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Investigation into Uneven Ripening Problem in Muscadine Grape for Enhancing Their Enological Characteristics

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Uniformly ripened, superior-quality grape berries are prerequisite to relishing them as table grapes; processing into juice, jam, and jellies; and also fermenting into wine. Muscadines are extensively cultivated in Florida and are native to southeastern United States. Although muscadine berries have a characteristic aroma and sweetness, there is occurrence of asynchronous ripening within the cluster, which prevents harvesting of the whole cluster at once. With this in view, an investigation was initiated to identify the gene/s associated with ripening and their regulation in muscadine (non-uniform ripening) and Florida hybrid bunch (uniform ripening) grape berry during different ripening stages to understand the molecular basis of ripening. Isolation and characterization of the transcripts expressed during different stages of berry ripening using differential display reverse transcription PCR revealed unique expression patterns of certain transcripts in muscadine and Florida hybrid bunch grapes. These transcripts were identified as calcium-dependent protein kinase (CDPKs) and protein phosphatase 2C (PP2C), which are known to regulate berry ripening process. Expression of PP2C was found only in the muscadine berries, which is a negative regulator of abscisic acid (ABA) signaling. ABA is a phytohormone involved in triggering ripening. Suppression of ABA in certain muscadine berries within the cluster may be the possible reason for its uneven ripening. Further analysis will help in developing muscadine grape cultivars with uniform ripening characteristics to increase product quality, growers' profits, and product marketability.

Grape (*Vitis* sp.) is one of the important fruit crops of the world. In the United States of America the grape industry is valued at $2.92 billion, making it the highest value and greatest use crop (Anonymous, 2002). Florida has a small grape industry with less than 1000 acres of grape cultivation, given the high incidence of pest and disease. These adverse conditions in this region have prevented the growing of the old world grape, also known as bunch or European grapes, which is preferred throughout the world. The incidence of Pierce's disease (PD) in particular has limited the growing of European grapes in Florida and southeastern United States. Therefore, only two types of grapes can be grown in the state today: Florida hybrid bunch and muscadine grapes. Florida hybrids produce berries in large clusters, while muscadines are noted for their small clusters with large berries. Compared to Florida hybrid bunch grapes, muscadines are popular in the South for their hardier nature, resistance to pest and diseases, and long vine life. All these characters make muscadine a perfect grape to be grown in southeastern U.S. Muscadine grapes are native to the southeastern U.S. and wine continues to be a major market for muscadine grapes. But establishment of a processing market for jam, jelly, juice, and wine has been a problem. This is because of the peculiar problem existing in certain commercially important muscadine cultivars related to ripening (ie., non-uniform ripening of berries within the cluster). The lack of uniform ripening within cluster is termed as "uneven ripening" and is characterized by the presence of green berries in an otherwise mature ripe cluster (Cawthon and Morris, 1983).

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Uneven ripening within the vineyard creates problems in the harvesting of the crop, either employing mechanical harvesters or "U-pick," as it is practically difficult to determine the best ripeness stage. This has limited the supply of sufficient quantities of raw material to the growing demand of the grape industry. Uneven ripening also affects quality of grape berries. Whole berries should ripen at once within the cluster to produce high-quality grape berries. Several factors have been reported to be responsible for uneven ripening, such as variation in bud breaking, flower opening, and fruit ripening (Haung and Lu, 2000). Muscadines are known to have long fruiting periods. With this in view an investigation was carried out for the first time at molecular level to understand the rationale of the ripening process in uniform ripening Florida hybrid bunch and non-uniform ripening muscadine cultivars. Analysis at molecular level using differential display reverse transcription-PCR (DDRT-PCR) will reveal unique transcripts expressed differentially between the muscadine and Florida hybrid bunch during ripening, which would help identify the molecular and cellular components associated with uneven ripening and promote uniform ripening in muscadine clusters.

Materials and Methods

Plant material. Muscadine and Florida hybrid bunch grape cultivars grown at the Center for Viticulture and Small Fruit Research in Tallahassee were used in this study. The whole berry of muscadine cv. Cowart and Florida hybrid bunch cv. Blue Lake at different stages (immature, mature, and ripened berries) of ripening were collected, frozen in liquid nitrogen and stored at –80 °C.

Berry RNA isolation. Berries have high levels of phenolics,

carbohydrates, and other compounds, which interfere during RNA isolation. RNA was extracted from the berries collected following the CTAB method (Reid et al. 2006).

Ripening-specific gene isolation. The uniquely expressed transcripts from uniform and non-uniform ripening grape cultivars were isolated using Differential Display RT-PCR technique using the above extracted total RNA. DDRT-PCR was carried out using RNAimage kit as per the manufacturer's instructions (GenHunter, Nashville, TN). Total RNA were reverse transcribed (RT) using T_{11} MN anchored primer (where M stands for dA, dC, dG and N stands for dA, dC, dG, dT as described) in presence of MMLV reverse transcriptase at 37 °C for 1 h. This mixture was used as template in the PCR reaction containing the same T_{11} MN anchored primer in combination with an arbitrary primer, 1 × PCR buffer and 1 unit of *Taq* DNA polymerase. The PCR conditions were as follows: 94 °C 30S, 40 °C 2 min, 72 °C 30S repeated for 40 cycles, followed by 72 °C for 5 min. The PCR product thus obtained were separated on 12% polyacrylamide gel and silver stained. The differentially expressed transcripts were eluted from the gel, re-amplified, cloned and sequenced. Re-amplification using the same original arbitrary primer avoided choosing any PCR artifacts.

Cloning and transformation. The differentially expressed genes were cloned into pGEM-T Easy vector (Promega). Recombinant colonies were selected based on blue/white selection. The plasmid was isolated using the miniprep (Sambrook et al., 2000) procedure. Plasmids with inserts were selected for further sequencing.

Sequencing of the cloned cDNA. Preparation of samples for sequencing reaction was carried out using Cycle sequencing kit as per manufacturer's instruction (Beckman Coulter). Thermal cycling was carried out at these conditions: 96 °C for 20 s, 50 °C for 20 s, 60 °C for 4 min for 30 cycles. The cDNAs were sequenced using the Beckman CEQ8800 Sequencer as per the manufacturer's (Beckman Coulter, Fullerton, CA) instructions using the preloaded software.

Data analysis. The raw sequence data obtained from the CEQ8800 were used for analysis. Both qualitative and sequence trimming were carried out to remove the junk and plasmid sequences from the raw sequence data. The sequence data thus obtained were analyzed for their identity using BLASTX (Nucleotide translated query vs. protein database) search for its homology with the known sequences of a gene recorded in the NCBI (National Center for Biotechnology Information) database.

PCR analysis. Specific primers were designed to the two unique genes differentially expressed in uniform and non-uniform ripening grape species. Oligonucleotide primers ranging from 17 to 20 bases were designed using commercial (Primer3) software. Care was taken to avoid possible secondary structure formation, primer dimer generation and false priming, and also to achieve appropriate melting temperature and internal stability during designing. A quantitative analysis for both the genes CDPKs (Left Primer: GGCAGTTTGGGACAACATTT and Right Primer: ATGCTTGCACAACACCTCTG) and PP2C (Left Primer: GCCATATACGCAAGGGAGAA and Right Primer: ATCATCCCTGATTCCACGAG) were carried out using PCR analysis at these conditions: initial heat activation of the DNA-polymerase was performed at 95 °C for 2 min. Thereafter 35 cycles at 95 °C (1 min), 55 °C (30 s) (for both genes), and 72 °C (1 min) were run, with final extension at 72 °C for 5 min during different ripening stages. The obtained PCR product was separated on 1% agarose gel, stained with Ethidium Bromide.

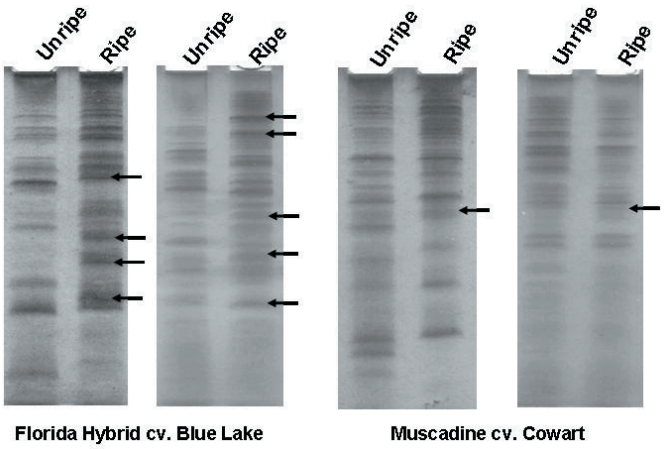

Fig. 1. Differential expression of transcripts in unripe and ripe grape berry (**A**) 'Blue Lake' (Florida hybrid bunch) and (**B**) 'Cowart' (Muscadine). Arrows indicate the differentially expressed transcripts.

Results and Discussion

High quality intact total RNA was obtained using CTAB extraction method from both mature and ripe grape berry tissue, which is prerequisite for any gene expression studies. The integrity of RNA was confirmed through agarose gel electrophoresis. Differential Display RT-PCR yielded several differentially expressed unique cDNA/s in uniform ripening Florida hybrid bunch grape ('Blue Lake') and in non-uniform ripening muscadine ('Cowart') grapes (Fig. 1). DDRT-PCR on an average yielded 10 to 12 unique transcripts in Florida hybrids compared with 4 to 5 in muscadine grape (Table 1). This indicates that there are certain sets of cDNAs which are uniquely expressed in Florida hybrids that might trigger uniform ripeness. Sequencing and characterization of selected transcripts revealed their role in signal transduction and defense (Table 2).

DDRT-PCR results revealed differential expression of two unique transcripts of 650 bp and 710 bp in muscadine and Florida hybrid bunch grapes, respectively (Fig. 2). Sequencing and NCBI -BLAST search of 650 bp sequence from muscadine revealed that it had 85% homology with protein phosphatase 2C (PP2C) and 710 bp sequence revealed it had 90% homology with calcium dependent protein kinase (CDPKs). Previously it has been reported that these genes are associated with berry development and ripening. Hence, these two genes were selected for further analysis. PCR analysis was carried out using primers specific to these genes and found differential expression of these genes both in muscadine and Florida hybrid bunch grape berries based on the intensity of the amplified bands. PCR results revealed that the expression of PP2C occurred only in immature and mature berries of muscadine, but was absent at ripened stages of muscadine. In contrast its expression was absent in all the stages of Florida Hybrid Bunch grape (Fig. 3). This shows that the PP2C acts as negative regulator of the ripening process. In contrary to PP2C expression, the expression of CDPKs was found significantly higher in all the stages of uniformly-ripening Florida hybrid bunch grape berries. However, in muscadine berries its expression was significantly higher at ripe and low at mature, and was completely absent at immature stage. This clearly shows that the CDPKs act as positive regulator of ripening process. Differential expression of PP2C

Table 1. Variation in the total number of cDNAs uniquely expressed in ripened Florida hybrid bunch and muscadine grape berries with selected primer combinations.

		Differentially expressed genes	
Anchored Oligo-dT primer	Arbitrary 13 mer primer	'Blue Lake' (Florida hybrid bunch)	'Cowart' (muscadine)
AAGCTTTTTTTTTTTA	AAGCTTAGTAGGC	14	6
	AAGCTTGCACCAT	9	5
	AAGCTTAACGAGG	10	4
	AAGCTTTTACCGC	10	2

*Primer Source: GenHunter Corporation, Nashville, TN.

Table 2. Nucleic acids uniquely induced in uniform ripening Florida hybrid bunch grape cv. Blue Lake.

Sl no.	Nucleotide similar to	Size (bp)	Organism matched	Function
1	Protein kinase	650	*Vitis vinifera*	ABA-signaling pathway
2	Protein phosphatase 2C	710	*Arabidopsis*	Negative regulator of ABA-signaling pathway
3	Sugar Kinase	410	*Arabidopsis*	Signal transduction
4	Lipid Transferase	540	*Vitis vinifera*	Defense
3	Transcription Factor	610	*Vitis vinifera*	Signal transduction
4	Unknown	992	---	---

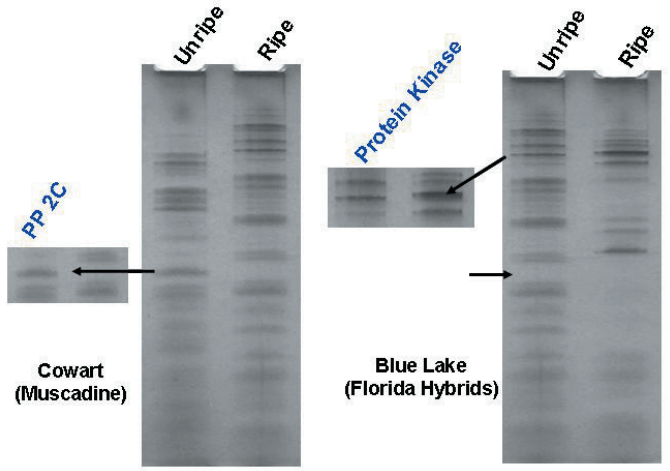

Fig. 2. Differential expression of protein phosphatase 2C (PP2C) and calcium-dependent protein kinase (CDPKs) genes in unripe and ripe grape berry of 'Cowart' (muscadine) and 'Blue Lake' (Florida hybrid bunch). Arrows indicate the differential expression.

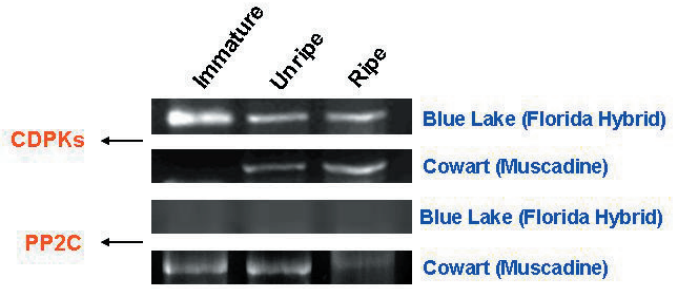

Fig. 3. Agarose gel depicting differential expression of calcium-dependent protein kinase (CDPKs) and protein phosphatase 2C (PP2C) at different stages of grape berry development.

and CDPKs genes may be responsible for varied maturity levels of berries within the cluster.

Abscisic acid is a key regulator of seed maturation and germination (Gosti et al., 1999), and it also promotes ripening (Cawthon and Morris 1983). Cawthon and Morris (1983) reported that the non-ripening fruits of 'Concord' grapes had low ABA content compared to ripened fruits. ABA level increased to 100 μg·100 g^{-1} fresh weight in ripe fruits. This clearly indicates the role of ABA in eliciting fruits to enter veraison. In another study they also investigated the influence of seed number and seed maturity on fruit maturation. Their results revealed that all berries containing mature seeds had entered veraison and the ones that had immature seeds were still in the pre-veraison category. With these data they have concluded that the ABA did not increase until all the seeds matured in the berry, suggesting an association of immature seeds with suppression of ABA accumulation and delay, which leads to uneven ripening. This research suggests that ABA first promotes seed maturation and then fruit ripening.

Calcium (Ca^{2+}) is known to play a central role in mediating ABA signaling (Yu et al., 2006). ABA in turn stimulates calcium dependent protein kinase (CDPKs), which encourages berry development (Yu et al., 2006). Yu et al. (2006) suggested that the application of other phytohormones such as auxin indoleacetic acid, gibberellic acid, synthetic cytokinin N-benzyl-6-aminopurine and brassinolide were found ineffective in stimulating CDPKs. CDPKs consequently trigger ethylene signaling and thus help in ripening of the berries (Hahn and Harter, 2009; Ludwig et al., 2005). Expression of CDPKs in uniform ripening Florida hybrid bunch grape cv. Blue Lake clearly shows that there is no impediment in ABA and ethylene signaling pathways.

On the contrary, expression of PP2C was found only in the immature and mature berries of muscadine cv. Cowart grape and was completely absent in the uniform ripening Florida hybrid bunch grape cv. Blue Lake. Protein Phosphatase 2C are known to regulate abscisic acid signaling and their by affecting ripening process. Gosti et al. (1999) reported that PP2C is a negative regulator of abscisic acid signaling and the loss of PP2C activity leads to an enhanced responsiveness to ABA. Thus, this clearly shows that the expression of PP2C in muscadine cv. Cowart affects ABA signaling, which in turn affects seed maturation and there by reducing ABA accumulation necessary for triggering calcium dependent protein kinase to induce ethylene signaling for uniform ripening process to take place. Expression of PP2C in muscadine cv. Cowart may be the possible reason for their uneven ripening problem within the cluster.

Conclusion

Several uniquely expressed genes have been isolated from uniform and non-uniform ripening grape species using differential display RT-PCR. Expression of PP2C in uneven ripening muscadine cv. Cowart alters ABA signaling thus affecting the ripening process. Further studies will be carried out to identify cDNA transcripts associated with enhancing and inhibition of ripening, and evaluated across the grape species, which will aid in developing uniform ripening muscadine grape cultivar.

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