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Vacuolar processing enzyme (VvβVPE) from *Vitis vinifera*, processes seed proteins during ovule development, and accelerates seed germination in *VvβVPE* heterologously over-expressed *Arabidopsis*

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ABSTRACT

Vacuolar processing enzymes (VPEs), belonging to cysteine protease, are responsible for processing seed protein during maturation. Stenospermocarpic grapes occur self-abortion in fertilized embryos during the ovule development, which affects the formation of matured seed proteins. However, little is known about VPE functions in ovule self-defeating. Here, we investigated the role of one seed-type VPE gene, *VvβVPE*. Sequence analysis showed that all ORFs (Open reading frames) of *VvβVPE* from 19 seed/seedless genotypes are highly conserved. At the transcriptional level, *VvβVPE* was specifically expressed during ovule development, with distinct expression patterns: it increased gradually in seeded grapes; while weakly expressed in seedless grapes. Whereas, at the translational level, 3 forms of VvβVPE were expressed during ovule development in seeded grape: precursor βVPE (pβVPE), intermediate βVPE (iβVPE) and finally, active mature βVPE (mβVPE). By contrast, in seedless grape, VvβVPE only exists as pβVPE at whole developmental stage of ovule. for confirming these expression patterns, 12 seeded/seedless genotypes were sampled and analyzed. Furthermore, VPE enzyme activity was increased in *Arabidopsis* overexpressing *VvβVPE*, leading to faster germination. Our study indicated that VvβVPE is essential for grapevine ovule maturation through various forms and is involved in seed germination.

1. Introduction

Legumain was classified as a member of clan CD (Caspase domain) and family C13 of cysteine proteases [1,2]. Legumain was called a vacuolar processing enzyme (VPE) or asparaginyl endopeptidase due to its localization and function in plants [3]. Therefore, VPE was localized in cell vacuoles and cleaves a peptide bond strictly at the C-terminal side of asparagines and aspartic acid. [4]. Initially, the VPE was thought to simply respond to most protein processing activity in seed storage vacuole through hydrolysing the peptide bond between the Asn and Gly to further produce functional proteins. [5]. In recent years, however, VPEs have been found to function in rupture of vacuole membrane leading to programmed cell death (PCD) in plants by promoting the hydrolysis of other vacuole proteins [6–8]. Following research revealed that VPEs also process enzymes that are involved in the PCD within the cytoplasm through the degradation of cell material [9]. They play a similar role and possess the same enzyme activity as caspase, which is well-known for regulating PCD in animal cells [10,11]. Therefore, VPE is generally considered to be the executor of the plant PCD process [9,12].

The biosynthesis and activation mode of VPEs have their own specific pattern. In the beginning, VPEs are synthesized in the endoplasmic reticulum as a pre-pro-protein precursor (ppVPE), including a signal peptide, short N-terminal propeptide, mature protease domain and long C-terminal propeptide. After co-translational removal of the signal peptide, the pro-protein precursor (pVPE) is self-catalytically activated and partial pVPEs are self-catalytically converted into the intermediate form (iVPE), in the case of the weak acidic condition (pH 5.5) after entering the vacuole [3]. Finally, the mature VPE (mVPE) was produced from the further hydrolysis of iVPE, which possesses a characteristic

Abbreviations: CBB, Coomassie brilliant blue; DAF, day after flowering; iVPE, intermediate VPE; mVPE, mature VPE; ORF, Open reading frame; PCD, programmed cell death; PN, cv.Pinot Noir; pVPE, precursor VPE; QRT-PCR, Quantitative Real-Time-PCR; (sq)RT-PCR, (semi quantitative) Reverse Transcript PCR; TS, Thompson Seedless; UTR, Untranslated region; VPE, Vacuolar processing enzyme; WT, Wild Type; YG, cv. Youngle

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catalytic dyad-cysteine/histidine in the active centre and a VPE substrate pocket similar to caspase-1 [4]. These active sites help VPEs to specifically recognize aspartic acid (Asp) or asparagine (Asn) sites in the peptide chain from corresponding substrates and, furthermore, to hydrolyse them from the C-terminal of recognition sites [4].

Four VPE family members have been identified in *Arabidopsis thaliana*, comprising αVPE, βVPE, γVPE and δVPE [13–15]. They are further divided into two subfamilies by homology and expression pattern analyses, including vegetative type αVPE and γVPE, and seed type βVPE and δVPE, respectively [16]. In the case of the vegetative type, both αVPE and γVPE participate in the maturation of proteins in typical lytic vacuoles for vegetative organs and in the PCD process induced by environmental stress and organ senescence [9,17]. Moreover, both αVPE and γVPE have been reported as playing a key role in the HR (Hypersensitive response) signal transduction pathway [17]. On the other hand, βVPE is one of the most essential enzymes to process seed storage proteins in *A. thaliana* [18], whereas there are fewer reports revealing whether βVPE is connected with regulating PCD during the seed development stage. Finally, δVPE is detected to express in the inner integument layer, where PCD occurs during the formation of seed coat. In addition, δVPE is the only enzyme from the VPE family localized outside cells [19].

All VPEs play a common important role in the processing of seed protein, especially the βVPE, which is responsible for processing most protein activity in the seed storage vacuole [10,18]. Shimada generated a series of VPE deletion mutants in *Arabidopsis* and found that more than 90% of VPE activity disappeared in the *βVPE*-deficient seeds, and these mutants resulted in the accumulation of precursor proteins [18]. In addition, VPE activity was almost undetectable in the αVPE/βVPE/γVPE/δVPE quadruple mutant [20]. However, compared with other single mutants (αVPE or γVPE) and double mutants (αVPE/γVPE), the quadruple mutant accumulates precursor protein in seeds Therefore, βVPE plays a dominant role in processing seed storage protein and the vegetative type of VPEs complement βVPE activity during this process [13].

Wang found a missense mutation of *OsVPE1* in rice, which is homologous to βVPE in Arabidopsis. Its Cys (269) site changes to Gly (269) and leads to the accumulation of glutelin precursors, demonstrating that *OsVPE1* plays an essential role in the maturation of rice glutelins [21]. Subsequently, Toshihiro and colleagues found that glutelin could not form a correct spatial structure in the protein storage vacuoles of this *OsVPE1*-deficient rice [22]. This result further suggests that *OsVPE1* is indispensable for glutelin crystal structure formation in seeds [22]. Radchuk and colleagues, considering a similar case in barley, proved that the VPE family mediates the PCD process of activating nucellus storage compounds by activating seven specifically expressed genes in the barley ovule, which suggests that VPE participates in normal growth and development processes [23].

Stenospermocarpic fruit is a vital economic trait in grapevines for producing seedless grapes [24]. The physiological process of stenospermocarpy has been described [25,26]. During the flowering process, the floral organ structure of the stenospermocarpic grapevine is normal and their pollination and fertilization occur regularly, but subsequently, the fertilized embryos undergo ovule abortion to various extents, resulting in little or even no perceptible seed trace left in mature fruits in high-quality seedless genotypes [27]. In addition, the ovule abortion of stenospermocarpic grapevines is highly stable and is determined mainly by their own genetic regulation independent of external environmental conditions. Therefore, the research into the mechanism of seedlessness and its utilizing value has remained a hot topic among grape researchers [28–31]. The endosperm nucleus generally starts to divide in the six to seven days after flowering (DAF); following that, cellularization begins around 15 to 20 DAF, and then the embryo sac is filled with endosperm in the 27 to 30 DAF. These processes are consistent with seeded grapevines. However, around 35 DAF, the endosperm of seedless grapes starts to abort and tissues also begin to disintegrate, then the whole ovule gradually degrades until, finally, only seed scars remain [25,32,33]. Each step of the process during the ovule development of seedless grapes is spontaneous and conducted in an orderly way, thus, the properties are in accord with the definition of PCD [26]. However, the mechanism of seedless ovule formation is still far from being completely understood.

To summarize, VPEs are a kind of cysteine protease which is involved in the processing of seed protein during the seed development stage to regulate the PCD process of plants. The agronomic traits of stenospermocarpic grapevines may form the abortion of fertilized embryos during ovule development, and this abortion phenomenon is similar to the PCD process, revealing that further study of VPEs in grapevines is a required way to explore the mechanism of the seedless traits of stenospermocarpic grapes. Our group previously identified three novel *VPE* genes from the grapevine [34]. Based on the sequence structure, the three *VPE* members were called *VvβVPE*, *Vvγ VPE* and *VvδVPE*. The results of quantitative real-time PCR (qRT-PCR) further showed that *VvβVPE* exhibits different expression patterns between seeded and seedless grapevines. In the present study, we use the critical organ of seedless traits, ovule (immature seeds) from seeded and seedless grapevine, as materials to clone *VvβVPE*, which is highly homologous to *βVPE* of *A. thaliana*. After further analysis via qRT-PCR technology, the result showed that the gene expressions at different stages of ovule development are significantly discrepant in the seeded genotype cv. Pinot Noir and seedless genotype cv. Thompson Seedless. More specifically, the relative expression level of *VvβVPE* in Pinot Noir increases rapidly, while in cv. Thompson Seedless, by contrast, the *VvβVPE* expressed at a relatively low level without increasing. Tissue-specific expression analysis of *βVPE* found that it is relatively specific and highly expressed in the ovule. Therefore, it can be concluded that *βVPE* might be an ovule abortion-related gene for stenospermocarpic trait grapes. Furthermore, exploration, such as *βVPE* cloning and sequence analyses in diverse grape genotypes, and expression analyses of *βVPE* at RNA and protein levels in eight tissues and organs during ovule development have been performed via bioinformation analysis, qRT-PCR and Western blot. Meanwhile, we also constructed plant expression vectors of seedless and seeded genotypes of *βVPE* which were then heterologously transformed into *Arabidopsis*. Transgenic plants were obtained and functional analysis was carried out to elucidate the seed development discrepancy among *βVPE* transgenic strains, wild type (WT) and related VPE mutants. Our findings provide an insight into the relationship between the seedless trait of stenospermocarpic grapevines and the bio-functions of the *βVPE* gene.

2. Materials and methods

2.1. Plant materials and sampling conditions

All grape plants were cultivated under a natural, field conditions germplasm resources orchard of Northwest A&F University, Yangling, Shaanxi, China. Seven seedless genotypes comprised the following cultivars Almo Seedless, Perlette, Dalihong Seedless, Sultanina Seedless, Thompson Seedless, Youngle and Fire Seedless. The eight seeded grapevines comprised Pinot Noir, Cabernet, 8th-Hutai, Carignane, Jiangxi-2, Tonghua-3, Purple Sweet [Zizhenxiang] and Muscat

The sampling method was used and described in detail by Zhang [26], with small modifications. The already opened flowers were firstly removed from the grapevine inflorescence to maintain the consistency of flowering. Grape bunches of seedless and seeded genotypes were then sampled after full-bloom stages of 10, 15, 20, 25, 30, 35, 40 and 45 days during ovule development, and the ovules were stripped out immediately and collected in Eppi tubes on ice. Subsequently, the tubes were quick-frozen in liquid nitrogen before storing at -80 ℃. The fresh tissues and organs of root, stem, leaf, alabastrum, floral, pericarp and pulp were sampled from the same plant, quick-frozen in liquid nitrogen and stored at -80 ℃.