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The *metacaspase* gene family of *Vitis vinifera* L.: Characterization and differential expression during ovule abortion in stenospermocarpic seedless grapes

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ABSTRACT

In both plants and animals, programmed cell death (PCD) is an indispensable process that removes redundant cells. In seedless grapes (*Vitis vinifera*), abnormal PCD in ovule cells and subsequent ovule abortion play key roles in stenospermocarpy. Metacaspase, a type of cysteine-dependent protease, plays an essential role in PCD. To reveal the characteristics of the *metacaspase* (*MC*) gene family and the relationship between *metacaspases* and the seedless trait, we identified the 6 *V. vinifera metacaspases VvMC1–VvMC6*, from the grape genome, using BLASTN against the 9 known *Arabidopsis metacaspases*. We also obtained full-length cDNAs by RT-PCR. Each of the 6 grape *metacaspases* contains small (p10-like) and a large (p20-like) conserved structural domains. Phylogenetic analysis of 6 grape and 9 *Arabidopsis metacaspases* showed that all *metacaspases* could be grouped into two classes: Type I and Type II. Each phylogenetic branch shares a similar exon/intron structure. Furthermore, the putative promoters of the grape *metacaspases* contained *cis*-elements that are involved in grape endosperm development. Moreover, expression analysis of *metacaspases* using real-time quantitative PCR demonstrated that *VvMC1* and *VvMC2* were able to be detected in any tissue, and *VvMC3*, *VvMC4*, *VvMC5* and *VvMC6* exhibited tissue-specific expression. Lastly, in cv. Thompson seedless grapes *VvMC1*, *VvMC3*, and *VvMC4* were significantly up-regulated at the 35 DAF during ovule development, roughly same stage as endosperm abortion. In addition, the expression trend of *VvMC2* and *VvMC5* was similar between cv. Pinot Noir and cv. Thompson grape ovule development and that of *VvMC6* was sustained in a relatively low level except the expression of cv. Pinot Noir significantly up-regulated in 25 DAF. Our data provided new insights into PCD by identifying the grape *metacaspase* gene family and provide a useful reference for further functional analysis of *metacaspases* in grape.

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1. Introduction

Programmed cell death (PCD) is a genetically programmed and highly ordered cell suicide process that removes unwanted or damaged cells. PCD is a fundamental requirement for stable growth and development in multicellular eukaryotes (Gadjev et al., 2008; Gunawardena, 2008). In animals, the molecular mechanisms of programmed cell death have been revealed in studies of *Caenorhabditis elegans* (Yuan et al., 1993). In animal cells, caspases, one kind of aspartic acid-specific cysteine protease, ultimately induce PCD and control the process (Cohen, 1997; Kitanaka and Kuchino, 1999; Aravind and Koonin, 2002). Caspase activation leads to cell structure and metabolic changes and mediates the occurrence of PCD (Earnshaw et al., 1999).

No orthologous caspases have been identified in plants, but PCD functions in plant disease resistance, growth and development (del Pozo and Lam, 1998; Solomon et al., 1999), including in xylem formation, embryogenesis, vascular development, plant regeneration, seed development, and leaf senescence (Bozhkov et al., 2005a; Filonova et al., 2000; Lim et al., 2007; Pennell and Lamb, 1997; Schindler et al., 1995). A family of cysteine-dependent proteases with “caspase-like” activity, termed *metacaspases*, was found in plants, fungi and protozoa (Uren et al., 2000). Metacaspase structures are similar to caspases but the two enzyme types are distantly phylogenetically related and differ in their substrate specificity. The predicted secondary structure of metacaspases contains conserved domains and motifs in all members of the caspase/metacaspase/paracaspase superfamily (Vercammen et al., 2007). Phylogenetic analysis of eukaryotic caspases, metacaspases, and paracaspases suggested that these groups are about equally distant from each other (Vercammen et al., 2007). In recent years, research on the biochemical characteristics and biological functions of metacaspase enzymes has become a hot topic, especially the question of whether

Abbreviations: At, *Arabidopsis thaliana*; cDNA, DNA complementary to RNA; DAF, day after full-bloom; ORF, open reading frame; PCD, programmed cell death; QRT-PCR, quantitative real-time PCR; RT-PCR, reverse transcription PCR; Vv, *Vitis vinifera*.

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metacaspases have caspase-like enzymatic activities (Carmona-Gutierrez et al., 2010; Enoksson and Salvesen, 2010). The substrates of most metacaspases also have not yet been identified. The recent identification of Tudor staphylococcal nuclease (TSN) as a common substrate for both the Norway spruce metacaspase *mcII-Pa* and the human caspase-3, but this phenomenon has not been identified in other plants (Sundström, 2009).

Plant metacaspases can be divided into two subclasses on the basis of similarities in amino acid sequence and general domain structure: Type I and Type II (Lam, 2004). Both kinds of metacaspases have putative small (p10-like) and large (p20-like) subunits, which contain the catalytic amino acid dyad histidine/cysteine. The catalytic histidine lies in the H(Y/F) SGHG sequence and the catalytic cysteine in the active-site pentapeptide DXCHS (where X is A or S) sequence (Piszczek and Gutman, 2007; Suarez et al., 2004). Type I plant pro-metacaspases contain a proline-rich or glutamine-rich N-terminal prodomain of about 80–120 amino acids. This prodomain contains zinc finger motifs, similar to the *Arabidopsis* regulatory protein LSD1 (LESIONS SIMULATING DISEASE 1) expressed during the hypersensitive response in plants (Coll et al., 2010; Watanabe and Lam, 2004). Type II metacaspases only exist in plants and lack the prodomain but harbor a linker region of about 90–150 amino acids between the putative large (p20-like) and small (p10-like) subunits (Rahman, 2010), whereas the linker region of Type I metacaspases is only about 30 amino acids. These metacaspases also have a relatively high degree of amino acid sequence identity, from 56 to 71% (Watanabe and Lam, 2005). Metacaspases play important roles in plant biotic or abiotic stress responses. Several *metacaspases* have been reported to involve PCD process from tomato, tobacco, arabidopsis and wheat (Coll et al., 2010; Hao et al., 2007; He et al., 2008; Wang, 2012; Watanabe and Lam, 2011a, 2011b). For example, mRNA levels of *LeMCA1*, a type-II *metacaspase* from tomato, rapidly increased upon infection of tomato leaves with *Botrytis cinerea* (Hoeberichts and Woltering, 2003). However, *LeMCA1* was not up-regulated during chemical-induced PCD in suspension-cultured tomato cells. These results demonstrate that plant PCD can be induced by metacaspase-dependent and -independent signaling pathways (Tsiatsiani et al., 2011). In most cases, *metacaspases* are involved in the process of PCD, but they do not display caspase-like activity, failing to cleave caspase-specific substrates. The results suggest that in plant cells other proteases probably have caspase activity (He et al., 2008; Vercammen, 2004; Watanabe and Lam, 2005).

Metacaspases initiate PCD during embryo patterning and provide a functional connection between PCD and embryogenesis in plants. RNA interference suppression of *mcII-Pa*, a Type II metacaspase in Norway spruce (*Picea abies*), led to failure of establishment of the embryonic mass and terminal differentiation of the embryo suspensor, which suggested that metacaspase-dependent programmed cell death is essential for plant embryogenesis (Suarez et al., 2004). Specific for caspase-6, VEIDase activity can be detected in living cells by using a fluorogenic cell-permeable peptidic substrate containing the — VEIDN-sequence. Silencing of *mcII-Pa* inhibited VEIDase activity, suppressed PCD in embryos, and blocked suspensor differentiation (Bozhkov et al., 2005b). Furthermore, VEIDase activity is an integral factor in the control of plant developmental cell death and essential for embryo pattern formation (Bozhkov et al., 2004). These results identified the effect of this metacaspase on suspensor PCD to establish a mechanistic link between PCD and plant embryo patterning.

In seedless grapes, two different types of seedlessness have been observed, parthenocarpy and stenospermocarpy (Stout, 1936). Parthenocarpy results from a failure of fertilization. By contrast, in stenospermocarpy, fertilization occurs normally but seeds fail to develop completely because of embryo and/or endosperm abortion two to four weeks after fertilization (Stout, 1936), leaving undeveloped seeds or seed traces in mature fruit. Most seedless grape varieties in production and cultivation, including cv. Thompson seedless, exhibit stenospermocarpy (Ledbetter and Burgos, 1994). Besides, the character of this phenotype is high self-initiated, stable and is not affected by the environmental factors. However, ovule abortion has not yet been characterized at the molecular level. Also, the process of ovule abortion in stenospermocarpy fits with the definition of PCD, and mechanisms of ovule abortion may involve abnormal PCD in ovule cells. Therefore, identification of the grape *metacaspase* gene family and examination of differential *metacaspase* gene expression during ovule abortion may provide key insights into regulation and execution of this important agronomic trait in *Vitis vinifera* L.

A total of 13 *metacaspase* family genes have been identified from *Arabidopsis thaliana* (9), tobacco (1), tomato (1), Norway spruce (1) and wheat (1), respectively. (Hao et al., 2007; Hoeberichts et al., 2003; Suarez et al., 2004; Tsiatsiani et al., 2011; Wang, 2012). To date, no *metacaspase* gene has been identified in grape. In this study, based on the 9 *metacaspase* sequences in *A. thaliana*, we identified *metacaspase* genes in the grape genome and obtained their full mRNA sequences. Phylogenetic analysis, exon/intron distribution analysis, and structural analysis of conserved protein motifs identified *metacaspase* sub-types. Finally, examination of expression profiles of *metacaspases* in different organs, tissues, and ovule development stages, compared between seed and seedless grape, by quantitative real-time PCR (QRT-PCR) analyses provided insights into the developmental functions of these enzymes and laid a foundation for further understanding of the stenospermocarpic seedless agronomic trait in grapes.

2. Materials and methods

2.1. Plant materials and sampling conditions

V. vinifera cv. Thompson seedless and cv. Pinot Noir were cultivated in the Grape Repository of Northwest A&F University, Yangling, Shaanxi, China under the natural environment with normal management. We used the sampling method of Shengyi Wang (Wang et al., 2011). To maintain the consistency of flowering, the already opened flowers and buds were removed from the grape inflorescence. Embryos of *V. vinifera* cv. Thompson seedless and cv. Pinot Noir were sampled after full-bloom stages of 10d, 15d, 20d, 25d, 30d, 35d, 40d, and 45d during embryo development, and then ovules were stripped out and collected in centrifuge tubes on ice. Subsequently, the tubes were quick-frozen in liquid nitrogen before storing at −80 °C. The fresh tissues and organs of root, stem, leaf, alabastrum, floral, pericarp, and pulp were sampled from the same plant of cv. Pinot Noir and also quick-frozen in liquid nitrogen and stored at −80 °C.

2.2. Identification and isolation of grapevine metacaspases

The nine known *metacaspase* cDNA sequences (*AtMC1–AtMC9*) in *A. thaliana* were downloaded from The *Arabidopsis* Information Resource (http://www.arabidopsis.org/) (Tsiatsiani et al., 2011) and used as queries for BLASTN searches for all sets of *metacaspase* genes in the high-quality, well-annotated grape genome (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/) and NCBI (http://www.ncbi.nlm.nih.gov/). This yielded non-redundant full mRNA sequences of *metacaspase* candidates. The ORFs of target mRNA sequences were determined by NCBI ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). ExPASy Proteomics Server (http://www.expasy.org/tools/#primary/) was used to identify their primary functional domains. Primer premier 5.0 was used to design primers to amplify the corresponding full ORFs (Supplemental table 1).

According to the improved SDS/phenol method previously described (Jinjin et al., 2003), total RNA was isolated from fresh cv. Pinot Noir tissues and organs of root, stem, leaf, alabastrum, floral, pericarp, pulp and ovules of each stage from *V. vinifera* cv. Thompson seedless and cv. Pinot Noir. Reverse transcription was performed with

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