Title: Development of a DNA marker for seedlessness in *Euvitis* × *Muscadinia* hybrids.

Final Report

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Research Proposal

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Objectives: 1) To evaluate the usefulness of DNA marker p3_VvAGL11 for predicting seedlessness in *Euvitis* × *Muscadinia* hybrids

Justification and Description

There are two types of seedlessness that can occur in grapes. In parthenocarpic seedlessness, the ovule develops without fertilization, while in stenospermocarpic seedlessness, the seeds abort a few weeks after fertilization (Bergamini et al., 2013). Therefore, stenospermocarpic grapes will generally have seed traces, which are more palatable than fully developed seeds, and parthenocarpic grapes will have no traces of seeds. Parthenocarpic grapes tend to be smaller than fertilized grapes of the same variety.

Currently, all seedless varieties of *Euvitis* with known pedigree are derived from the cultivar 'Sultanina' (also known as 'Thompson Seedless'), which is stenospermocarpic seedless (Karaagac et al., 2012). Genetic control of seedlessness is a matter of some contention, but the most widely accepted theory is that seedlessness is conditioned by the presence of a dominant allele at a single locus named *SDI* for "seed development inhibitor". *SDI* inhibits the development of the seed by regulating three recessive genes (Mejia et al., 2011). QTL studies have confirmed that seedlessness is primarily regulated by SDI which is responsible for 50% to 70% of total phenotypic variance for the trait. New seedless cultivars can be obtained by crossing a seeded parent to a seedless self-fertile pollen parent. These crosses typically produce from 10% to 40% seedless progeny depending upon the definition of seedless used by the breeder. In order to improve the percentage of seedless progeny and reduce the size of seed traces, many breeding programs have begun crossing two seedless cultivars and progeny are obtained through embryo rescue and tissue culture.

Breeding seedless muscadines has been hampered by the lack of a source of stenospermocarpic seedlessness. The only seedless member of *Muscadinia* is 'Fry's Seedless', which is both parthenocarpic and male-sterile, and therefore cannot be used as a parent in breeding (Basiouny and Himelrick, 2001). Additionally, this cultivar is largely fruitless and

produces very small berries. The introduction of seedlessness from *Euvitis* is hampered by the difference in chromosome numbers between *Euvitis* and *Muscadinia* (*Muscadinia* has 40 chromosomes while *Euvitis* has 38). However, recently the UGA breeding program has obtained complex *Euvitis*-*Muscadinia* hybrids which are able to serve as bridges between the two subgenera. Using these hybrids, a breeding program has begun with the goal of introgressing stenospermocarpic seedlessness from *Euvitis* into new seedless muscadine grape cultivars.

Previous research has located a functional candidate gene, *VvAGL11*, for stenospermocarpic seedlessness in *Vitis vinifera* (Mejía et al., 2011). The STS marker p3_VvAGL11 was designed to perform genetic analysis of INDELs in the regulatory region of *VvAGL11*. It marks a (GAGA)n motif and has a high correlation with seedlessness (K = 73.3%, 69.8%, and 78.3%) across all three seasons of the study performed by Mejía et al. (2011). Furthermore, p3_VvAGL11 segregates 1:2:1 (*ab* x *ab*) and displayed no false positives or negatives in the homozygous genotypes.

Subsequent tests on *V. vinifera* germplasm by Bergamini et al. (2013) to validate the p3_VvAGL11 marker found only 8 of 475 genotypes that were seeded but carried the dominant allele associated with seedlessness. Additionally, each seedless variety that was tested did carry the seedlessness-associated allele. The lack of false negatives and very low rate of false positives indicate that this marker is suitably effective for performing early negative selection of stenospermocarpy. Analyses performed to test the discriminating power of p3_VvAGL11 across different genetic backgrounds indicated three alleles in the regulatory region of *VvAGL11*, 194, 206, and 216 bp in size, with cultivars containing the 216 bp allele being seedless.

Materials and Methods

Plant Material: Three female parents (NC74CO49-10, DRX 60-40, and Fennell's 3-way Hybrid) were used in crosses with seedless *Euvitis* pollen parents to generate seedless segregating *Euvitis* × *Muscadinia* progenies. NC74CO49-10 and DRX 60-40 are complex hybrids with a mixture of *Euvitis* and *Muscadinia* background, and Fennell's 3-way Hybrid is a mixture of all three *Muscadinia* species. Due to the high mortality and sterility of these progenies, genotypic and phenotypic data was collected from fourteen separate progenies and pooled together to examine the effect of the *VvAGL11* STS marker on seed and berry weights.

Genotyping: DNA was extracted from the *Euvitis* pollen parents, *Euvitis* × *Muscadinia* hybrid progenies, and a wide array of muscadine germplasm. The *VvAGL11* STS marker was amplified from this material using the methods of Bergamini et al. (2013), and the marker was sized at the Georgia Genomics Facility using a Fragment Analyzer™ Automated CE System.

Phenotyping: Berry samples were taken from all fruiting plants hybrid progenies segregating for seedlessness. For each sample of berries (up to twenty berries per plant, as available), the total berry mass, seed tissue mass, and number of seeds and seed traces was determined. These measurements were used to calculate average seed tissue weight, average seed weight per berry, average berry weight, and percent berry weight composed of seed tissue.

Data Analysis: Microsoft Excel was used to create histograms for each of the measurement categories for the hybrid progenies, as well as to perform one-way ANOVAs for phenotypic data.

Results

The *VvAGL11* STS marker primers of Bergamini et al. (2013) were able to amplify markers from all tested *Euvitis* and *Muscadinia* samples. All seedless *Euvitis* samples possessed

a single 214 bp allele, and this allele was not found in any seeded *Euvitis* or *Muscadinia* samples (Table 1). The 214 bp allele appears to be identical to the 216 bp allele which was associated with the seedless allele in *Euvitis* (Bergamini et al. 2013). The 2 bp difference in size of this allele is likely due to differences in the sizing equipment and size standards used in the two experiments. The most common *Muscadinia* allele size was 196 bp, a size which was not found in any *Euvitis* samples. Importantly, the 214 bp seedlessness associated allele was not found in any *Muscadinia* samples. This indicates that this marker can be used to follow the seedlessness allele as it is introgressed into *Muscadinia* germplasm without interference from a muscadine allele of the same size.

The UGA breeding program has generated a wide array of *Euvitis* x *Muscadinia* hybrid progenies which use seedless *Euvitis* cultivars as parents. Seedling death and sterility were common in these progenies, and relatively few seedlings produced fruit, forcing us to combine seedlings from a large number of progenies together in order to examine the effect of the 214 bp allele on seed and berry traits. The presence of the 214 bp allele was associated with a reduced seed weight, seed weight per berry, percent berry weight composed of seed, and berry weight (Table 2). Hybrid seedlings which lacked the 214 bp allele occasionally had very low weights for each phenotypic category and these seedlings likely represent sterility and parthenocarpic fruit set, rather than stenospermocarpic seedlessness. Thus the use of the marker allowed an easy differentiation between parthenocarpic and stenospermocarpic seedlessness.

Table 1. Allele frequencies of the VvAGL11 STS marker in tested germplasm.

Allele size	*Muscadinia*		Seeded *Euvitis*		Seedless *Euvitis*	
	Count	Frequency	Count	Frequency	Count	Frequency
192	0	0	2	6.3%	0	0
194	15	12.7%	6	18.8%	0	0
196	93	78.8%	0	0	0	0
198	0	0	1	3.1%	0	0
200	10	8.5%	1	3.1%	1	3.9%
204	0	0	18	56.3%	7	26.9%
208	0	0	2	6.3%	1	3.9%
212	0	0	2	6.3%	4	15.4%
214	0	0	0	0	13	50.0%
TOTAL	118 (59 cultivars)		32 (16 cultivars)		26 (13 cultivars)	

Table 2. Effect of the 214 bp seedlessness associated allele on seed weight, total seed weight per berry, berry weight, and percent berry weight composed of seed in *Euvitis* × *Muscadinia* hybrid seedlings.

Allele	Individual seed weight (mg)	Total seed tissue weight per berry (mg)	Berry weight (g)	Percent berry weight composed of seed tissue
- 214	51.6	62.4	1.7	3.6%
+ 214	20.2	25.9	1.3	1.9%
Sig.	$P<0.001$	$P<0.001$	$P<0.05$	$P<0.001$

Conclusions

1. The 214bp allele for the *VvAGL11* STS marker only appeared in stenospermocarpic seedless germplasm.
2. The 214 bp seedlessness associated allele was not found in any tested *Muscadinia* germplasm.
3. Hybrid seedlings with the 214 bp allele had smaller seeds (or seed traces), slightly smaller berries, and less seed tissue per berry than those without it.
4. Continuing to use the *VvAGL11* marker to screen for seedless individuals will improve the efficiency of the breeding program and allow the breeding program to separate stenospermocarpic seedless seedlings from seedlings with parthenocarpic berry set.

Impact Statement

The data from this experiment indicate that the *VvAGL11* STS marker will be useful to assist the introgression of the dominant SDI gene into muscadine germplasm to produce seedless muscadine cultivars. This marker will allow us to remove approximately 50% of the seedlings in these crosses which do not inherit the SDI gene. It also has the added benefit of allowing us to differentiate between parthenocarpic seedless vines and stenospermocarpic seedless vines. We will begin to test all seedless segregating progenies in 2016 with this marker while seedlings are still in the greenhouse, allowing us to remove seeded vines before planting them in the field. This will allow us to grow larger progenies and improve our chances of finding seedlings with desirable combinations of traits. It will also allow us to choose parents with the SDI gene more quickly use in the next round of crosses, facilitating introgression of the SDI gene into muscadine germplasm.

Literature Cited

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