues. In this case, our focus is the study of the responses of pedicel tissue to GA₃ treatment evaluated through a transcriptomic platform, and how this could contribute to understand a complex trait such as berry drop.

The availability of contrasting phenotypes for postharvest berry drop may serve as a useful reference to understand this phenomenon [11]. L23, a line obtained in the framework of INIA's table grape breeding program, is a genotype with high susceptibility to berry drop compared to cv. Thompson Seedless under similar agroclimatic and management conditions. By capturing transcriptional variation in response to GA₃ treatments from two contrasting genotypes for berry drop, valuable information can be extracted to unveil the underlying differential response through the identification of differentially expressed genes. The objective of this study was to characterize the early phenotypic and transcriptional responses to GA₃ application of two genotypes that show contrasting susceptibility to berry drop and to identify the main biological processes involved in each condition.

Results

2.1 Genotype L23 is more susceptible to postharvest berry drop than cv. Thompson seedless under GA₃ treatment conditions

To compare the differential response of L23 and cv. Thompson Seedless in terms of postharvest berry drop, these genotypes were characterized across three seasons following GA₃-treatment (Fig. 1). The expected effect on berry enlargement after GA₃ application was confirmed (Fig. 1A). Significant differences in berry drop among conditions by season followed the same trend in all cases; L23 showed higher levels of berry drop than cv. Thompson Seedless but only under GA₃ treatment conditions (Fig. 1B). In the first season L23 was up to 5.97-fold more susceptible to berry drop. Values for the second and third seasons were 4.11 and 3.11-fold, respectively.

2.2 GA₃ treatment for berry enlargement promotes pedicel growth and enhances primary cell wall yield

Since GA₃ treatment is associated with postharvest berry drop, phenotypic variables of treated plants were observed during several developmental stages. Results of the first season are shown in Fig. 1C; two attributes considered as maturity indicators in the study were firmness and total soluble solids of berry samples. As can be seen, the expected effect on berry enlargement was observed under GA₃ treatment conditions for berry diameter and length. A similar response of the pedicel to GA₃ treatment was detected, particularly in the thickness of this tissue.).

Changes in pedicel dimensions induced by GA₃ have already been reported [11], and were observed in this study (Additional file 1: Fig. S1A). In our study, enlargement of cortex cells and transcript levels of monolignol biosynthesis-related genes (*4CL*, *CAD6* and *CCR1L*) were upregulated by GA₃ treatment. Transcriptional induction of lignin biosynthesis genes along with increased cell size could be related to the greater stiffness of pedicels as proposed in [6]. To test if the amount of lignin in samples treated with GA₃ is really higher in these groups, lignin concentration was determined by the acetyl bromide (AB) method [21]. Successful validation of this method adapted to pedicel tissue was performed using the same reference tissue as in the original research article (Additional file 1: Fig. S1B and S1C).

As Fig. 2 shows, lignin concentration in treated groups was unexpectedly lower than in control samples; this difference was more evident at early stages of development, particularly following GA₃ application (2A). The difference between control and treated conditions in lignin was less accentuated in the véraison stage (2B), and at harvest the observed variation was too great to detect significant differences among conditions (2C). Since the method quantifies the amount of soluble lignin obtained from a protein-free cell wall fraction, measurements are relative to the dry matter obtained in each sample.

Considering that pedicel dimensions differed between control and treated groups, changes in dry matter among conditions were recorded. Table 1 reports changes induced by GA₃ related to dry matter and cell wall accumulation. Dry matter increased in both genotypes; for cell wall yield, which is proportional to the protein-free cell wall fraction obtained from the AB protocol, GA₃-treated samples showed a significant difference for the L23 genotype.

In summary, the results suggest that phenotypic changes in the pedicel induced by GA₃ treatments could be related to promotion of primary cell wall yield and secondary cell wall modification-related processes.

2.3 Early transcriptional responses of pedicel to GA₃ are characterized by an enrichment of processes such as cell cycle, photosynthesis, cell wall modification, phenylpropanoid metabolism and xylem development

To characterize the early transcriptional responses of pedicel tissue to GA₃ treatment a transcriptomic profiling of both genotypes to this growth regulator was implemented. Information about treatments and timing of samples is summarized in Fig. 3A (the pipeline followed to perform differential expression analysis is illustrated in Additional file 2: Fig. S2).

The number of differentially expressed genes was obtained as indicated in the methods section. The number of genes identified after testing relative to a fold-change threshold [22] was used (Additional file 3: Fig. S3). Differential expression analysis revealed a total of 1,281 and 1,787 upregulated genes for L23 and cv. Thompson Seedless, respectively (Fig. 3B). A total of 1,202 and 1,317 downregulated genes were detected for L23 and cv. Thompson Seedless (Fig. 3B). This is the first report about differential gene expression in GA₃ treatments in genotypes with contrasting susceptibility to berry drop (See report for differential expression analysis in Additional file 4: Data S1).

To test if the differentially expressed genes (DEGs) were significantly linked to specific biological processes, gene set enrichment analysis was conducted in gene ontology (GO) annotation terms. Significant GO terms revealed numerous functions associated with enhanced primary cell growth, cell wall modification, histone and chromatin modification, among many others. GO functions of repressed genes were enriched in stress and defense-related processes (See full report in Additional file 5: Data S2).

To reduce redundancy and detect meaningful information among the GO terms obtained, the method described by Supek *et al.* [23] was followed. Enriched GO terms for upregulated genes in L23 is shown in Fig. 4. The same analysis conducted on cv. Thompson Seedless can be seen in Additional file 6: Fig. S4 (a list with detailed terms and p-adjusted values is given in Additional file 7: Table S1 for L23 and Additional file 8: Table S2 for cv. Thompson Seedless). Several GO terms grouped to cell growth and DNA replication processes. Other relevant functions were related to photosynthesis, xylem development, phenylpropanoid and flavonoid metabolism and cell wall modification processes, among many others.

To corroborate data from transcriptome profiling and validate the results, transcript abundance detected by RNAseq analysis for eighteen genes was compared to the measurements obtained by quantitative PCR (qPCR). Genes sampled from RNAseq analysis are given in Additional file 9: Table S3 along with the primers designed for qPCR. Results for the correlation tests between these techniques are given in Additional file 9: Fig. S5; qPCR correlated significantly with RNAseq measurements ($p= 1.368e-13$, $R^2=0.80$), validating the transcriptomic assay.

In summary, transcriptomic responses to GA₃ are mainly associated with the regulation of primary cell wall metabolism and cell cycle regulation, which could support primary growth. Other features such as histone modification, positive regulation of photosynthesis-related genes, flavonoids and phenylpropanoid metabolism, along with xylem development were strongly regulated by GA₃ in pedicel tissue.

2.4 Genes related to phenylpropanoid, photosynthesis and cell wall pathways were identified in the highly susceptible L23 background as possible biomarkers of berry drop.

To gain insight about the highly regulated genes in the susceptible L23 background, as an approach to associate berry drop and gene regulation, a list of thirty highly regulated genes was obtained from the transcriptomic data. For this, the DEGs grouping under functional categories were identified through the method proposed by [24]; results can be seen in Additional file 10: Fig. S6. Relevant categories were cell wall, along with lignin and some secondary metabolism-related genes. The main reason for the selection of these categories was their high representation as functional categories in terms of associated probability values; all significant categories identified by this method can be seen in Fig. 5. Quantitative transcriptome values of gene expression were fitted to a linear model, considering lowest to highest levels of observed berry drop values (cv. Thompson Seedless untreated, L23 untreated, cv. Thompson Seedless treated, L23 treated) [25]. Regression analysis was performed, and twelve genes were selected (Additional file 11: Fig. S7).

2.5 Expression studies of L23 showed predominant regulation of flavonoids, xylem-related genes and secondary cell wall formation.

The list of the twelve genes proposed to be studied at several developmental stages comparing the two genotypes is given in Table 2, along with the primers used to measure the abundance of the transcripts by quantitative PCR. Fig. 6 shows that the expression of candidate genes in pedicel samples collected from several stages revealed genes related to secondary cell wall formation such as cellulose synthase subunit-7 (*CESA7*) and endoglucanase (*GUN10*) that are overexpressed under GA₃ treatment conditions, particularly in the L23 background. Phenylpropanoid-related genes such as phenylalanine ammonia lyase (*PALY*), caffeoyl CoA O-methyltransferase (*CCoAOMT*) and cinnamoyl CoA-reductase (*CCR*) were highly expressed in cv. Thompson Seedless and L23. These are also positively regulated by GA₃ (*7DAT*) as well as by cold treatment (30 days at 0 °C), most notoriously for the *PALY* gene.

Flavonoid 3'-hydroxylase (*F3H*), an enzyme belonging to the flavonoid biosynthesis pathway, is highly expressed in the L23 genotype and upregulated by GA₃. The expression pattern of this gene in the susceptible genotype is strikingly different from cv. Thompson. Some laccases, also differentially expressed, such as Laccase-4 (*LAC4*) and Laccase-17 (*LAC17*) may exert an important role in lignin condensation, although their expression was relatively low. Other genes such as Nepenthesin-1 (*NEP1*), Xylem serine proteinase (*XSP1*) and Chlorophyll a-b binding protein 40 chloroplastical (*CB12*) had higher basal levels in the L23 background than in cv. Thompson Seedless and were regulated positively by GA₃.

These sets of genes responsive to GA₃ showed distinctive expression patterns between genotypes and treatments across several stages of development.

Discussion

Gibberellins are the most used growth regulators in table grape production; particularly in seedless varieties, in which the endogenous generation of the hormone is reduced to basal levels and so the application of GA₃ is mandatory to obtain berries of marketable size [26]. However, there is also a detrimental effect of GA₃, which increases postharvest berry drop, a problem well known for a long time for some cultivars such as Thompson Seedless [7]. Some authors have proposed that the rachis and pedicel could be one of the main structures influencing berry drop [6, 10, 11]. But scarce information exists about the transcriptional response of this tissue to GA₃ treatments.

This is the first study to characterize the transcriptomic response of table grape pedicel to GA₃ in two genetic backgrounds with contrasting susceptibility to berry drop. The main findings were: (i) GA₃ treatments induced major phenotypical changes in the pedicel, by increasing its size and modifying cell-wall properties; (ii) accumulation of lignin was negatively affected by an early response of cell wall augmentation, resulting in an initial dilution effect with lower concentration of the polymer. As a consequence, lignin concentration reached similar values for both treated and untreated groups at advanced stages such as harvest time; (iii) transcriptome profiling of samples after treatment provided cues on the main biological processes upregulated by GA₃, which can be summarized as promotion of vegetative growth through positive regulation of cell growth, cell wall modification, xylem development, phenylpropanoid and flavonoid metabolism, along with downregulation of stress-related responses; and (iv) core responses are similar between the genotypes, one more tolerant and one more susceptible to postharvest berry drop, although there are a number of DEGs that are specific to each one. Genes which showed contrasting expression patterns were identified and studied at several stages; this set of genes, or some of them, could be used potentially as indicators or biomarkers of postharvest berry drop. However, to obtain robust indicators it is necessary to generate information in the context of segregating populations to understand the underlying genetics of the shattering trait and subtraits. With this information, comprehensive models can be inferred which could serve to predict the occurrence of this trait in wider genetic backgrounds.

Berry drop in seedless varieties has been linked to growth regulator treatments, most of them consisting of the application of the gibberellic acid-3 (GA₃) isoform for berry size enlargement [3, 5, 7, 27, 28]. Researchers have reported the effects of this growth regulator on several tissues and conditions, such as a positive regulation by GA₃ on lignin biosynthesis, cell expansion and xylem development [9, 11, 28]. The present study supports these findings with the identification of upregulated genes with significant annotations for these processes. Variation in the response to GA₃ treatments was detected between the genetic backgrounds assessed. Measurement of lignin was coherent with other techniques performed on similar tissues: for example, lignin content in grape stalks has been estimated to be 17.4%

by the Klason method (corrected previously by tannin, polyphenols and other substances) [29]. In our study, the range for reported lignin content was 15.7%-20.4%. Diluted content at early stages of GA₃ application could suggest a strong response in cell wall synthesis that sustains the growth of cell components through creep processes [30]. A small but significant difference in cell wall yield was observed between genotypes. Cellulose synthase subunit-7 (*CESA7*) has been described as a gene involved in secondary cell wall synthesis [31]; in this study this gene was highly expressed in the pedicel of L23 background along with several genes encoding other CESA-related subunits (GSVIVG01023643001, GSVIVG01021248001, GSVIVG01028402001, GSVIVG01033278001; see additional file 3 for more detailed results). Further analyses will be required to evaluate the specific effects of these genes on cell wall synthesis and its interactions with GA₃. It would also be interesting to determine if there are sequence differences in these genes and their regulatory upstream regions between cv. Thompson Seedless and L23 backgrounds, to uncover possible regulation motifs or structural variations associated with the different transcription levels detected by transcriptomics and qPCR methods.

Responses of seedless berry to GA₃ treatments are strongly oriented towards hormone crosstalk, as observed in cv. Centennial Seedless, which ultimately orchestrates a general response of cell wall relaxation that promotes berry size enlargement [20]. The pedicel, however, showed more enrichment in 'effector-related' responses than in hormone-related pathways in the same time frame of seven days after GA₃ treatment (7DAT). Remnants of seed tissues in the berry may play a major role in the response to exogenous hormone applications, and since the pedicel is a vascular and supporting tissue of berry growth, the responses of the latter to GA₃ applications could be focused on downstream pathways, whereas the former is integrating the external stimuli (in this case, mostly GA₃) to the signals given by the embryo remnants. Results of the gene set enrichment analysis of the pedicel to GA₃ (Additional file 5: Data S2) showed positive responses on plant cell wall loosening (GO:0009828) and organization (GO:0071669), among other cell wall-related and cell growth processes, which can be attributed to tissue enlargement similar to what has been described in berry tissue. In contrast, hormone-related terms showed scarce enrichment, although response to gibberellin (GO:0009739) was detected in positive DEGs. Other responses related to hormones in negative DEGs were related to jasmonic acid (GO:0071395), salicylic acid (GO:0009754, GO:0009863, GO:0071446), ABA (GO:009738) and ethylene (GO:0009723, GO:0009692, GO:0009693). It is worth mentioning that in the present study the response to GA₃ treatment was only evaluated at seven days after application. As can be seen in [20], sequencing of samples from earlier stages of evaluation (one and three days after treatment) showed that hormone-related responses were richer. Thus, it is important to consider how the response to hormone signaling develops over time; however, the aim of this study was to evaluate how different is the response to GA₃ between two genetic backgrounds with contrasting performance in berry drop.

Enrichment in processes such as photosynthesis, phenylpropanoids and flavonoids highlights interesting features on how pedicel tissue is regulated by GA₃. Although this study did not include measurements of photosynthetic variables, transcriptomics showed that many photosynthesis-related

genes were upregulated by GA₃ treatment. KEGG analysis based on KEGG Orthologs showed several genes involved in photosynthesis reactions (Additional file 12: Fig. S8A). Non-foliar photosynthesis has been discussed as an important alternative source of carbon acquisition [32]. The green shoots from annual woody species, known as stalks, diminish CO₂ efflux through photosynthesis in their chlorenchyma cells [33]. Therefore, it may be suggested that photosynthetic positive responses of pedicels to GA₃ could be favorable in the context of sustaining general growth, although the extent of CO₂ assimilation and its contribution to pedicel and berry growth remains to be further analyzed. The phenylpropanoids pathway showed positive regulation of genes related to the synthesis of these metabolites, except for those involved in the final steps of monolignol biosynthesis (Additional file 12: Fig. S8B). These results suggest that phenylpropanoids are upregulated towards the flavonoid pathway in response to GA₃ (Additional file 12: Fig. S8C). Polyphenolic compounds derived from plant secondary metabolism, such as phenylpropanoids and flavonoids, include a wide spectrum of structurally and functionally rich compounds, to which a high antioxidant capacity has been attributed [34]. The role of these compounds in the pedicel and its interaction with GA₃ seems to be related to a general vegetative response, and further metabolomics analysis could help to identify the variety of secondary metabolites present in this tissue. The abundance of these metabolites is suggested to be higher in treated samples, since polyphenolic compounds interfere with RNA extraction as was observed in this study prior to the optimization of extraction methods for pedicels (data not shown).

Open questions remain about the long-term response of GA₃-treated tissues, such as the influence of cold treatment during storage conditions and their gene activity, which have not yet been addressed under postharvest conditions. Besides, even though a genotype-dependent factor has been suggested in berry drop, there are no genetic studies linking traits such as berry drop in segregating populations, due to the complexity of phenotyping this disorder. Several efforts have focused on increasing the precision of measurement of berry drop [35]. Hopefully, more studies will help to understand the genetic factors and conditions determining berry drop in table grapes.

Conclusions

This is the first study to investigate the effects of GA₃ treatment on the pedicel transcriptomic response of genotypes with different susceptibility to postharvest berry drop. This phenomenon in table grape is related to GA₃ treatments, which increase pedicel dimensions due to promotion of cell wall and phenylpropanoid metabolism. This is consistent with the differentially expressed genes and gene families found here by a transcriptomic approach. Molecular responses to GA₃ treatment varied according to the genetic background; genes involved in cell wall synthesis, phenylpropanoid, flavonoid and laccase-related processes were highly upregulated in the L23 susceptible background, which showed a higher incidence of berry drop compared to cv. Thompson Seedless. Positive regulation of these genes could partially explain the differences observed in cell wall yield-related results. Moreover, a strong response of photosynthesis-related genes was detected by gene ontology analysis, suggesting a possible role of the pedicel in non-foliar photosynthesis for both genotypes. Considering that GA₃ is a widely used

growth regulator for table grape production, the differentially expressed genes identified here could be valuable candidate genes for assisted selection in breeding programs.

Methods

5.1 Plant material

This study was performed at La Platina Research Center that belongs to the Instituto de Investigaciones Agropecuarias (INIA), located in Santiago, Chile (33°34'20"S; 70°37'32"W; 630 m.a.s.l.), during the 2015-2016, 2016-2017 and 2017-2018 seasons. The plant material (adult plants grown on their own roots, conducted with the Spanish trellis system and managed under standard irrigation, fertilization and pest management programs) of two seedless table grape varieties, cv. Thompson Seedless and L23 (*'Line #23'*: F1 from cv. Ruby Seedless x cv. Centennial Seedless). Six individual plants from each genotype were used for qPCR experiments; four biological replicates were used for RNAseq experiments. Each plant had treated (GA₃) and untreated (Control) clusters to reduce any bias related to individual variation.

Timing of samples was based on the Einhorn-Lorenz phenology system adapted for *Vitis vinifera* L. by Coombe, 1995 [36]. Stage(s) evaluated in each experiment are indicated in each figure.

Thompson Seedless (also known as 'Sultanina') is a free, ancestral variety, supposedly native to somewhere in Iran; it is said it was introduced into Chile by the beginning of XX century from California. L23 is a segregant belonging to INIA's breeding program and derived from the crossing 'Ruby Seedless' x 'Centennial Seedless'. These parental varieties were both originally released by UC Davis, CA, but are currently free. As 'Thompson seedless', they were also introduced into Chile many years ago and kept through vegetative propagation.

5.2 Growth regulator treatment

Clusters of cv. Thompson Seedless and L23 were subjected to the following growth regulator treatments: (1) Thompson Seedless were sprayed five times: once with 10 ppm gibberellic acid (GA₃, Pro-Gibb 40% Valent Biosciences Chile S.A., Santiago, Chile) during pre-bloom for bunch elongation; 15 ppm at full bloom for thinning and 200 ppm for berry enlargement (three doses of 40, 100 and 60 ppm with seven-day intervals) when berries reached 4~5 mm diameter [11]. (2) L23 were sprayed with 120 ppm GA₃ (3 x 40 ppm, intervals of seven days) for berry enlargement when berries reached 4~5 mm diameter. Dosage for cv. Thompson Seedless and L23 was defined in previous assays, optimizing berry enlargement at the lowest possible value of berry drop.

5.3 Physiological parameters

The parameters measured were berry and pedicel diameter (mm), soluble solids (% w/w g sucrose per 100 g solution), titratable acidity (g L⁻¹ tartaric acid) and firmness (g mm⁻¹) of 10 bunches collected from

different plants. For each variable, 30 healthy and homogenous berries with their cap stems were randomly sampled per cluster. Diameter was measured with a digital caliper. Soluble solid content of fruit was measured with a refractometer (ATC-1E, Atago, Tokyo, Japan). Firmness of berries (considering skin and flesh) was determined using a firmness tester (Firmtech II, BioWorks, Wamego, KS). Titratable acidity was measured in the pooled juice of 10 berries per cluster by titration with 0.1 N NaOH (pH 8.2).

Random samples of pedicels from clusters were pooled (300 ~ 500 pedicels). Plant material was immediately frozen with liquid nitrogen and stored at -80 °C for molecular analysis.

5.4 Berry drop

Clusters were harvested based on the average soluble solids content (18° Brix). For postharvest storage experiments, 10 clusters per treatment (control and GA₃-treated) were stored after harvest for 15, 30 and 45 days at 0 °C and then berry drop was measured. To determine percentage of berry drop (shattering), 10 bunches were weighed before and after being shaken for 30 s; weight of detached berries was recorded. Berry drop percentage was calculated as $BD\% = [(DB + SB)/TB] \times 100$, where DB represents the weight of dropped berries during storage time at 0 °C, SB represents the weight of berries dropped after shaking of bunches and TB represents the total weight of the bunch.

5.5 Determination of lignin

For lignin estimation, quantitative determination by the acetyl-bromide method [21] was followed with slight modifications. Pedicels were dried at 105 °C for 16 hours in an oven and cooled in a vacuum desiccator until the next step. Dry samples (0.3 g) were homogenized in 50 mM potassium phosphate buffer (5 mL, pH 7.0) using an Ultra Turrax T25 homogenizer (IKA-Werke GmbH & Co., Staufen, Germany). The solution was centrifuged (1400xg, 5 min) and the pellet was washed by successive stirring and centrifugation as follows: twice with phosphate buffer (pH 7.0; 7 mL), three times with 1% (v/v) Triton X-100 in pH 50mM potassium phosphate 7.0 buffer (7 mL), and six times with acetone (5 mL). All supernatants were monitored by spectrophotometric measurements at 280 nm to ensure no contamination with protein and UV-absorbing materials in downstream steps. The pellet was dried in an oven (60 °C, 24 hours) and kept cool in a vacuum desiccator until further reaction with acetyl bromide. Samples and a blank solution were prepared as follows: dry matter obtained from the previous step (considered as a protein-free cell wall fraction) was placed in a screw cap 15 mL centrifuge tube (20 mg per sample) containing 0.5 mL 25% acetyl bromide (v/v in glacial acetic acid) and incubated at 70 °C for 30 min. After digestion, samples were cooled in an ice bath and mixed with 0.9 mL of 2 M NaOH and 0.1 mL of 5 M hydroxylamine-HCl. Samples and blank solution were solubilized in 8 mL of glacial acetic acid and absorbance of supernatant was measured at 280 nm immediately after centrifugation (1400xg, 5 mins). Dilution in glacial acetic acid was performed to avoid signal saturation. The standard curve was constructed using alkali lignin (Aldrich 37, 096-7) with a reported absorptivity value of 23.03 g⁻¹ L cm⁻¹ (Additional file 1: Fig. S1B). Results were expressed as mg lignin g⁻¹ cell wall as appears in the cited protocol [21].

5.6 Dry Matter

For the determination of changes in pedicel dry matter accumulation elicited by GA₃ treatments, samples from different conditions at harvest were measured in an analytical balance. Samples taken from bunches when berries reached an average of 18 °Brix were pooled into a group of 30~60 pedicels and weighed for fresh weight determination (at least 0.3 g fresh weight to avoid technical error). Then replicates were dried at 105 °C in an oven for 16 hours and dry weight was measured [21]. Percentage of dry matter and weight of pedicels was determined, considering the number of pedicels weighed in each batch.

5.7 RNA isolation

Total RNA was isolated from 1.0 g frozen tissue using a modified hot borate method [37], following all the indications listed in the cited protocol with the only exception that sample tissue was reduced to a third of the recommended amount and polyvinylpyrrolidone was doubled to avoid phenolic compound contamination before RNA isolation. The quantity and quality of RNA were assessed with Qubit® 2.0 fluorometer (Invitrogen™ by Life Technologies, Singapore). Spectrophotometric determination of A_{260/280} and A_{260/230} ratios and electrophoresis in 1.2% formaldehyde-agarose gels verified the quality and integrity of extracted RNA.

5.8 Library synthesis and sequencing

For the RNAseq experiment, pooled pedicel samples of individual plants were obtained seven days after the last GA₃ application (corresponding to EL31 stage according to the Einhoch-Lorenz phenology system [36], plus seven days—defined in this study as 7DAT). Two levels were used in the treatment factor, control and GA; and two levels were considered in the genotype factor, cv. Thompson Seedless and L23. Four biological replicates were used for each condition, summing to 16 libraries which were synthesized and sequenced.

Prior to library synthesis, total RNA was assessed by fragment analyzer PROSize® 2.0 version 1.3.1.1 (Advanced Analytical Technologies, Inc., Ames, IA, USA). Mean RNA quality in all samples was 7.35 (SD: 0.56), confirming the integrity of extractions (refer to Table SIV for individual scores). Then 2.5 µg aliquots were used to isolate poly(A) mRNA for preparation of libraries using TruSeq RNA Sample Prep Kit v2, following the manufacturer's instructions described in the TruSeq RNA Sample Preparation v2 Guide, Part #15026495 Rev. F (Illumina, Inc.). Libraries were sequenced using the HiSeq 4000 platform (Illumina). Libraries were sequenced as paired-end data (2 x 100 bp).

5.9 Pre-processing of reads

Low-quality reads (Phred score Q<25, nucleotides with undefined base assignment N>1 and read length<50) were removed using the wrapper tool Trim Galore! [38]. Contamination with Illumina adapters was handled with the same tool, removing sequences matching the adapters.

5.10 Mapping and matrix count extraction

Alignment of clean reads to the *V. vinifera* L. PN40024 12X reference genome [39] was performed using STAR software, considering paired-end data default parameters [40]. Uniquely mapped reads were kept for generation of a count matrix using HTseq [41]. The gene model was extracted from the CRIBI database. Genes reported were based on Genoscope structural annotation. Summarized mapping results are given in Table S4.

Data from the count matrix of uniquely mapped reads were visualized to confirm major effects expected from the experimental design. Assessment using multidimensional scaling and hierarchical clustering based on counts per million reads showed no anomalies; differences were given mainly by genotype and treatment factors, with low variability of replicates within groups (for experimental details please see Fig. S10).

5.11 Differential expression analysis

Count matrices were analyzed with EdgeR package [42] under R 3.4.4 software. Correction for composition bias in each sample was handled with the CalcNormFactor option. Then limma-voom transformation of data with was performed with the limma package [25]. Since the number of DEGs was relatively high, an additional filter of \log_2 -fold-change was established. To keep a low false discovery rate, the method described in [22] was followed, considering a prior \log_2 -fold-change of 1. For differential expression analysis considered an adjusted p-value (FDR) < 0.05.

5.12 Gene set enrichment analysis and Revigo

Gene set enrichment analysis was conducted on the Agrigo platform v2.0 [43] using hypergeometric distribution of data; p-values were adjusted by the Benjamini-Yeukiteli method and significant terms (FDR<0.05) were analyzed with the Revigo algorithm to reduce redundancy of terms based on semantic relatedness. The cutoff value used was C: 0.7 [23].

5.13 MapMan analysis

Mapping of differentially expressed genes as determined in 2.8 was performed using MapMan software (X4 version). Annotation of *V. vinifera* L. was downloaded from BioMart of the Phytozome v12.1 resource page; amino acid sequences were fed into the Mercator 4 online tool, creating an up-to-date mapping file. The Wilcoxon test with adjusted p-value by the Benjamini-Yeukiteli method was used to detect significant differences among BIN categories provided by MapMan [24].

5.14 cDNA synthesis

Synthesis of cDNA was performed by reverse transcription with 2 μ g of total RNA as template using MMLV-RT reverse transcriptase and oligo dT primers (Promega, Madison, WI), according to standard procedures. Concentration of cDNA was assessed by measuring the absorbance at 260 nm.

5.15 Real-time qPCR assays

Transcript abundance was analyzed by qPCR with the LightCycler® 96 system from Roche (Roche Diagnostics, Mannheim, Germany), using SYBR Green to measure the amplified RNA-derived DNA products as described in [44]. Gene-specific primers (Table IV) were designed using the online software Primer3plus [45] and were synthesized by IDT (Integrated DNA Technologies). The qPCR assays were performed on six biological samples in duplicate.

The reference gene was GSVIVG01011810001 – probable fructose-bisphosphate aldolase 3 chloroplastic (AFLC3). This gene was identified based on RNAseq results; all non-differentially expressed genes were filtered and ranked according to the lowest variance and coefficient of variation, considering the 16 libraries. Five genes were selected, and primers were synthesized (Additional file 14: Table S5). AFLC3 gene values were used in downstream calculations related to relative expression.

5.16 Experimental design and statistical analyses

A fully randomized experimental design was used. The two main factors considered were Genotype factor (two levels: cv. Thompson Seedless and L23) and Treatment factor (two levels: Control and GA₃). Statistical tests and parameters are shown in each figure.

Abbreviations

°C: degrees on Celsius scale; *4CL*: 4-coumarate-CoA ligase; *7DAT*: Seven days after GA₃-treatment; $A_{260/230}$: Ratio of the absorbance at 260nm and 230nm; $A_{260/280}$: Ratio of the absorbance at 260nm and 280nm; AB: Acetyl bromide; *AFLC3*: probable fructose-bisphosphate aldolase 3 chloroplastic; ANOVA: Analysis of variance; *CAD6*: cinnamyl-alcohol dehydrogenase 6; *CB12*: chlorophyll a-b binding protein 40 chloroplastic; *CCoAOMT*: caffeoyl-CoA O-methyltransferase; *CCR*: cinnamoyl-CoA reductase; *CCR1L*: cinnamoyl-CoA reductase 1-like; *CESA7*: cellulose synthase A catalytic subunit 7 [UDP-forming]; *CHS*: chalcone synthase; CO₂: carbon dioxide; DEGs: Differentially expressed genes; EL17: Eichhorn-Lorenz phenological stage 17; *F3H*: flavonoid 3'-hydroxylase; FC: Fold-change; FDR: False discovery rate; g: gram; GA: Gibberellic acid; GO: Gene ontology; *GUN10*: endoglucanase 10; KEGG: Kyoto Encyclopedia of Genes and Genomes; *LAC17*: laccase-17; *LAC4*: laccase-4; mg: milligram; mL: milliliter; mM: millimolar; mm: millimeter; *MTDH*: probable mannitol dehydrogenase; *NEP1*: aspartic proteinase nephentesin-1; nm: nanometer; *PALY*: phenylalanine ammonia-lyase; *PMR5*: powdery mildew resistance 5; ppm: parts per million; qPCR: quantitative Polymerase Chain Reaction; RNAseq: Ribonucleic acids sequencing (whole transcriptome shotgun sequencing); Ta: annealing temperature; *TIP11*: aquaporin tonoplast intrinsic protein 1-1; Tukey HSD test: Tukey's Honest Significant Difference test; v/v: volume of solute to volume of solution ratio; *XSP1*: xylem serine proteinase 1.

Declarations

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Author's contributions

MM, MGR, PH and BD planned and designed the research. MM and MGR performed the experiments and analyzed the data concerning phenotyping, lignin measurement and qPCR. MM and TCV analyzed the transcriptomic data. MGA assisted on qPCR-related experiments. MM and PH wrote the manuscript. CM, CME, RI, PH contributed to the critical discussion, interpretation of data and edited the manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The author declare that they have no competing interest

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Tables

Table 1 Dry matter and cell wall deposition are increased in pedicel samples from GA₃ treated clusters.

| Genotype | Treatment | Dry matter (%) | Pedicel fresh weight (mg/pedicel) | Pedicel dry weight (mg/pedicel) | Protein-free cell wall fraction yield (%)* |
|-------------------|-----------|-------------------------------|-----------------------------------|---------------------------------|--|
| Thompson Seedless | Control | 31.0 ± 1.82 ^a (16) | 12.6 ± 3.90 ^a (16) | 3.86 ± 1.03 ^a (16) | 45.4 ± 8.96 ^{ab} (6) |
| | GA | 34.5 ± 1.52 ^b (16) | 73.4 ± 14.8 ^b (16) | 25.1 ± 4.27 ^b (16) | 52.2 ± 5.18 ^{ab} (6) |
| L23 | Control | 24.5 ± 1.05 ^c (16) | 15.2 ± 2.34 ^a (16) | 3.71 ± 0.45 ^a (16) | 41.8 ± 7.33 ^a (8) |
| | GA | 33.8 ± 1.04 ^b (16) | 73.2 ± 12.0 ^b (16) | 24.8 ± 4.55 ^b (16) | 56.2 ± 5.89 ^b (6) |

Mean ± standard deviation is shown in each cell. Number of replicates is presented in parenthesis. Significant differences between conditions in each column are reported as letters (Tukey HSD test: p<0.05). *Starting material corresponded to dried weight of pedicels (~0.3 g per sample).

Table 2 Primer list of candidate genes to characterize the underlying responses to GA₃ in pedicel samples from genotypes with contrasting susceptibility to berry drop.

| Gene id | Description | Ta (C°) | Primer | Sequence (5' -> 3') |
|-------------------|--|---------|-------------|-----------------------------------|
| GSVIVG01009881001 | Endoglucanase 10 (<i>GUN10</i>) | 54 | VvGUN10-s | TCG GAC TGC AAA GCT ATC CT |
| | | | VvGUN10-a | GCA TTG GGG TCC TTT GAT TT |
| GSVIVG01031715001 | Caffeoyl-CoA O-methyltransferase (<i>CCoAOMT</i>) | 60 | VvCCoAOMT-s | TCA AGC TCA TCA ATG CCA AG |
| | | | VvCCoAOMT-a | AGT CAA TCT TGT GGG CAA CC |
| GSVIVG01023643001 | Cellulose synthase A catalytic subunit 7 [UDP-forming] <i>CESA7</i> | 54 | VvCESA7-s | GTC ATT GGT GGT GTG TCA GC |
| | | | VvCESA7-a | GGA TAA GGA GGG TGG TCC AT |
| GSVIVG01032968001 | Chalcone synthase (<i>CHS</i>) | 60 | VvCHS-s | CCC GGT GCT GAC TAT CAA CT |
| | | | VvCHS-a | AAT CCA GGT GGG TGT CAG AG |
| GSVIVG01002109001 | Probable mannitol dehydrogenase (<i>MTDH</i>) | 54 | VvMTDH-s | TGG TGT TGG GTG TAT GGT TG |
| | | | VvMTDH-a | TGT GAT CCC AGC ACA TAG GA |
| GSVIVG01033677001 | Aquaporin TIP1-1 (<i>TIP11</i>) | 54 | VvTIP11-s | AAG AAG GGC AAT TTG GGA AT |
| | | | VvTIP11-a | CCT CGT ACA CAA GTC CAG CA |
| GSVIVG01027584001 | Xylem serine proteinase (<i>XSP1</i>) | 54 | VvXSP1-s | TCA GAT ACC GGT TCG GAG AG |
| | | | VvXSP1-a | TTG TAT GTG GCG CTG TTG TT |
| GSVIVG01025703001 | Phenylalanine ammonia-lyase (<i>PALY</i>) | 60 | VvPALY-s | AAT TGC AGC CAT TGG AAA AC |
| | | | VvPALY-a | GTG TTG CTC AGC ACT TTG GA |
| GSVIVG01034003001 | Laccase 4 (<i>LAC4</i>) | 54 | VvLAC4-s | CTC CCC CAT CGC AGT AGA TA |
| | | | VvLAC4-a | TTT GGC TGG GTA CTT TTT GG |
| GSVIVG01016100001 | Aspartic proteinase nepenthesin-1 (<i>NEP1</i>) | 54 | VvNEP1-s | CTC TGA AGG CCG AGT TTC TG |
| | | | VvNEP1-a | AGC AAG AGC CAA ACA CAC CT |
| GSVIVG01001005001 | powdery mildew resistance 5 (<i>PMR5</i>) | 54 | VvPMR5-s | GGC ATG ATT CAC TGG GTT CT |
| | | | VvPMR5-a | GCT TCC ACC TCC ATT TCT CA |
| GSVIVG01029789001 | Chlorophyll a-b binding protein chloroplastic (<i>CB12</i>) | 54 | VvCB12-s | CCG GTG ACT ATG GCT TTG AT |
| | | | VvCB12-a | AGG AGT TGG GTT CCA AGG AG |
| GSVIVG01011810001 | Probable fructose-bisphosphate aldolase 3 chloroplastic (<i>ALFC3</i>) | 54 | VvAFLC3-s | GAT GGG GAT CAC CCA ATT GAT |
| | | | VvAFLC3-a | ATT TGG CGA TGG TCT CTG GA |

Genes of interest were selected to generate a group of markers to study its variation along several stages of development in genotypes with contrasting berry drop phenotypes. Gene name and description comes from basic

local alignment using the Swissprot database.

Figures

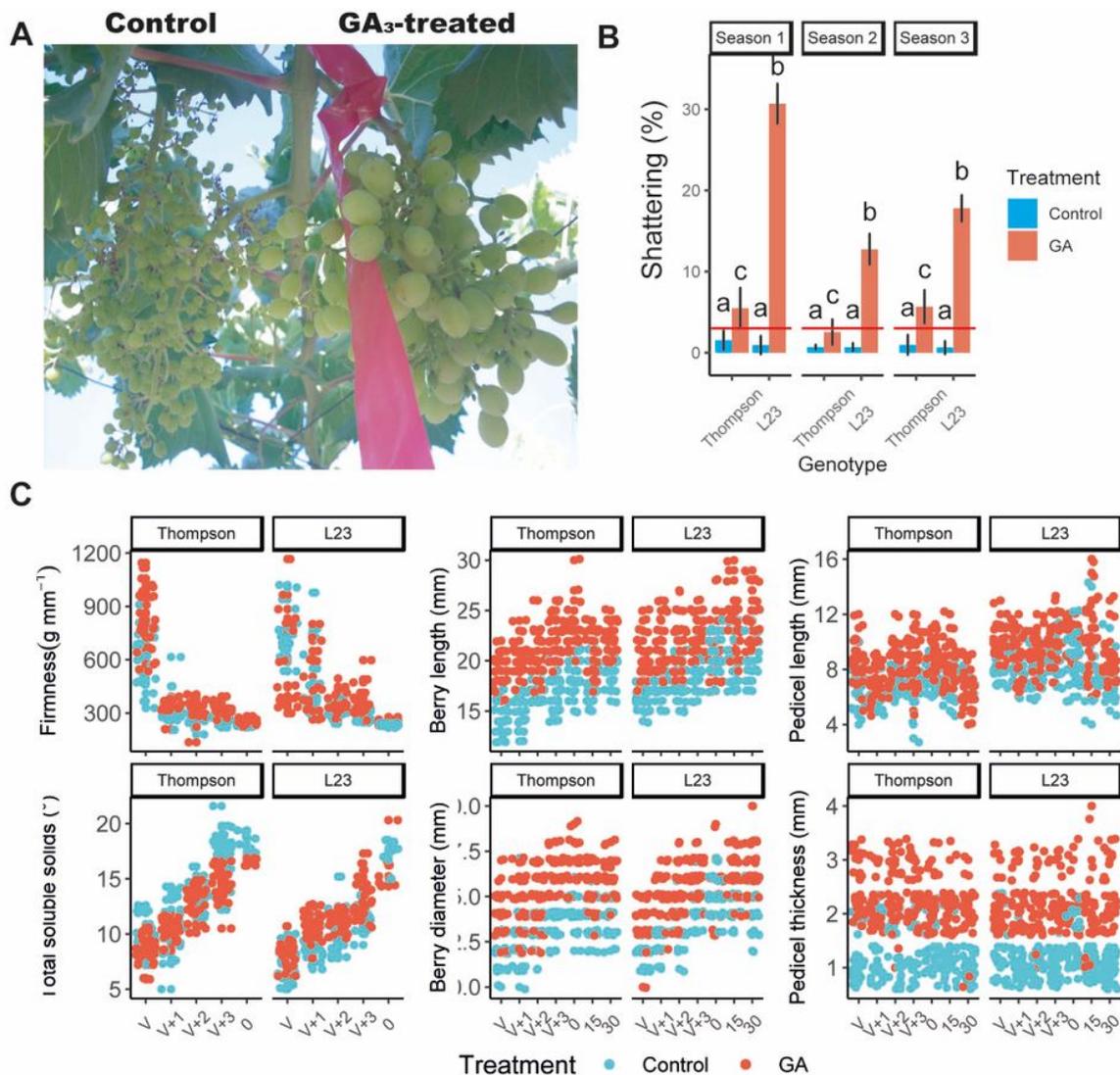


Figure 1

GA₃ not only increases berry and pedicel dimensions but also exacerbates postharvest berry drop. A Effects of GA₃ application on bunches from cv. Thompson Seedless. The image illustrates the notorious increase on berry size produced by GA₃ compared to a non-treated cluster of the same vine. B L23 exhibits high susceptibility to postharvest berry drop under GA₃ treatment conditions. Total weight of berries separated from cap stems against total weight of bunches after shaking is reported. Mean and standard deviation are shown on each bar and error bar (replicates for Season 1, Season 2 and Season 3 were 15, 8, 8, respectively, on each point). Red abscissa line in the graph shows 3% berry drop, a recommended tolerance value for high quality table grape exportation. Data were analyzed with ANOVA and Tukey HSD tests to find significant differences between conditions ($p < 0.05$). C Pedicel dimensions

increase in GA3-treated samples. Scatter plots of variables show differences in dimension of berry and pedicel. Total soluble solids and firmness were used as indicators of phenological development. V+n: Number of weeks (n) passed since veraison (V). The numbers '0', '15', '30' represent the number of days spent in cold storage after harvest time (n=30).

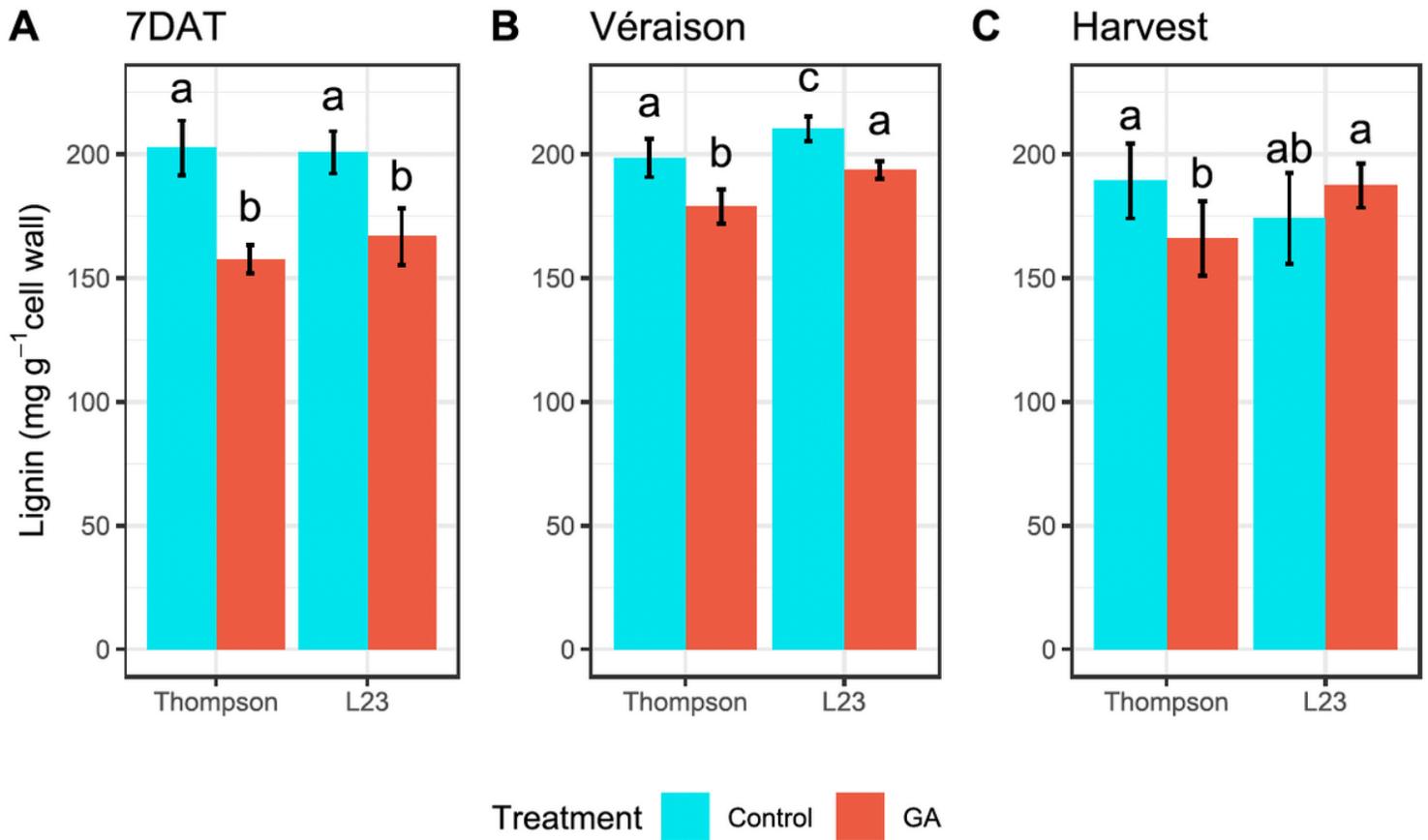


Figure 2

Lignin concentration in pedicels diminishes after GA3 treatment. Quantification of soluble lignin by acetyl bromide method of pedicels sampled from three developmental stages. Mean and standard deviation are shown on each bar and error bar, respectively (n=8). ANOVA followed by Tukey tests were performed to detect significant differences between conditions (p<0.05). Stages of evaluation were: A Seven days after treatment, B veraison and C Harvest. Homoscedasticity of data was previously verified by the Levene test, with p-values of 0.6583 (A), 0.1799 (B) and 0.1252 (C).

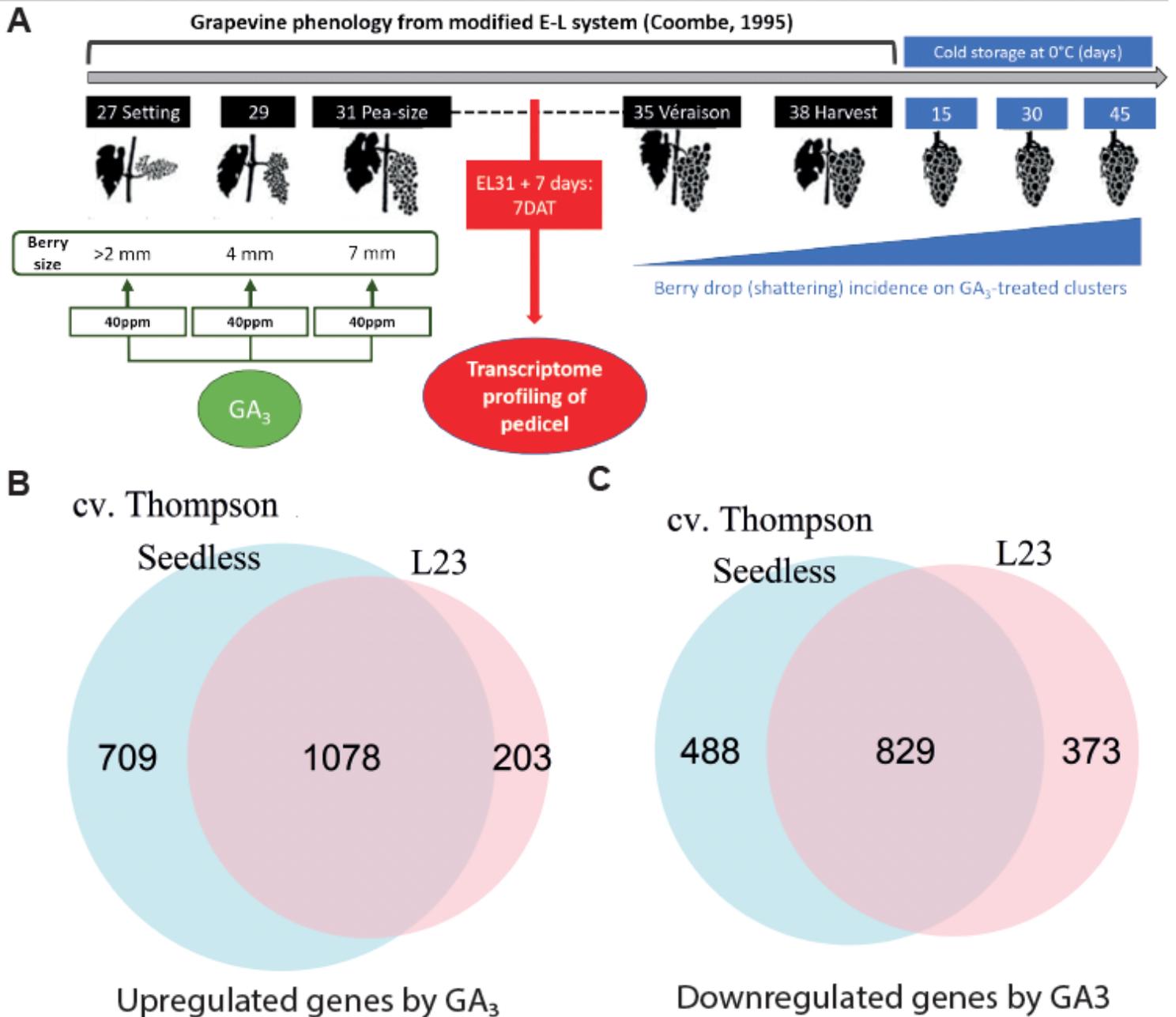


Figure 3

Characterization of the early response to GA₃-treatment in two backgrounds with different susceptibility to berry drop. A The scheme illustrates the rationale behind the timing of pedicel samples since GA₃-treatment is a major factor involved in berry drop incidence. Phenological-based sampling was critical since the L23 and cv. Thompson Seedless fruit development time frames differ slightly (2-3 weeks). The reference time frame was based on the study of [16]. B Upregulated genes in response to GA₃ treatments. The number of DE genes was 1,787 and 1,281 in cv. Thompson seedless and L23, respectively (FDR<0.05). C Repressed genes in response to GA₃ treatments. The number of DE genes was 1,317 and 1,202 in cv. Thompson seedless and L23s, respectively (FDR<0.05).

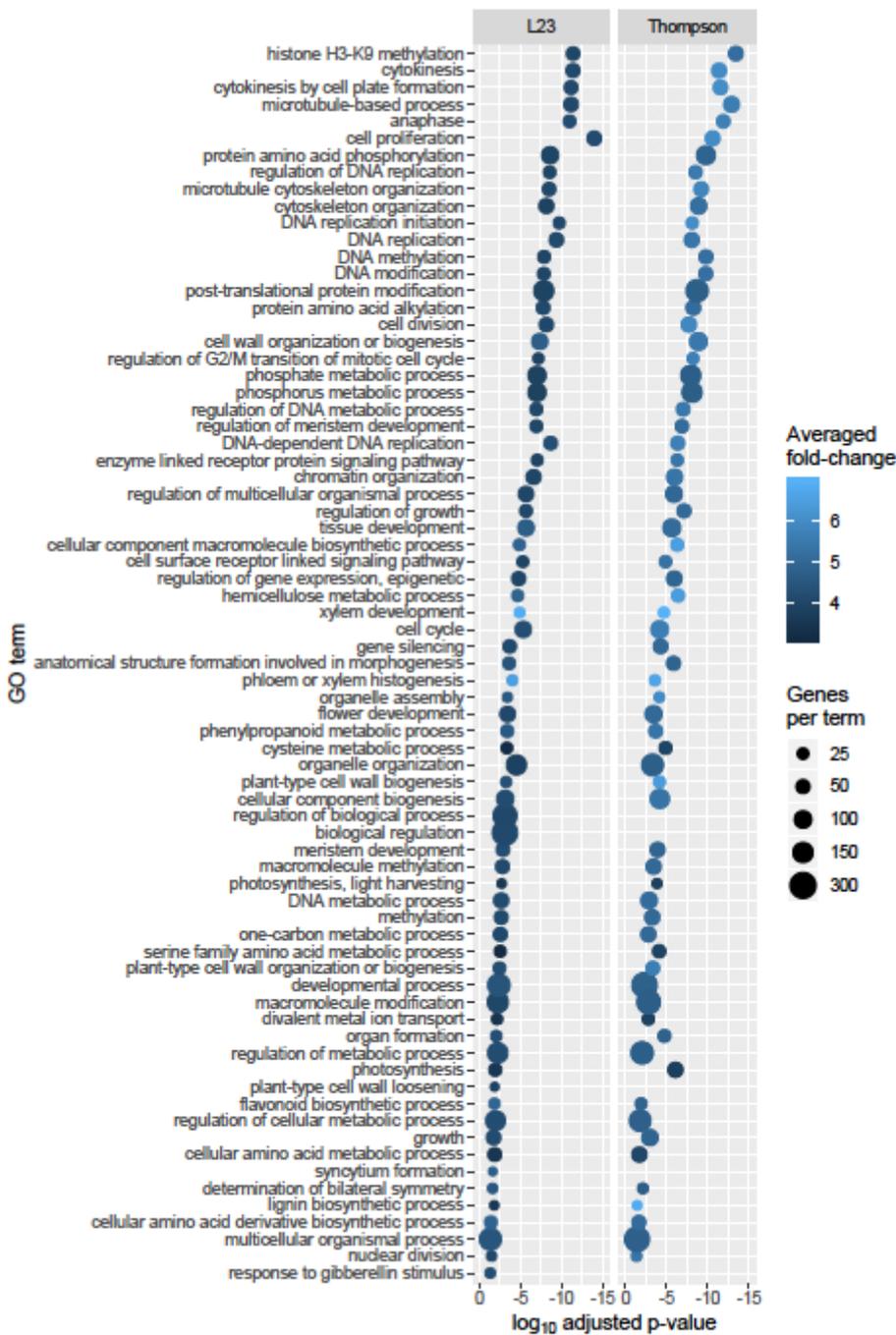


Figure 4

Comparison between genotypes of GO terms enrichment analysis for the upregulated genes response to GA3. A list of the 73 most prominent and least redundant GO accessions obtained from Revigo analysis of L23 (Cutoff value: 0.7) is shown here; this set of GO accessions is compared to the results obtained in cv. Thompson seedless. The \log_{10} adjusted p-value is shown here; the size is relative to the number of query items matching the corresponding GO accession obtained from the single enrichment analysis. The fold-change of every DE gene matching each GO accession by genotype was averaged and is shown according to the scale color gradient depicted in the figure. Input of DE upregulated genes was 1,281 and 1,787 for L23 and cv. Thompson seedless, respectively.

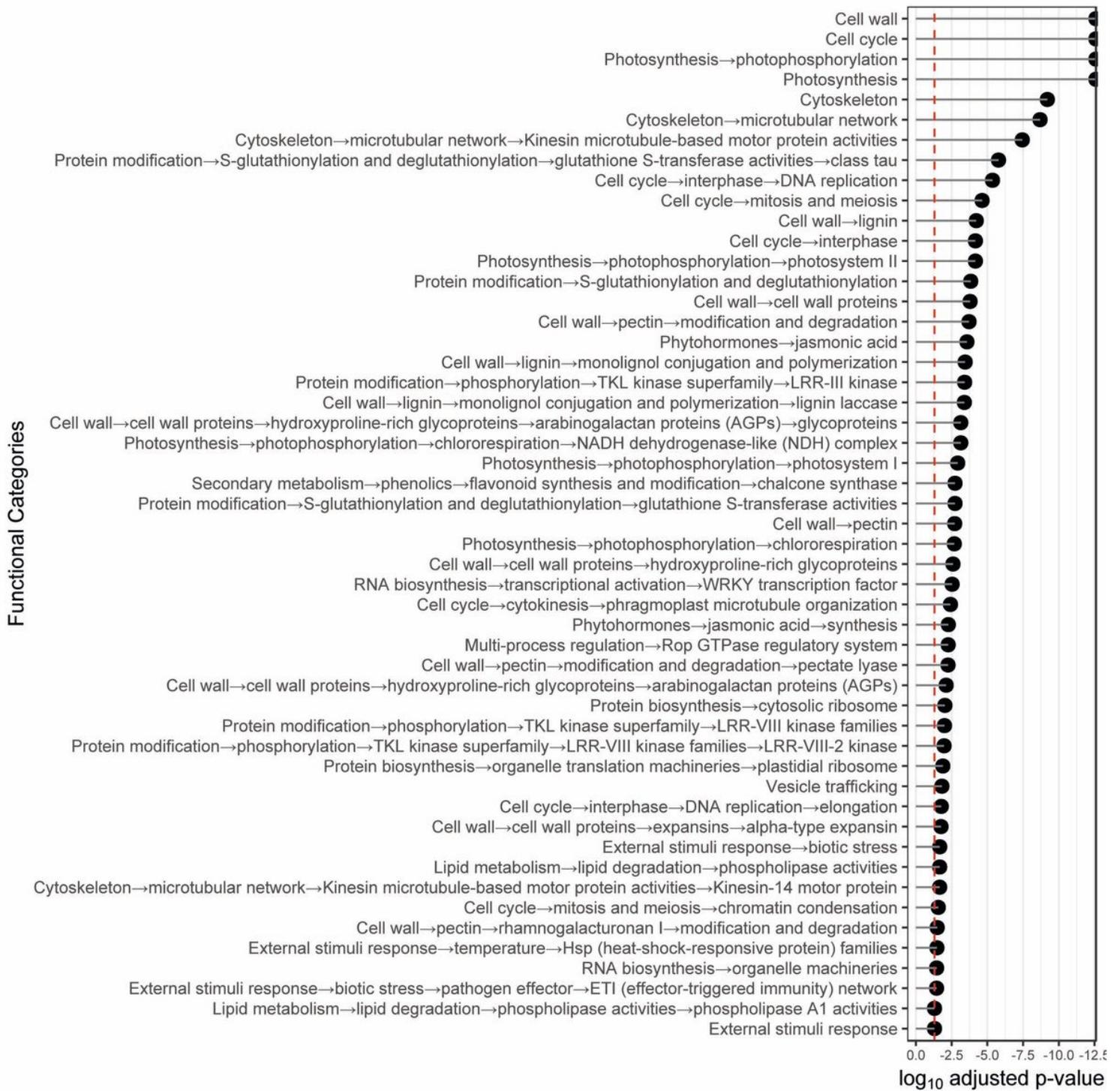


Figure 5

Enriched functional categories in L23 background in response to GA3. All significant categories detected by the Wilcoxon test with p-value adjusted by Benjamini-Yeuketeli are shown on the Cleveland dot plot. Red segmented vertical line depicts threshold significance value (p adjusted value < 0.05). Functional categories were consistent with the results given by the gene ontology analysis and were used as reference in the identification of possible biomarker genes according to the genetic background assessed for the berry drop trait.

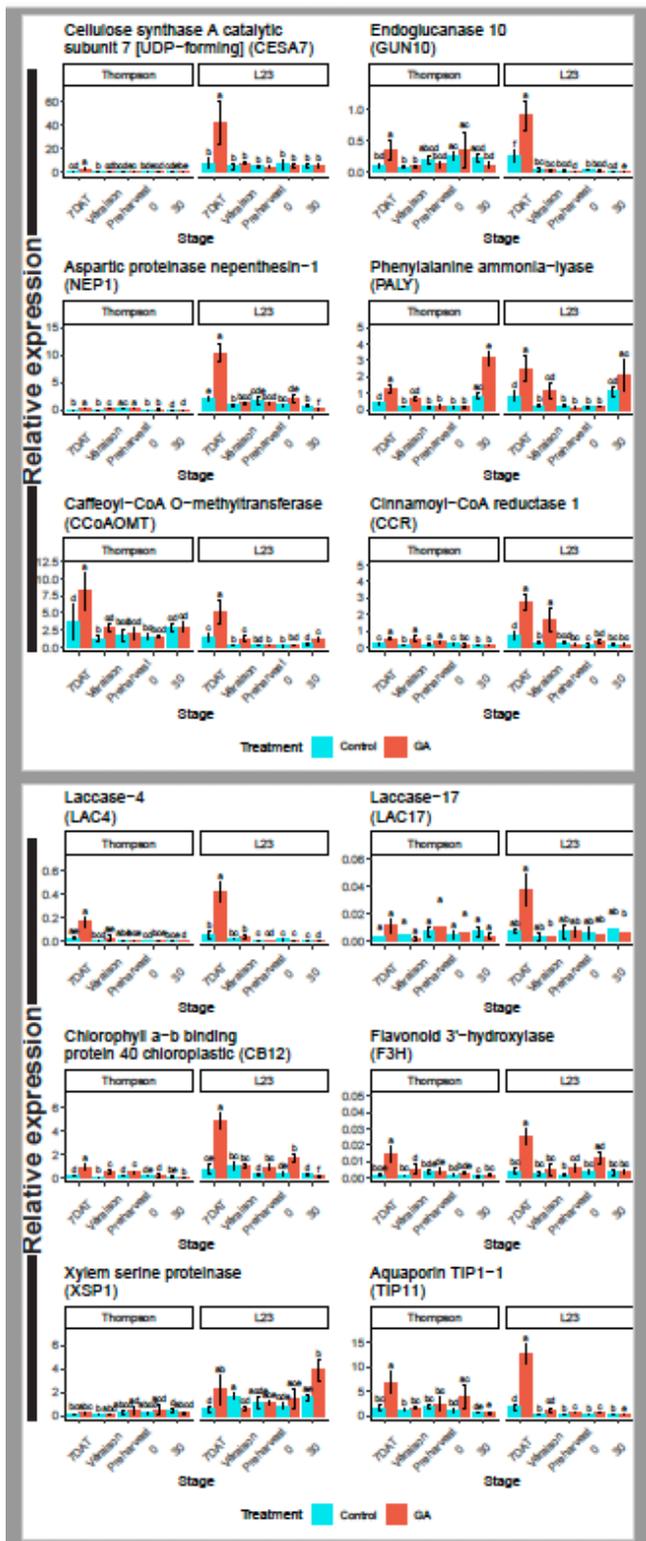


Figure 6

Gene expression changes in genes proposed as candidate biomarkers reveal strong regulation by GA3 treatment. Mean and standard deviation are shown on each bar and error bar, respectively (n=6). The reference gene used in this study was AFLC3 (GSVIVG01011810001 – possible fructose-bisphosphate aldolase 3 chloroplastic), identified from RNAseq data. Common letters on top of each bar indicate no

significant differences between conditions and were identified based on Tukey's post-hoc test on log2-transformed expression data ($p < 0.05$).

Supplementary Files

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