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Proteomic analysis of grape berry skin responding to sunlight exclusion

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ABSTRACT

The most obvious effect of sunlight exclusion from grape clusters is the inhibition of anthocyanin biosynthesis in the berry skin so that no color develops. Two-dimensional gel electrophoresis coupled with mass spectrometry was used to characterize the proteins isolated from berry skins that developed under sunlight exclusion versus those from sunlight-exposed berries. Among more than 1500 spots resolved in stained gels, the accumulation patterns of 96 spots differed significantly between sunlight-excluded berry skin and that of sunlight-exposed control berries. Seventy-two proteins, including 35 down-regulated and 37 up-regulated proteins, were identified and categorized. Proteins involved in photosynthesis and secondary metabolism, especially UDP-glucose:flavonoid 3-*O*-glucosyltransferase (*UFGT*), the key step for anthocyanin biosynthesis in grape berry skin, were accumulated less in the absence of sunlight. Several isoforms of heat shock proteins were also down-regulated. The proteins that were over-accumulated in sunlight-excluded berry skin were more often related to energy production, glycolysis, the tricarboxylic-acid cycle, protein synthesis and biogenesis of cellular components. Their putative role is discussed in terms of their relevance to sunlight exclusion processes.

Introduction

The anthocyanin level in grape berry skin is one of the most important criteria to assess grape quality. Well-colored table grapes owe their consumer appeal to a cultivar-specific color, and wine grapes must have sufficient anthocyanins if used for red wine production. Anthocyanin biosynthesis has been one of the most studied secondary pathways in grape berries. The transcriptional level of the structural genes that encode the enzymes in the pathway appears to primarily regulate anthocyanin biosynthesis (Davies and Schwinn, 2003). Previous studies have concluded that, among structural genes, UDP-glucose:flavonoid 3-*O*-glucosyltransferase (*UFGT*) is critical for anthocyanin biosynthesis in the grape berry (Boss et al., 1996a,b; Kobayashi et al., 2001). The structural genes are activated by a transcription complex composed of *R2R3 MYB*, β helix–loop–helix (*bHLH*, also known as *MYC*), and *WDR* families (Boss and Davies, 2009). The *MYB* family has been the most extensively analyzed and was reported to be involved in the regulation of *UFGT* (Kobayashi et al., 2002), and a retrotransposon-induced mutation in *MYBa1* was shown to be associated with the lack of anthocyanins in white grapes of *Vitis vinifera* (Kobayashi et al., 2004).

Though genetically controlled, anthocyanin biosynthesis in grape berry skin is greatly influenced by one of the most important environmental factors, sunlight (Boss and Davies, 2009). The effects of shading clusters or excluding sunlight from clusters has been extensively documented, with sunlight exclusion generally resulting in inferior color development and decreased anthocyanin concentration in black and red grapes (Jeong et al., 2004; Cortell and Kennedy, 2006). Sunlight exclusion was also found to have a negative effect on the related structural and regulatory genes chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), dihyoflavonol reduetase (*DFR*), leucoanthocyanidin dioxygenase (*LDOX*), *UFGT* and *MYBa1* in berry skin of the black grape 'Cabernet Sauvignon' (Jeong et al., 2004). For 'Lambrusco f.f.' grape seedlings, transcripts for *CHS*, *CHI*, *F3H*, *DFR*, *LDOX* and *UFGT* were present at a very low level in the darkness and dramatically increased after 6 h of irradiation, but phenylalanine ammonia lyase (*PAL*) was constitutively expressed both in darkness and light (Sparvoli et al., 1994).

Despite these insights, previous studies have been more often confined to the level of individual genes or small groups of genes involved in anthocyanin biosynthesis. Via cDNA microarray analysis of 'Shiraz', Waters et al. (2005) showed a group of differentially expressed genes between green and colored berry skins, a group composed of a diverse range of genes with unknown functions.

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Proteomic analysis provides the capacity to objectively analyze many data points at levels that approach the whole proteome. Analysis of the protein composition of grape berries has been used to examine developmental differences as well as environmental effects in grape (Deytieux et al., 2007; Grimplet et al., 2009; Martínez-Esteso et al., 2011). Thus far, no proteomic analysis has been performed to investigate the complex protein variation following sunlight exclusion. In this study, we excluded sunlight from clusters of a red grape from fruit set until maturity, and proteomic profiles were compared at véraison, when anthocyanins sharply accumulated in sunlight-exposed grape berry skin.

Materials and methods

Plant material and treatment

Vines of the red grape 'Jingxiu' (*Vitis vinifera*) grafted on 'Beida' rootstocks planted in 1994 in the experimental vineyard of the Institute of Botany, Chinese Academy of Science were used in 2010. The vines were trained to a trellis, spaced 1.5 m in the row and 2.5 m between rows with a north–south row orientation and maintained under routine cultivation, including irrigation, fertilization, soil management, pruning and disease control.

Vines were selected on the basis of uniformity of shoot growth and cluster development, and shoots were thinned to one cluster per shoot. To exclude sunlight, clusters were enclosed in an opaque black bag with a layer of aluminum foil to ensure ambient gas exchange, reduce the absorbance of solar energy and eliminate any potential temperature difference inside versus outside the bag. Sunlight was excluded from clusters from 20 days after anthesis (DAA) until maturity. During the growing season, the temperature inside and outside the bag was monitored with Temperature and Humidity Data Loggers (ZDR-20h, Zeda, Hangzhou, China). The temperature range within the bag was not significantly different from the ambient external temperature at $P < 0.05$ (±2.0 °C, data not shown). Quantum light sensors were also placed inside the bag, and the opacity coefficient was 99.75%. The clusters exposed to full sunlight throughout the growing season were considered the sunlight exposure control. Three replicates (5 clusters each) of both treatments were sampled every 5–7 days from 40 DAA (about 10 days pre-véraison) until maturity. Berry maturity was based on seed color turning dark brown without senescence of berry tissue, and on previous records and experience. Immediately after each sampling, berry skin was peeled, frozen in liquid nitrogen and stored at −80 °C for anthocyanin measurement and proteomic analysis.

Anthocyanins measurement via HPLC

Anthocyanins were extracted and quantified as described by Liang et al. (2008).

Total protein extraction

Proteomic profiles in berry skin were investigated for the control and sunlight exclusion treatment at 50 DAA, when total anthocyanin showed a sharp accumulation for the control, but no increase in sunlight-excluded berry skin. The frozen berry skin (4 g) was ground into a fine powder with liquid nitrogen using a pre-cooled pestle and mortar, and 12 ml of extraction buffer (0.1 M Tris–HCl pH 7.5, 5 mM EDTA, 10 mM PMSF, 2% β-mercaptoethanol, 0.1 M KCl, 0.7 M sucrose, 1% PVPP) was added to the powder (Deytieux et al., 2007). The mixture was vortexed for 30 s and suspended at 4 °C for 1 h. An equal volume of phenol–Tris–HCl at pH 7.5 was added, and then the mixture was vortexed for 30 s and suspended at −20 °C for 1 h. The homogenate was centrifuged (30 min, 10 000 × *g*, 4 °C) and the phenol phase was collected. The aqueous phase was re-extracted with 4 ml of extraction buffer and 4 ml of phenol–Tris–HCl at pH 7.5, vortexed for 30 s, suspended for 30 min and centrifuged. The supernatants were combined. Then, the extracted proteins were precipitated by adding five volumes of ice-cold 0.1 M ammonium acetate in methanol to the phenol phase, vortexing, and incubating at −20 °C overnight. The next day, the precipitated proteins were recovered by centrifugation (30 min, 35 000 × *g*, 4 °C) and the pellets were washed once with ice-cold 0.1 M ammonium acetate in methanol, once with ice-cold methanol, once with ice-cold acetone, and finally, once with ice-cold 80% acetone. The final pellet was dried under vacuum, and dissolved in 800 μL IEF solubilization buffer [7 M urea, 2 M thiourea, 40 mM DTT, 2% (w/v) CHAPS, 2% (v/v) IPG buffer, 1% (w/v) Triton X-100] by vortexing for 30 s and incubating for 4 h at room temperature. The resulting mixture was centrifuged (20 min, 40 000 × *g*, 4 °C), and the supernatant was transferred to a new tube. Two independent protein extractions for each of three biological replicates were combined. The total protein concentration was determined according to the Bradford method (Bradford, 1976) using a protein-dye reagent (Bio-Rad) and bovine serum albumin (BSA) as a standard.

Two-dimensional electrophoresis

Each biological replicate was run. Isoelectrofocusing (IEF) was carried out with 1200 μg of total protein extract using an IPG phor II electrophoresis system at 20 °C with a current limit of 50 μA/strip and immobiline dry strips (24 cm-long), a linear pH gradient of 4–7. Protein was loaded during the passive rehydration step for 16 h. IEF was then performed with the following settings: 2 h at 150 V, 1 h at 500 V, a 5 h gradient at 1000 V, a 3 h gradient at 8000 V and 5 h at 8000 V. Prior to second dimension electrophoresis, the gel strips were equilibrated for 15 min in an equilibration buffer containing 0.05 M Tris–HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 0.125% (w/v) DTT, followed by another 15 min equilibration in the same buffer containing 125 mM iodoacetamide without DTT as described by Sheoran et al. (2006). After equilibration, the strips were applied to vertical SDS-PAGE (12.5% acrylamide resolving gels) at 50 V for 1 h and then 200 V for 10 h at 15 °C until the bromophenol blue ran off using a ETTAN DALTsix apparatus. Protein markers were loaded besides the strips before sealing. All the materials and instruments were from GE Healthcare, Amersham Biosciences (Little Chalfont, UK).

Protein staining and analysis of 2D gels

After second dimension electrophoresis, gels were stained with colloidal Coomassie brilliant blue (CBB R-250). The stained gels were scanned at a 300 dpi resolution with a UMAX Power Look 2100XL Scanner (Maxium Technologies, Taipei, China). Image and data analysis of the scanned gels were performed using the Image Master 2D Platinum, version 5.01 (GE Healthcare Amersham Biosciences), which allows spot detection, quantification, background subtraction, and spot matching among multiple gels. Automatic matching by the software was complemented by manual matching, and the relative volume of each spot was calculated and normalized against total spot volume. Only spots whose presence was detectable in at least two gels out of the total three biological replicates and whose relative volume was >0.1% in at least two gels were considered acceptable. Relative volumes of the three biological replicated gels between the control and sunlight exclusion treatment were compared, and were statistically evaluated using Student's *t*-test to verify whether they were significantly different ($P < 0.05$). Only spots with relative volumes that varied significantly by at least a ratio of 2 were sequenced.

In-gel digestion and MALDI-TOF MS analysis

Protein spots were excised from the CBB stained 2D-gels. The proteins were then digested as follows: each small gel fragment was washed three times with MilliQ water (Millipore) and then destained with solution containing 50 mM NH_4HCO_3 and 50% v/v acetonitrile (ACN) for 20 min at 37 °C. The destaining step was repeated until the gel fragment was colorless. The gel fragments were subsequently dehydrated and dried by 100% ACN. They were rehydrated with 0.01 μg/μL sequencing grade modified trypsin (Roche) in 25 mM NH_4HCO_3 at 4 °C for 1 h, and then submitted to overnight in-gel digestion in the same solution at 37 °C. The next day, the protein peptides were collected, and the gels were washed with 0.1% TFA in 50% ACN three times to collect the remaining peptides. The peptide solution was concentrated to 10 μL and co-crystallized with one volume of saturated CHCA in 50% (v/v) ACN containing 1% TFA. Tryptic peptide masses were measured with a MALDI-TOF mass spectrometer (SM, Shimadzu Biltech, Kyoto, Japan).

Protein identification and functional classes

The resulting PMF data were searched against the NCBI database using the MASCOT software available at (http://www.matrixsciende.com). Viridiplantae (Green plants) was chosen as the taxonomic category. The search parameters were as follows: modifications were carbamidomethyl and oxidation; the enzyme was trypsin; the maximum number of missed cleavages was 1 and 100 ppm mass endurance. To determine the confidence of the identification results, the following criteria were used: in addition to a minimum of 50 in the MOWSE score, sequence coverage of the protein was no less than 12% of the matching peptides. Only the best matches with high confidence levels were selected (Yang et al., 2007). Theoretical mass and p*I* of identified proteins were calculated from sequence data with the ExPASy Compute p*I*/Mw tool at http://www.expasy.org/tools/pi_tools.html. The identified proteins were assigned a Gene Ontology (http://www.geneontology.org) (GO) term according to their molecular function, and were grouped into functional categories using the 'GO-MIPS funcat conversion table' (http://geneontology.org/external2go/mips2go) set up at the Munich Information Center for Protein Sequences (MIPS Institute). In some cases, where the GO term assigned to the protein appeared too broad, proteins were assigned to MIPS funcats (http://mips.helmholtz-muenchen.de/proj/funcatDB/) according to their roles described in the literature.

Results and discussion

Seasonal changes of total anthocyanin concentration

Before véraison (40 and 45 DAA), there was no significant difference in total anthocyanin concentration between the control and sunlight exclusion treatment (Fig. 1). Starting at 45 DAA, the total anthocyanin concentration in berry skin exposed to sunlight sharply increased followed by a gradual and continuous increase throughout berry ripening. However, the total anthocyanin concentration maintained a very low level throughout the berry ripening period in sunlight excluded berry skin. At maturity, the total anthocyanin concentration of control berry skin reached 74.90 mg/kg FW. In contrast, sunlight-excluded berry skin did not show any increase in total anthocyanin (1.36–5.99 mg/kg FW). These findings are in accordance with the results of Jeong et al. (2004) and Cortell and Kennedy (2006), i.e., excluding sunlight suppressed anthocyanin biosynthesis.

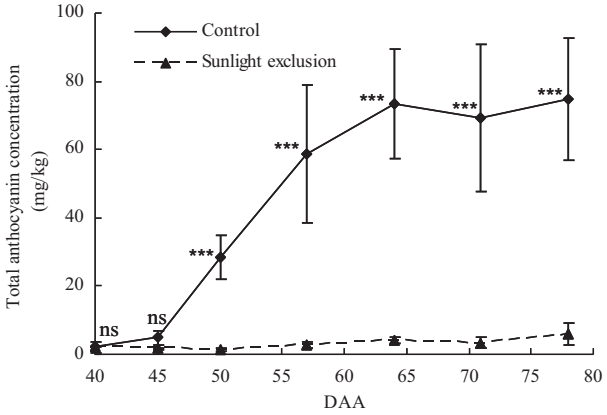

Fig. 1. Dynamic changes of total anthocyanin concentration in 'Jingxiu' berry skin exposed to sunlight (control) and excluded from sunlight (sunlight exclusion) from 20 days after anthesis (DAA) until maturity during grape berry development. Bars represent standard deviation. ns and *** indicate no difference or a significant difference at $P < 0.001$ via *t*-test between the control and sunlight exclusion on each date.

Comparative analysis of proteome profiles

Véraison is known to be a critical period for a grape berry, representing the transition from berry growth to berry ripening, and many changes in berry development start at véraison. Sugar and color accumulate rapidly, and the concentration of organic acids declines (Harris et al., 1968). In this study, proteomic analysis was performed at véraison (50 DAA), when the sharp increase in anthocyanins occurred in berry skin exposed to sunlight but did not in sunlight-excluded berry skin.

Three two-dimensional electrophoresis gels were prepared for the control and sunlight exclusion treatment (Fig. S1). Each gel was stained with colloidal blue, then scanned and analyzed. One thousand to 1500 spots were visible on the gels. Spots, whose presence was detectable in at least two gels out of the total three biological replicates, whose relative volume was >0.1% in at least two gels, and were in at least a 2:1 ratio significantly between the control and sunlight exclusion treatment, were chosen for identification. Ratios were calculated using the average volume of the spots on the three gels. A total of 96 protein spots showed a modified accumulation pattern between the control and sunlight exclusion treatment. Compared to the control, the number of down-regulated proteins (46) in sunlight-excluded berry skin was similar to that of up-regulated proteins (50). These 96 differentially displayed spots were excised from the gels manually and subjected to in-gel digestion to identify them through MS analysis and database searches. Among them, 72 proteins in the represented gels (Fig. 2) were successfully identified and categorized, and included 35 down-regulated and 37 up-regulated proteins (Table 1, Fig. 3).

Supplementary material related to this article found, in the online version, at doi:10.1016/j.jplph.2012.12.020.

Proteins involved in metabolism and energy

It is widely acknowledged that anthocyanins are secondary metabolites and are part of the large phenolic family collectively known as the flavonoids (Holton and Cornish, 1995). Two isoforms of *UFGT* (6, 7) involved in secondary metabolism were down-regulated by sunlight exclusion. In grape berry skin, only the *UFGT* step was regulated independently of the other structural genes of the anthocyanin biosynthetic pathway (Boss et al., 1996a; Kobayashi et al., 2001), suggesting the coloration of grape berry skin was determined at the *UFGT* step. The mRNAs of *CHS*, *CHI*, *F3H*, *DFR* and *LDOX*, except *UFGT*, accumulated in the early

Table 1
Proteins differentially accumulated in sunlight excluded 'Jingxiu' berry skin compared to sunlight exposed control.

Regulated [a]	Function category[b]	Accession no.	Protein name	Organism	% coverage	Score	Theo[c] mass (kDa)/p*I*	Exp[c] mass (kDa)/p*I*	Relative volume (%)		Spot no.[e]
									Control[d]	SE[d]	
Secondary metabolism											
–	01.20	gi|13620859	UDP-glucose:flavonoid 3-O-glucosyltransferase	*Vitis vinifera*	38	144	50/5.98	54/6.28	0.10	ND[f]	6
–	01.20	gi|13620855	UDP-glucose:flavonoid 3-O-glucosyltransferase	*Vitis labrusca* × *Vitis vinifera*	29	71	51/5.87	53/6.06	0.03	ND	7
C-compounds and carbohydrate metabolism											
–	01.05	NP_001148959.1	NAD-dependent epimerase/dehydratase	*Zea mays*	52	133	28/5.85	27/5.85	0.58	0.10	5
–	01.05	NP_001148959.1	NAD-dependent epimerase/dehydratase	*Zea mays*	49	138	28/5.85	27/5.79	0.19	0.09	20
–	01.05	XP_002519002.1	Phosphoribulose kinase	*Ricinus communis*	27	67	46/5.83	46/5.26	0.03	0.01	14
+	01.05	AAB47171.1	Vacuolar invertase 1, GIN1	*Vitis vinifera*	15	62	61/4.45	68/4.58	0.01	0.19	40
+	01.05	gi|1839578	Vacuolar invertase 1, GIN1	*Vitis vinifera*	16	82	72/4.60	67/4.54	0.02	0.06	41
Amino acid metabolism											
+	01.01	XP_002510270.1	Acylamino-acid-releasing enzyme	*Ricinus communis*	28	99	76/5.34	81/5.46	ND	0.01	37
+	01.01	BAH89272.1	Serine carboxypeptidase-like acyltransferase	*Diospyros kaki*	18	57	54/5.46	30/4.73	ND	0.05	65
Glycolysis and gluconeogenesis											
+	02.01	gi|209917124	2,3-Bisphosphoglycerate-independent phosphoglycerate mutase	*Vitis amurensis*	27	79	34/5.45	71/5.67	0.02	0.09	48
+	02.01	XP_002510526.1	Hexokinase	*Ricinus communis*	33	56	45/5.03	56/5.14	ND	0.03	57
Tricarboxylic-acid pathway											
+	02.10	ACF17659.1	3-Isopropylmalate dehydrogenase small subunit	*Capsicum annuum*	45	85	27/7.04	18/5.31	ND	0.02	45
+	02.10	gi|113170491	NADH dehydrogenase subunit 9	*Ostreococcus tauri*	44	56	22/6.60	37/4.61	ND	0.02	62
Photosynthesis											
–	02.30	BAA96365.3	Oxygen evolving enhancer protein 1 precursor	*Bruguiera gymnorhiza*	44	93	35/6.77	30/5.32	0.06	0.03	2
–	02.30	ADZ75466.1	Oxygen evolving enhancer protein 1	*Litchi chinensis*	53	178	33/5.87	32/5.36	0.53	0.26	17
–	02.30	XP_002523404.1	Rubisco subunit binding-protein beta subunit	*Ricinus communis*	41	123	65/5.80	67/5.44	0.04	ND	11
Fermentation											
–	02.16	gi|7264740	Alcohol dehydrogenase 6	*Vitis vinifera*	42	97	42/5.85	49/6.18	0.05	0.02	1
–	02.16	XP_002511512.1	Short chain alcohol dehydrogenase	*Ricinus communis*	48	88	28/5.74	26/5.80	0.13	0.02	4
+	02.16	ACN87275.1	Short chain dehydrogenase/reductase	*Nandina domestica*	55	134	33/5.42	31/5.35	ND	0.03	66
Transcription											
–	11	XP_002510403.1	Pentatricopeptide (PPR) repeat-containing protein	*Ricinus communis*	17	62	82/5.24	15/4.96	0.06	0.01	25
–	11	NP_193101.2	Pentatricopeptide (PPR) repeat-containing protein	*Arabidopsis thaliana*	27	71	47/7.16	13/5.52	0.17	ND	33
+	11	XP_002510403.1	Pentatricopeptide (PPR) repeat-containing protein	*Ricinus communis*	18	65	82/5.24	29/5.25	ND	0.01	67

Table 1 (*Continued*)

Regulated [a]	Function category[b]	Accession no.	Protein name	Organism	% coverage	Score	Theo[c] mass (kDa)/p*I*	Exp[c] mass (kDa)/p*I*	Relative volume (%)		Spot no.[e]
									Control[d]	SE[d]	
Protein fate											
–	14	P30236.1	22.0 kDa class IV heat shock protein precursor	*Glycine max*	43	105	21/5.89	17/5.76	0.10	0.03	10
–	14	P30236.1	22.0 kDa class IV heat shock protein precursor	*Glycine max*	41	60	21/5.89	17/6.05	0.06	ND	27
–	14	NP_175665.1	26.5 kDa heat shock protein, mitochondrial	*Arabidopsis thaliana*	40	77	26/7.78	24/5.65	0.21	0.04	21
–	14	gi|269838634	Small heat shock protein 17.1 kDa	*Vitis vinifera*	33	77	17/5.80	14/5.80	1.03	0.24	35
–	14	XP_002521274.1	Heat-shock protein	*Ricinus communis*	42	91	21/5.89	17/6.30	0.31	0.13	28
+	14	XP_002513744.1	Protein disulfide isomerase	*Ricinus communis*	35	150	56/4.93	70/4.98	0.02	0.04	43
+	14	gi|225470846	Nascent polypeptide associated complex alpha	*Vitis vinifera*	34	68	22/4.34	27/4.53	0.01	0.04	64
+	14	XP_002516232.1	GroES chaperonin	*Ricinus communis*	50	71	26/8.96	24/5.44	ND	0.04	71
Protein synthesis											
–	12	gi|225452282	Elongation factor Tu, chloroplastic	*Vitis vinifera*	45	143	53/6.41	49/5.67	0.05	0.01	13
+	12	ABI84255.1	Translationally controlled tumor-like protein	*Arachis hypogaea*	42	60	18/4.63	16/4.53	0.01	0.05	44
+	12	O65751.1	40S ribosomal protein SA	*Cicer arietinum*	50	159	34/5.43	47/5.55	0.03	0.10	59
+	12	O65751.1	40S ribosomal protein SA	*Cicer arietinum*	33	84	34/5.43	47/5.58	0.01	0.03	60
Protein activity regulation											
–	18	ADB07168.1	DEAD-box RNA helicase-like protein	*Prunus persica*	61	181	47/5.38	55/5.53	0.02	ND	12
+	18	Q6L3H0	Putative receptor kinase	*Vitis vinifera*	18	53	38/9.37	88/5.13	ND	0.01	36
Protein with binding function or cofactor requirement											
+	16	ZP_04631332.1	Iron-sulfur cluster-binding protein	*Yersinia frederiksenii* ATCC 33641	69	64	3/10.83	33/4.63	ND	0.01	63
Cellular transport, transport facilitation and transport routes											
+	20	AAD03392.1	Mitochondrial ATPase beta subunit	*Nicotiana sylvestris*	50	171	59/5.90	61/5.23	0.04	0.11	47
+	20	XP_002511778.1	Arsenical pump-driving ATPase	*Ricinus communis*	36	79	40/4.76	48/4.97	0.01	0.02	54
Cell cycle and DNA processing											
–	10	gi|30697295	ADF3 (actin depolymerizing factor 3)	*Arabidopsis thaliana*	71	72	16/5.93	41/5.13	0.11	0.05	16
Cell rescue, defense and virulence											
–	32	XP_002524812.1	Endo-1,3(4)-beta-D-glucanase	*Ricinus communis*	45	75	26/5.55	26/5.53	0.04	0.01	3
–	32	Q9SEC2.1	Methionine sulfoxide reductase	*Lactuca sativa*	47	82	23/6.30	24/6.58	0.03	0.01	9
+	32	gi|225431844	Pathogenesis-related protein 10	*Vitis vinifera*	70	192	17/5.95	15/6.36	0.45	1.29	46
Cellular communication/signal transduction mechanism											
+	30	XP_002524007.1	Phospholipase C3 precursor	*Ricinus communis*	25	114	60/5.71	65/5.69	ND	0.04	50
Cell fate											
+	40	XP_002525369.1	Nucleoredoxin	*Ricinus communis*	35	164	66/4.98	74/5.13	0.01	0.04	38
+	40	XP_002525369.1	Nucleoredoxin	*Ricinus communis*	48	216	66/4.98	74/5.19	0.02	0.15	39
Biogenesis of cellular component											
+	42	gi|255538950	Tubulin beta chain	*Ricinus communis*	37	178	51/4.75	60/5.09	0.05	0.17	52
+	42	gi|224056825	Tubulin beta chain	*Populus trichocarpa*	45	184	51/4.76	60/5.12	0.05	0.11	53
+	42	gi|15222873	Tubulin beta	*Arabidopsis thaliana*	42	214	51/4.68	60/5.00	ND	0.03	51
+	42	gi|32186896	Actin	*Gossypium hirsutum*	54	217	42/5.44	52/5.05	0.01	0.06	56
+	42	gi|2253219	Actin 2	*Podocarpus macrophyllus*	57	177	37/5.36	51/5.17	0.02	0.04	58

Table 1 (*Continued*)

Regulated [a]	Function category[b]	Accession no.	Protein name	Organism	% coverage	Score	Theo[c] mass (kDa)/p*I*	Exp[c] mass (kDa)/p*I*	Relative volume (%)		Spot no.[e]
									Control[d]	SE[d]	
Unknown function											
–	Unknown	gi|159472342	Predicted protein	*Chlamydomonas reinhardtii*	29	62	32/8.57	24/5.96	0.12	0.02	8
–	Unknown	gi|224140303	Predicted protein	*Populus trichocarpa*	71	59	13/4.97	15/5.02	0.22	0.11	26
–	Unknown	gi|303289367	Predicted protein	*Micromonas pusilla* CCMP1545	9	52	140/8.56	12/4.87	0.03	ND	31
–	Unknown	gi|168022278	Predicted protein	*Physcomitrella patens* subsp. *patens*	36	65	29/8.80	13/5.48	0.12	0.02	32
–	Unknown	gi|242117507	Hypothetical protein	*Oryza sativa* Indica Group	20	61	35/12.10	22/5.05	0.03	ND	22
–	Unknown	gi|302758992	Hypothetical protein SELMODRAFT_78306	*Selaginella moellendorffii*	18	63	93/6.03	24/5.27	0.01	ND	19
–	Unknown	gi|242074838	Hypothetical protein SORBIDRAFT_06g033510	*Sorghum bicolor*	61	65	18/5.52	22/5.15	0.26	0.11	23
–	Unknown	gi|242057429	Hypothetical protein SORBIDRAFT_03g016730	*Sorghum bicolor*	13	66	86/6.37	16/6.22	0.05	ND	29
–	Unknown	gi|297725675	Os07g0486000	*Oryza sativa* Japonica Group	28	53	28/8.76	48/5.32	0.04	0.02	15
–	Unknown	gi|238011182	Unknown	*Zea mays*	37	52	16/12.33	21/6.33	0.07	ND	24
–	Unclassified	gi|13509221	N1-D protein	*Linum usitatissimum*	13	61	127/6.33	28/5.10	0.41	0.12	18
–	Unclassified	gi|144601014	Self-incompatibility RNase	*Sorbus aucuparia*	41	60	22/9.39	14/5.34	0.09	ND	30
–	Unclassified	NP_849875.1	MLP28 (MLP-LIKE PROTEIN 28)	*Arabidopsis thaliana*	32	65	35/6.64	13/5.56	0.17	0.03	34
+	Unknown	gi|326494016	Predicted protein	*Hordeum vulgare subsp. vulgare*	24	55	37/9.17	26/5.33	ND	0.02	70
+	Unknown	gi|302846015	Hypothetical protein VOLCADRAFT_106419	*Volvox carteri f. nagariensis*	25	53	38/8.77	65/4.83	ND	0.01	42
+	Unknown	gi|302814505	Hypothetical protein SELMODRAFT_427612	*Selaginella moellendorffii*	21	52	61/6.65	22/4.75	ND	0.03	69
+	Unknown	gi|302789191	Hypothetical protein SELMODRAFT_416278	*Selaginella moellendorffii*	24	58	58/6.84	22/5.22	ND	0.03	72
+	Unknown	gi|125524127	Hypothetical protein OsI_00094	*Oryza sativa* Indica Group	21	67	11/6.25	63/5.62	ND	0.04	49
+	Unknown	gi|297600719	Os03g0277300	*Oryza sativa* Japonica Group	29	76	44/4.88	51/5.02	ND	0.01	55
+	Unknown	gi|116780837	Unknown	*Picea sitchensis*	43	50	18/5.89	38/4.50	ND	0.03	61
+	Unclassified	XP_002524839.1	Carboxymethylenebutenolidase	*Ricinus communis*	38	73	26/5.04	26/4.94	0.01	0.05	68

[a] Proteins were up-regulated (+) or down-regulated (–) in sunlight-excluded 'Jingxiu' berry skin compared to a sunlight-exposed control.

[b] Function category was based on the 'GO-MIPS funcat conversion table' set up at the Munich Information Center for Protein Sequences (MIPS Institute).

[c] Theo and exp represented theoretical and experimental mass (kDa)/p*I*.

[d] Control, sunlight exposure control; SE, sunlight exclusion treatment.

[e] Spot numbers identified from 2D gel presented in Fig. 2.

[f] ND, not detectable.

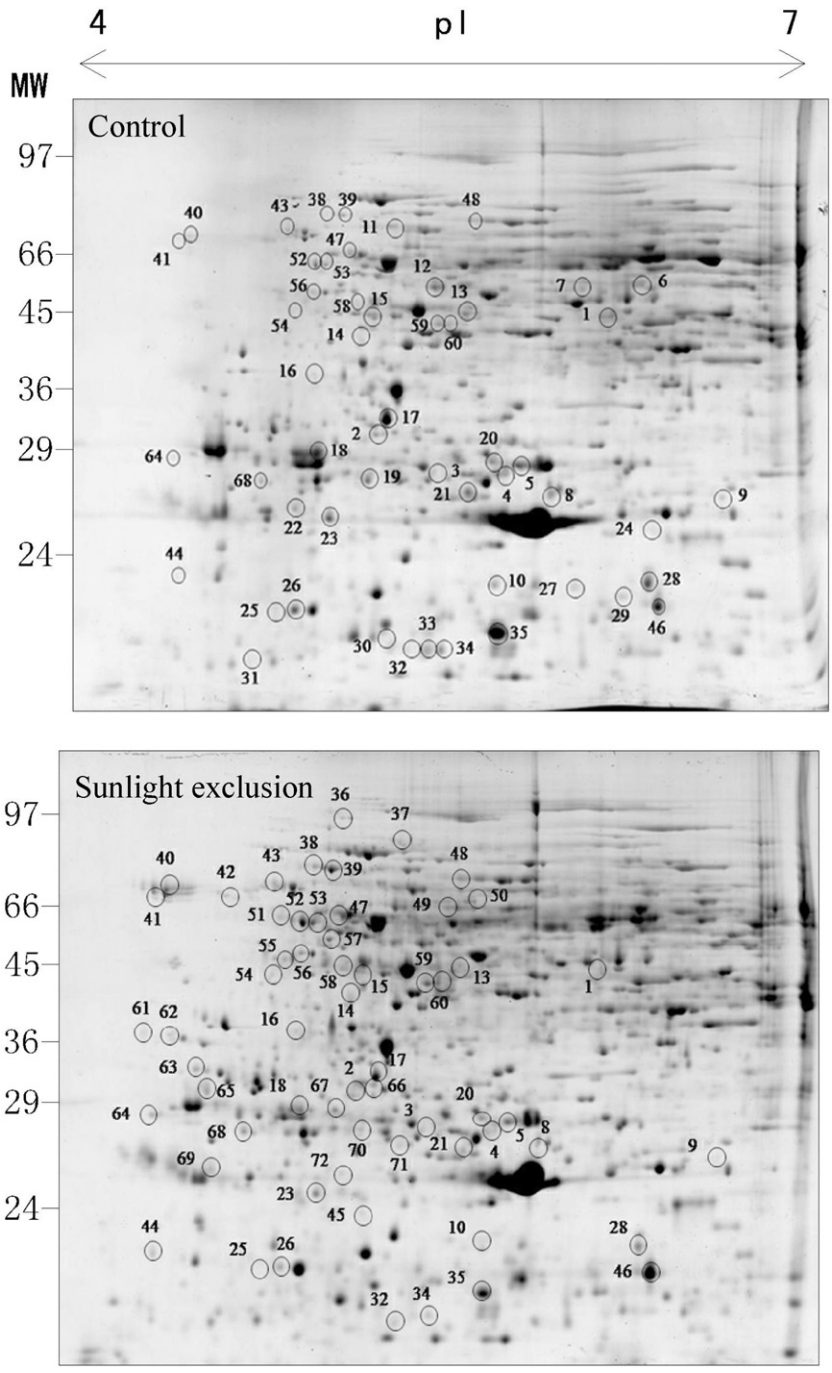

Fig. 2. 2D PAGE analysis of the proteins in 'Jingxiu' berry skin exposed to sunlight (control) and excluded from sunlight (sunlight exclusion) at véraison (50 days after anthesis). The differentially accumulated proteins between control and sunlight exclusion are indicated by circles and standard spot numbers on a representative gel. See Table 1 for a detailed list of proteins.

developmental stage and decreased until véraison, and then the mRNA levels of all the genes including *UFGT* increased in the coloring stage of red grape cultivars (Boss et al., 1996a). The mRNA of *UFGT* was also detected only in the berry skin of red cultivars (Boss et al., 1996b; Kobayashi et al., 2001). However, Jeong et al. (2004) showed that cluster shading decreased the transcription of not only *UFGT*, but also *CHSs*, *CHIs*, *F3Hs*, *DFR*, *LDOX* in 'Cabernet Sauvignon' grape. For 'Lambrusco f.f.' grape seedlings, transcripts for *CHS*, *CHI*, *F3H*, *DFR*, *LDOX* and *UFGT* were present at a very low level in darkness and all dramatically increased after 6 h of irradiation induction, except that *PAL* was constitutively expressed both in darkness and light (Sparvoli et al., 1994). In this study, UFGT proteins were not detectable in sunlight-excluded berry skin, corresponding to low total anthocyanin concentration and the absence of the red color. Among the anthocyanin biosynthetic enzyme genes, only *UFGT* was found to negatively respond to sunlight exclusion, which may indicate that the other genes were not, or were much less, affected by sunlight exclusion in 'Jingxiu' berry skin at the protein level.

NAD-dependent epimerase/dehydratase (5, 20) utilizes NAD as a cofactor and nucleotide-sugar as substrates for a variety of chemical reactions (Thoden and Holden, 2005). Phosphoribulose kinase (14) participates in carbon fixation. Rubisco (11) is a well-known enzyme involved in the first major step of carbon fixation, and is probably the most abundant protein on earth. Oxygen-evolving

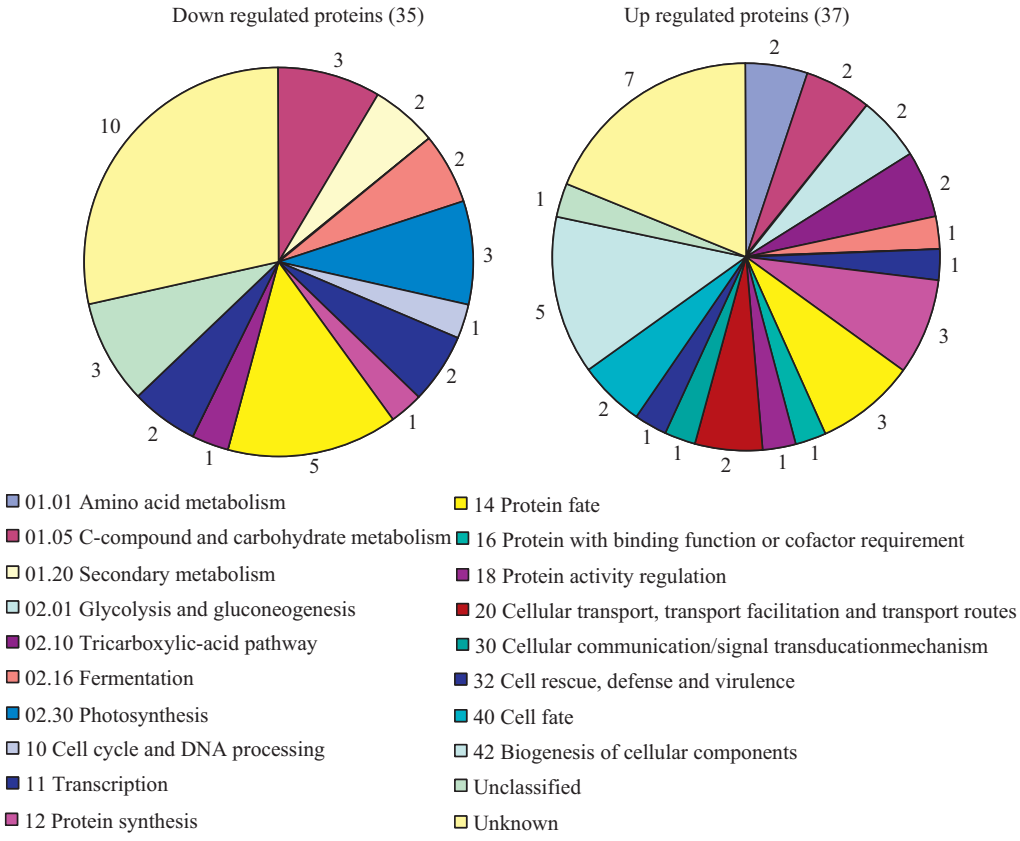

Fig. 3. Functional categorization of the differentially accumulated proteins in sunlight-excluded 'Jingxiu' berry skin compared to sunlight-exposed berry skin at véraison (50 days after anthesis), showing the number of proteins belonging to each category.

enhancer proteins (2, 17) are auxiliary components of the photosystem II manganese cluster (Heide et al., 2004). These proteins, involved in carbohydrate metabolism and photosynthesis, were accumulated significantly less in sunlight-excluded berry skin compared to the control. Deytieux et al. (2007) found that proteins involved in photosynthesis and carbohydrate metabolism were accumulated less at the end of color change (100% red) than at its onset (10% red), suggesting that the berry skin is a source of carbohydrates for the skin itself at the onset of color. In this study, the absence of sunlight would have reduced berry skin photosynthesis, and subsequently resulted in low carbohydrate metabolism, though chlorophyll pigment levels were still high in berry skin. No proteins involved in secondary metabolism and photosynthesis were up-regulated in sunlight-excluded berry skin compare to the control. However, among enzymes of carbohydrate metabolism, vacuolar invertase (40, 41), which can break down sucrose into glucose and fructose, was more abundant in sunlight-excluded berry skin, which might accelerate the accumulation of glucose and fructose in the approach to berry maturity in sunlight-excluded berries.

Amino acid metabolism, glycolysis and the tricarboxylic-acid pathway were more active in sunlight-excluded berry skin. For example, acylamino-acid-releasing enzyme (37) and serine carboxypeptidase-like acyltransferase (65) participate in amino acid metabolism; 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (48) is an enzyme of the glycolytic pathway that catalyses the interconversion of 3-phosphoglycerate and 2-phosphoglycerate (Grana *et al.*, 1992); hexokinase (57) mostly uses glucose as a important substrate and produces glucose-6-phosphate; 3-isopropylmalate dehydrogenase (45) and NADH dehydrogenase subunit 9 (62) take part in the tricarboxylic-acid pathway, and the latter is the first enzyme of the mitochondrial electron transport chain; ATPase (47, 54) is essential in the dephosphorylation reaction for releasing energy, though they were classified into cellular transport, transport facilitation and transport routes here (function 20). The enhancement of these proteins might benefit for compensating the loss of energy caused by the absence of sunlight.

In the group of fermentation proteins, three isoforms of short chain dehydrogenase/reductase (1, 4, 66, called alcohol dehydrogenases also) were identified. Alcohol dehydrogenases catalyze a reaction as part of fermentation to ensure a constant supply of NAD^+ (Thompson et al., 2010). In this study, these were either accumulated less (spots 1 and 4) or over-accumulated (spot 66) in sunlight-excluded berry skin.

Proteins involved in transcription

Many transcription factors belonging to *R2R3 MYB*, β helix–loop–helix (*bHLH*, also known as *MYC*), and *WDR* have been reported to be correlated with anthocyanin biosynthesis in grape berry skin, and some significantly respond to cluster shading (Sparvoli et al., 1994; Jeong et al., 2004). Specially, *MYBa* is closely related with the expression of *UFGT* (Kobayashi et al., 2002). However, none of them were differentially accumulated between the control and sunlight exclusion treatment, probably because transcription factors could function at low levels so that proteomic analysis would not detect differences. Only three pentatricopeptide (PPR) repeat-containing proteins (25, 33, 67) were observed in the transcription group, and they were either up- or down-regulated by sunlight exclusion in berry skin. Pentatricopeptide repeat (PPR) proteins are a huge family in plants (450 members in *Arabidopsis* and 477 in rice), and have been shown to play crucial roles in virtually all stages of organellar gene expression (O'Toole et al., 2008).

Their roles in the response of berry skin to sunlight exclusion need study.

Proteins involved in protein synthesis, fate, and activity regulation and proteins with binding function

Five proteins were identified as heat shock proteins (10, 21, 27, 28, 35). Heat shock proteins can stabilize proteins and are involved in the folding of denatured proteins. They have generally been found to accumulate when plants are exposed to elevated temperatures, abscisic acid, water stress or other stresses (Hu et al., 2010). Abscisic acid has a well-known positive relationship with grape ripening, especially anthocyanin synthesis (Peppi et al., 2008). In this study, heat shock proteins were less accumulated in sunlight-excluded berry skin, probably due to less abscisic acid or less thermal radiation. Two other proteins related to protein synthesis and regulation were less accumulated, the elongation factor thermo unstable (EF-Tu, 13) protein, which plays a role in protein biosynthesis through promoting the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes, and the DEAD box helicase-like protein (12), which is a family of proteins whose purpose is to unwind nucleic acids (Aubourg et al., 1999).

Comparatively, the other proteins related to protein synthesis, fate, and activity regulation, and the proteins related to binding function, were up-regulated in sunlight-excluded berry skin. The translationally controlled tumor protein (TCTP, 44) is highly regulated by a wide range of extracellular signals, and has been implicated in important cellular processes, such as cell growth, cell cycle progression, and in the protection of cells against various stress conditions and apoptosis (Bommer and Thiele, 2004). Iandolino et al. (2008) detected a relatively high abundance of TCTP when the grape berry was green and hard based on frequency analysis of ESTs and MPSS signatures. The 40S ribosomal protein SA (59, 60), a large complex molecule, is responsible for protein synthesis. Protein disulfide isomerase (PDI, 43) catalyses the formation, isomerization and reduction/oxidation of disulfide bonds, and was reported to show increased accumulation under Fe deficiency in cucumber roots (Donnini *et al.*, 2010). Nascent polypeptide associated complex alpha (NACA, 64) can prevent nascent ribosome-associated polypeptides from inappropriate interactions with cytosolic proteins (Beatrix et al., 2000). GroES chaperonin (71), together with GroEL, is commonly required for the proper folding of many proteins (Keskin et al., 2002). Iron–sulfur cluster-binding protein (63) is best known for its role in the oxidation–reduction reactions of mitochondrial electron transport, and some Fe—S proteins regulate gene expression. The receptor kinase protein (36) is commonly and broadly used in responding to vast arrays of stimuli to modulate gene expression (Shiu and Bleecker, 2001). Over-accumulation of these proteins may be a protective precaution so that the grape has a pool of proteins present to rapidly respond to the absence of sunlight.

Proteins involved in cell rescue, defense, virulence, cell fate, cell cycle and DNA processing, cellular communication, and biogenesis of cellular component

Only two enzymes related to cell rescue, defense and virulence were accumulated less in response to sunlight exclusion, endo-1,3(4)-beta-D-glucanase (3) and methionine sulfoxide reductase (9). Endo-1,3(4)-beta-D-glucanase is a specific enzyme that catalyzes the hydrolysis of cellulose (Robert et al., 2005). Methionine sulfoxide reductase fulfills an essential physiological function during environmental constraints through a role in protein repair and in protection against oxidative damage (Tarrago et al., 2009). In addition, an actin depolymerizing factor (ADF, 16), belonging to cell cycle and DNA processing, was down-regulated by sunlight exclusion. Some reports have linked ADFs with mediation of defense signaling (Miklis et al., 2007; Tian et al., 2009) in plants. In grape, it was induced in grape cuttings during new root formation (Thomas and Schiefelbein, 2002), and over-expressed at berry maturity (Deytieux et al., 2007), but there is no clear information on this protein in response to sunlight.

Many more proteins were over-accumulated in sunlight-excluded berry skin than the control. Pathogenesis-related protein (46) was strongly present when sunlight was excluded. Phospholipase (50) is an enzyme that hydrolyzes phospholipids into fatty acids and other lipophilic substances. The phospholipase D gene family members and their evolutionary relationship were recently studied in grape (Liu et al., 2010), while little has been reported on grape phospholipase C (50). Phospholipase C has been described to play an important role in cellular responses to a variety of extracellular signals in mammals (Katan and Williams, 1997). Both pathogenesis-related protein and phospholipase C may be connected with rapid responses to external signals, though the real contribution of these proteins in grape berry ripening is still unclear.

Both tubulin (52, 53) and actin (56, 58) are known to participate in many important cellular processes, including cell motility division, cytokinesis and signaling. During grape berry development, tubulin and actin are present since the huge changes in berry cell volume must be supported by changes in the cytoskeleton structure (Giribaldi et al., 2007). In addition, nucleoredoxin (38, 39) belongs to the thioredoxin family, and is involved in cell growth and differentiation (Funato and Miki, 2007). In this study, the abundance of tubulin, actin and nucleoredoxin indicated that the cell cytoskeleton may be rearranged to adapt to sunlight exclusion.

Concluding remarks

This proteomic approach to grape berry skin provides a great deal of information that enhances our understanding of the effects of sunlight exclusion on berry skin, especially related to anthocyanin biosynthesis. Proteins involved in various functions were found to be differentially accumulated between sunlight-exposed and sunlight-excluded berry skin. Generally, proteins involved in photosynthesis and secondary metabolism (especially *UFGT*), as well as heat shock proteins, were accumulated less in sunlight-excluded berry skin. The proteins related to glycolysis, the tricarboxylic-acid cycle, protein synthesis and biogenesis of cellular components were generally over-accumulated in sunlight-excluded berry skin. Thirteen of 35 down-regulated proteins and 8 of 37 up-regulated proteins were unknown and unclassified. The presence of these proteins with no clear functions indicated there is much to learn about the molecular and proteomic events that surround stopping color development in grape berry skin caused by sunlight exclusion. A further study on a comparative genome-wide transcriptome analysis may be included, and whether the observed protein variations reflect changes in gene expression and changes in some enzyme activities was also needed to be further confirmed. In addition, some proteins that were reported to respond to light signals, such as phytochromes (PHY) and cryptochromes (CRY) (Jiao et al., 2007), were not detected as expected, which could be due to either or both transcription and/or translation, and would call for improved techniques in proteomic analyses.

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