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CONTENTS

Genes involved in regulation of peach fruit development and their role in split-pit formation

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Abstract

Expression of MADS-box regulatory genes involved in peach fruit development was examined in split-pit sensitive and tolerant varieties, as well as, after common treatments in peach cultivation practice. Expression of FRUITFUL that is required for the expansion and differentiation of the fruit after fertilization in Arabidopsis displayed a similar pattern in both plants. SHATERPROOF that is required for the differentiation and lignification of the dehiscence zone in Arabidopsis was not detectable in peach, indicating either that another homologous gene may be expressed or that more sensitive methods are needed for assessment of its expression. Further experiments are underway to unravel peach fruit development and the cause of split-pit that deteriorates fruit quality.

Introduction

Peach cultivation of clingstone varieties is important for Greek agriculture and canning industry, as Greece has been the second, next to USA, canned peach producer worldwide, and the first exporter among EU countries. This robust agro-industrial enterprise faced severe problems the last 2-3 years, as a result of adverse weather conditions, but also from deterioration of canned fruit quality due to the presence of small pit fragments originated from split-pits during processing. Split-pits are a recurring problem in peaches due to genetic as well as environmental factors. The term "split-pit" refers to the opening of the pit at the stem end and splitting of the fruit. Any treatment that promotes fruit growth at the start of pit hardening tends to increase the number of split-pits. Split-pits may result from early excessive thinning caused by frost followed by irrigation or rains. Girdling of tree limbs by wire and excessive nitrogen may also promote the problem. Early maturing varieties are particularly prone to split pits, as are varieties in which pit hardening is relatively late. Understanding the genetic factors underlying split-pit sensitivity in peach may provide the tools for breeding resistant varieties and molecular markers for better management of existing cultivars.

Split-pit occurs at a specific zone of the pit destined to open for the release of the seed. Fruits have evolved to mediate the maturation and dispersal of seeds. Fruit dehiscence is a strategy that many fruits adopt to achieve seed dispersal. The dehiscence process involves the differentiation of specialized cell types and a tight co-ordination of molecular and biochemical events that eventually lead to a cell separation process that frees the seeds once they have matured. In the last few years, great progress has been made in identifying the molecular mechanisms underlying fruit dehiscence in the model plant *Arabidopsis thaliana* (Roberts et al., 2002).

The types of cell layers that contribute to the opening of the fruit are: the seperation layer, the lignified valve layer and the lignified margin layer. The first genes shown to be required for both the differentiation of separation layer and the lignification of the specific zone were the MADS-box genes *SHATTERPROOF1* (*SHP1*) and *SHATTERPROOF2* (*SHP2*) (Ferrandiz et al. 2000; Liljegren et al., 2000). Another gene involved in the differentiation of the separation layer is

ALCATRAZ (*ALC*; bHLH transcription factor) (Rajani and Sundaresan, 2001). Additionally, the gene *INDEHISCENT* (*IND*; bHLH transcription factor) is involved in the differentiation of all three layers (Liljegren et al. 2004). On top of all, the gene *FRUITFUL* (*FUL*; MADS-box gene) negatively regulates *IND*, *ALC*, *SHP1*, and *SHP2* to ensure that valve margin differentiation occurs at the edge of the valve (Gu et al. 1998; Liljegren et al. 2004).
Anatomical and physiological comparisons reveal that peach pericarp is an organ analogous to *Arabidopsis* valves having both originated from the carpel of the ovary, and split-pit occurs exactly at the separation layer of the endocarp that is analogous to the dehiscence zone of the valve's endocarp. This prompted us to examine if peach genes homologous to the *Arabidopsis* genes involved in dehiscence, play a role as genetic factors affecting split-pit formation in peach. More specifically in this study we characterized the expression and examined whether there are any differences in *FUL* and *SHP* transcription in split-pit sensitive in comparison to resistant varieties and if cultivation practices affect the expression of these genes.

Materials and Methods

Plant material: Samples from *Prunus persica* var. Andross and var. Katerina (susceptible and resistant to split-pit, respectively) were collected from Veria every week after anthesis until the collection of ripened fruits. Moreover, samples from Andross which were treated with phytohormones: Perlan (gibberilin+cytokinin), chemical compounds: Ca acetate, Chelan (CaO 15%), Profical (CaO 15%+ MgO), Bcazin (CaO 10%, B 1%, Zn 1%) and manipulations (early or late pruning, excess N_2 fertilizing) that seem to influence the phenomenon of stone cracking, were collected for pilot experiments. Samples were frozen in liquid nitrogen and stored at –80 ^{o}C until used.

Est clones: Est clones were from a peach mesocarp library curated at Clemson University (www.genome.clemson.edu/gdr/projects/prunus/abbott/PP_LEa/index.shtml). The library was searched for MADS-box gene ESTs, and those homologues to *Arabidopsis SHP* (BU046256), *FUL* (BU039475) and *SEP* (BU047495) were obtained. After sequencing of the clones we were able to identify the full length of each EST and based on this information BLAST search against GenBank was performed.

RNA isolation, cDNA synthesis, and Reverse transcriptase (RT)-PCR: One μg of total RNA isolated from various tissues using the RNeasy plant RNA isolation kit (Qiagen) was used for first strand cDNA synthesis. The cDNA was synthesized using 1 μg 3' RACE adapter Primer 5'-GGCCACGCGTCGACTAGTAC$(T)_{17}$-3' (Gibco-BRL), 1 mM dNTPs and 200 U M-MuLV reverse transcriptase (NEB) in 50 μl total volume. This cDNA served as a template in PCR reaction using 0.2 pmol gene-specific primers, 0.2 mM dNTPs and 1 U DyNAzyme II DNA polymerase (Finnzymes). Gene specific primers were designed from the ESTs found at Genome Database for Rosaceae (Clemson University Genomics Institute) for *SHP*, *FUL*, *SEP* and *β-actin* of peach.
Both actin and sepallata were used for RT-PCR controls. Sepallata1 gene is expressed throughout flower development. The thermocycler program was: 30 cycles of 30 s at 94 ^{o}C, 30 s at 54 ^{o}C, 30 s at 72 ^{o}C, preceded by 2 min at 94 ^{o}C, and followed by 10 min at 72 ^{o}C. Fragments were of predicted lengths. Control PCR reactions contained the RNA that was used as a template in the cDNA synthesis.

Comparison and phylogenetic analysis: The nucleotide and deduced amino acid sequence of the peach *FUL, SHP*, and *SEP* were used for BLAST analysis on the EBI databases, and among the best BLAST hits, genes for which were published reports, were selected for comparison. Sequence names were changed to include initials were needed. The sequences for *FUL* were: *Betula verrucosa* BvMADS5 (CAA67969.1), *Petunia hybrida* FBP26 (AF176783), *Malus domestica* MdMADS2 (AAC83170.1), *Nicotiana tabacum* NtMADS11 (AF385746), *Petunia hybrida* PFG (AF177682), *Petunia hybrida* PhFUL (AY306172), *Solanum tuberosum* POTM-1 (U23758), *Vitis vinifera* VvFUL (AY538747), *Arabidopsis thaliana* AGL8/Fruitful (U33473). The sequences for *SHP* were: Rosa rugoza RrMasakoD1(AB025643), *Petunia hybrida* PhFBP6 (X68675), *Malus domestica* MdMADS14 (AJ251117), *Ipomonea nil* InPeony (AB006183), *Petunia integrifolia* Pi PAGL1 (L33973), *Panax ginseng* PgGAG2 (Z46612), *Vitis vinifera* VvMADS1 (AF265562), *Arabidopsis thaliana* AGL1/Shatterproof (M55550). The sequences for *SEP* were: *Malus domestica* MdMADS8 (AJ001681), *Petunia hybrida* PhMADS12 (AAQ72506), *Petunia hybrida* PhFBP5 (AF335235), *Malus domestica* MdMADS1 (U78947), *Cucumis sativus* CsCAG2 (AF135962), *Lycopersicum esculentum* LeTAGL2 (AY098738), Asparangus officinalis AoM1 (AAQ83834), *Vitis vinifera* VvMADS2 (AF373601), *Arabidopsis thaliana* Sepallata1 (M55551). The deduced amino acid sequences of each set of the genes were aligned using the multiple sequence alignment program Clustal W (Thompson et al., 1994). Phylogenetic and molecular evolutionary analyses were conducted using the MEGA 2.1 software (Kumar et al., 2001) by the Neighbor-Joining Method with Poisson correction (Saitou and Nei, 1987). The phylogenetic tree was tested by the bootstrap method (Dopazo, 1994)

Results

EST clones for the peach *FUL, SHP*, and *SEP* genes were sequenced and full-length open reading frames were identified. The *FUL* sequence was aligned with the closest matching homologous genes from other plant species and the phylogenetic tree is presented in Figure 1. Data indicate that the closest homologous to the peach gene is a gene from apple, as could be expected. Similar results were obtained for the other two genes.

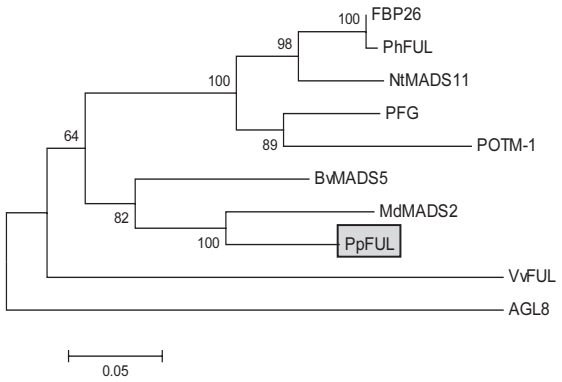

Figure 1. Phylogenetic relationship of the peach FUL protein sequence (in gray box) with related sequences from other species identified as best scores in BLAST searches. Sequences were aligned with CLUTAL-W and the trees were generated by the Neighbor-Joining method using the Poisson Correction distances. Bootsatrap values (1000 replications) are shown at the nodes of clades. Branch distance denotes amino acid substitutions.

Expression analysis of *SEP* and *FUL* revealed that transcripts were present in peach pericarp throughout the examined developmental period, which included early (after anthesis) to late (maturation) stages of fruit development.. No *SHP* expression could be observed during the same period. Various treatments of the trees did not affect expression patterns of these genes neither to the sensitive nor to the resistant variety. Representative results of the RT-PCR analysis are shown in Figure 2.

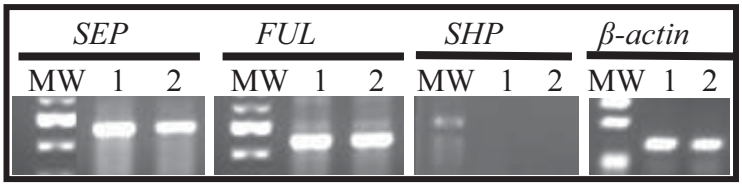

Figure 2: RT-PCR results using gene-specific primers for the genes *SEP*, *FUL*, *SHP* and *β-actin* examined at an early stage of fruit development. Lanes are, 1: Andross (split-pit sensitive), 2: Katerina, (split-pit resistant). MW. Molecular weight marker

Discussion

The fruit that mediates the maturation and dispersal of seeds in flowering plants is an organ derived from the female reproductive structure. Fruit development has been the subject of extensive studies in *Arabidopsis* and key regulatory steps with the genes involved have been recognized and their role characterized (Ferrandiz et al., 1999). Though peach and *Arabidopsis* fruits are different, anatomical and physiological analogies exist between *Arabidopsis* valves and peach pericarp. This prompted us to identify genes and examine their expression patterns in peach, based on their homology with *Arabidopsis* genes known to regulate fruit development.

In *Arabidopsis FUL* expression is required for the expansion and differentiation of the fruit valve after fertilization (Gu et al., 1998). It is expressed throughout the valves, except a narrow strip where the dehiscence zone is formed. FUL is a negative regulator of several genes involved in the dehiscence zone development including SHP, and its absence cause valves to tear open in a fashion similar to split-pit in peach (Fernandiz et al., 2000). We examined *FUL* expression in peach and our results indicated that *FUL* was normally expressed during fruit development. There were no differences in *FUL* expression between a sensitive to split-pit and a tolerant variety, neither its expression was affected by treatments with a variety of agents nor cultivation practices. We also examined the expression of *SHP* that controls dehiscence zone differentiation and promote lignification of adjacent cells in *Arabidopsis* (Liljegren et al., 2000). Expression of *SHP* in *Arabidopsis* is restricted to the dehiscence zone and is negatively regulated by *FUL* in other tissues, while its ectopic expression can cause valve opening and results in a phenotype of *Arabidopsis* fruit similar to split-pit in peach. Our results indicated that no detectable *SHP* expression could be observed during fruit development in peach. This could be due to either that another homologous gene may be expressed and being redundant to the one examined is enough for normal fruit development, or that more sensitive methods are needed for assessment of its expression. Further experiments are underway to unravel peach fruit development and the cause of split-pit that deteriorates fruit quality.

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