DOI: 10.1007/s10535-014-0417-6 BIOLOGIA PLANTARUM 58 (3): 531-538, 2014

Involvement of the vacuolar processing enzyme γVPE in response of *Arabidopsis thaliana* to water stress

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Abstract

Plant vacuoles play several roles in controlling development, pathogen defence, and stress response. *γVPE* is a vacuolar-localised cysteine protease with a caspase-1 like activity involved in the activation and maturation of downstream vacuolar hydrolytic enzymes that trigger hypersensitive cell death and tissue senescence. This work provides evidence that *γVPE* is strongly expressed in *Arabidopsis* guard cells and is involved in water stress response. The *γvpe* knock-out mutants showed reduced stomatal opening and an increased resistance to desiccation suggesting a new role of *γVPE* in control of stomatal movements.

Additional key words: abscisic acid, guard cells, knock-out mutant, RT-qPCR, relative water content, stomata.

Introduction

Vacuolar processing enzymes (VPEs) were originally related to the maturation of seed storage proteins (Hara-Nishimura *et al*. 1991, 1998a,b, Kinoshita *et al*. 1995a,b, 1999) and subsequently characterised for their role in programmed cell death (Hara-Nishimura *et al*. 2005, Yamada *et al*. 2005). VPEs are endopeptidases with a caspase-1-like activity and substrate specificity towards asparagine residues (Hara-Nishimura *et al*. 2005). They are synthesised as inactive pro-protein precursors and they are self-catalytically activated by sequential removal of the N-terminal and C-terminal peptides at acidic conditions (Li *et al*. 2012). The *Arabidopsis* genome has four *VPE* homologues traditionally distributed into seeds, *βVPE* and *δVPE*, and vegetative tissues, *αVPE* and *γVPE* (Kinoshita *et al.*1995b, 1999, Hara-Nishimura *et al*. 1998a, Gruis *et al.*2002). However, a reinvestigation of *αVPE* and *γVPE* expression revealed that *γVPE* is the only *VPE* gene for which a significant transcription is detected in vegetative tissues (Gruis *et al.* 2004, Misas-Villamil *et al.* 2013) and that significant amounts of *α*- and *γ-VPE* transcripts are present in developing seeds (Gruis *et al.* 2004). The βVPE accumulates in protein storage vacuoles and has the main responsibility for the maturation of seed proteins (Yamada *et al*. 2005). VPE-mediated processing has been demonstrated to be the key machinery for proper processing storage proteins in *Arabidopsis* seeds, as the lack of the *βVPE* gene causes the accumulation of the precursors of storage proteins in mutant seeds (Shimada *et al*. 2003, Yamada *et al*. 2005). However, the analysis of various mutants lacking *α*- and *γ-VPE* genes demonstrated their involvement in this process together with *βVPE* (Shimada *et al*. 2003) in agreement with their expression in seeds (Gruis *et al.* 2004). On the contrary, *δVPE,* despite being abundantly expressed in seed coat, has no detectable impact on seed protein processing (Shimada *et al*. 2003, Gruis *et al*. 2004), whereas it is a key player in the developmental programmed cell death of cell layers of the inner integument in seed coat (Hara-Nishimura *et al*. 2005). The activities of *α*- and *γ-VPE* are also associated with cell death processes; they are localised in the lytic vacuoles of vegetative tissues (Kinoshita *et al*. 1999) in

Submitted 4 June 2013, *last revision* 17 December 2013, *accepted* 24 January 2014.
Abbreviations: ABA - abscisic acid; RT-qPCR, real-time quantitative polymerase chain reaction; RWC - relative water content; VPE - vacuolar processing enzyme.
Acknowledgments: This work was supported by grants from the "Fondo per la Promozione di Accordi Istituzionali", project BIOGESTECA 15083/RCC. A. Albertini and F. Simeoni contributed equally to this work.
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which senescence occurs and their expression is up-regulated by wounding and by treatments with ethylene and salicylic acid (Yamada *et al*. 2004, 2005). Lytic vacuoles are responsible for several types of plant programmed cell death, including hypersensitive cell death, tissue senescence, and differentiation of tracheary elements (Kuriyama and Fukuda 2002, Lam 2004, Van Doorn and Woltering 2005). Different results have demonstrated the role of *α*- and *γ-VPE* in vacuole-mediated hypersensitive cell death (Hatsugai *et al*. 2004, 2006, Kuroyanagi *et al*. 2005, Li *et al*. 2012) through the activation of hydrolytic enzymes that firstly cause the collapse of the lytic vacuoles and, when released into the cytosol, attack various organelles and nuclear DNA, crucial events in plant cell death (Hara-Nishimura *et al*. 2005, Yamada *et al.*2005, Hatsugai *et al*. 2006). So far, the research of plant VPEs has mainly focused on terminal differentiation, plant senescence, and pathogen-induced hypersensitive cell death.

By contrast, the role of VPEs in response to abiotic stresses that adversely affect growth, metabolism, and yield (recently reviewed by Hussain *et al*. 2011, Thapa *et al*. 2011), is poorly understood (Li *et al*. 2012). Only the involvement of *Arabidopsis* γVPE and of its putative radish orthologue RsVPE1 has been recently demonstrated in response to heat shock stress (Li *et al*. 2012, Zhang *et al*. 2013a).

The complex interaction between biotic and abiotic stresses has been extensively investigated (Atkinson and Urwin 2012, Lee and Luan 2012, Roberts *et al*. 2013, Zhang *et al*. 2013b). In the natural environment, plants are constantly challenged with biotic and abiotic stresses and they activate specific stress responses when subjected to multiple stresses (Atkinson and Urwin 2012). Biotic and abiotic stress responses are controlled by a range of molecular mechanisms that act together in a complex regulatory network (Atkinson and Urwin 2012). Stomatal movements represent an example of the complex crosstalk of biotic and abiotic stress responses, in fact opening and closure of stomata not only regulate water loss during abiotic stress, such as drought, but also serve as defense mechanism in preventing pathogen invasions (Lee and Luan 2012). Although in the past, stomata have been considered as passive portal for plant pathogen entrance, Melotto *et al*. (2006) demonstrated an active role for stomata in the plant defence response, as they closed in response to the presence of different bacteria, restricting the pathogenic invasion.

A transcriptomic analysis of *Arabidopsis* guard cells revealed that within the four *VPE* genes, *γVPE* is the most expressed in these cells (Bauer *et al*. 2013), supporting another recent study of the *Arabidopsis* guard cell transcriptome that indicates *γVPE* as one of the 50 most abundant guard cell transcripts (Bates *et al*. 2012). The aim of this paper was to show that the γVPE protein is involved in water stress response. To support the idea of the involvement of VPEs in biotic and abiotic stress responses, the knock-out mutants *γvpe* were used.

Materials and methods

All *Arabidopsis thaliana* L. plants were of the Col-0 background. Two mutant alleles of the *γVPE* gene were used: the *γvpe::T-DNA1* (SALK_010372), previously described by Gruis *et al*. (2004), and the *γvpe::T-DNA2* (SALK_024036), both provided by the *NASC European Arabidopsis Stock Center* (Nottingham, UK). Plants were grown in soil containing a loam sandy soil and peat (4:1, v/v), in a growth chamber at a temperature of 22 °C, a relative humidity of 70 %, a 16-h photoperiod, and an irradiance of 100 μmol m^{-2} s^{-1} except where otherwise stated. Homozygous *γvpe::T-DNA1* and *γvpe::T-DNA2* mutants were isolated by PCR genotyping (data not shown). The position of the T-DNA insertion was determined by sequence analysis of the T-DNA/gene junctions.

Three rosette leaves were detached from four 3-week-old wild type and mutant plants and dehydrated on *Whatman 3MM Chr* filter paper at 22 °C and an irradiance of 100 μmol m^{-2} s^{-1}. After 1/2, 2, 4, 6, and 8 h, the leaves were weighed and the water content was expressed as $[(Mt_0 - Mt_n) / Mt_0] \times 100$, where Mt_0 corresponds to the mass of the leaf at time 0 and Mt_n to the mass of the leaf at time point n.

The wild type, *γvpe::T-DNA1*, and *γvpe::T-DNA2* mutant plants were grown in pots and well irrigated with tap water for 24 d. Before starting a drought stress, the pots were covered with a *Saran* wrap to avoid water loss from the soil. Water was withheld for 10 d and the plants were harvested for a relative water content (RWC) analysis. Leaves from the rosette were excised with a razor blade and immediately weighed (fresh mass, FM). The samples were rehydrated for 24 h in the dark on a water-saturated tissue paper in a close Petri dish (cut surface down) and then weighted again (turgid mass, TM). Finally, the leaf samples were dried in a 65 °C oven for at least 48 h and weighed (dry mass, DM). The RWC was estimated as: $RWC = [(FM - DM)/(TM - DM)] \times 100$.

A stomatal density and a stomatal index were determined on abaxial epidermis strips from seven 6-week-old wild type and mutant plants grown under conditions previously described. The stomatal density was expressed as number of stomata per mm^2, whereas the stomatal index was expressed as number of stomata/(number of stomata + number of epidermal cells) × 100. The measurements of the stomatal density and index were performed using a *DM2500* (*Leica*, Solms, Germany) optical microscope. Stomatal aperture measurements were performed on abaxial epidermis strips from 4-week-old wild type and mutant plants, incubated in Petri dishes in 10 mM KCl, 25 mM MES-KOH, pH 6.1, and exposed to an irradiance of 300 μmol m^{-2} s^{-1} in a growth chamber at 22 °C for 1 h to

assure that most stomata were open before beginning the experiments. To test the stomatal closure induced by dark, the Petri dishes were moved to the darkness for 1 h. For the abscisic acid (ABA; *Sigma*, Milano, Italy) treatment, the epidermis peels were incubated for 2 h with 10 μM ABA (or the same volume of ethanol as solvent control) added to the MES buffer. Also, whole young leaves were excised and incubated in the same buffer and conditions as described above. For the ABA treatment, the leaves were incubated for 2 h with 10 μM ABA added to the MES buffer. The leaves were then peeled and analysed. The measurements of stomatal aperture and pore area were performed using a *Leica DM2500* optical microscope and the *LAS Image Analysis* (*Leica*) software.

For the ABA treatment, the wild type plants were grown in a growth chamber under a 16-h photoperiod and after four weeks, they were sprayed with 100 μM ABA. Whole leaves were harvested at different time points. For guard cell purification leaves from 6-week-old wild type and mutant plants were harvested and mid veins were removed with a razor blade. The leaves were whisked with a blender in ice-cold deionised water with crushed ice for 1 to 2 min and then filtered through a 210 μm nylon net. After two further rounds of whisking, the dark green tissue fragments, remaining on the nylon net and containing different cell types, were removed with tweezers, whereas the light green epidermal fraction, enriched in guard cells, was analyzed. By vital staining, more than 90 % of the living cells were identified as guard cells (Geiger *et al*. 2011). Total RNA from different samples was extracted using a *E.Z.N.A.* plant RNA kit (*Omega Bio-tek Inc.*, Norcross, USA) and reverse transcription was performed using 5 μg of DNase-treated RNA with RT *Superscript™ II* (*Life Technologies*, Paisley, UK), according to the manufacturer's instructions. The RT-qPCR reaction was performed using the fluorescent intercalating dye *SYBR-Green* with the *Cfx96TM BioRad®* real time system, according to the manufacturer's protocol, in a final volume of 0.02 cm^3, containing cDNA samples diluted 1:20, 0.3 - 0.4 μM primers, and 0.01 cm^3 of 2× *SOS Fast™ EVA-Green® Supermix* (*BioRad*, Hercules, CA, USA). *VPEs* expression was analysed using primers γVPE-F and γVPE-R for *γVPE* (*At4g32940*), αVPE-F and αVPE-R for *αVPE* (*At2g25940*), βVPE-F and βVPE-R for *βVPE* (*At1g62710*), and δVPE-F and δVPE-R for *δVPE (At3g20210*). *ACTIN2* (*At3g18780*) and *PP2AA3* (*At1g13320)* were used as references for normalisation (Table 1). All primers gave the expected amplification on wild type genomic DNA (data not shown). A relative quantification was analysed using the *iCycler™ iQ* optical system software *v. 3.0a* (*BioRad*). PCR experiments were repeated three times. The protocol used was as follows: 95 °C for 2 min, followed by 55 cycles of 95 °C for 15 s, 60 °C for 30 s. A melt curve analysis was performed following each run to ensure a single amplified product for each reaction (from 55 to 95 °C, increment 0.5 °C for 10 s). Data analyses were performed as previously reported (Giuntini *et al*. 2008).

Table 1. The sequence of oligonucleotides used in this study.

Genes	Forward primers(5'-3')	Reverse primers (5'-3')
γVPE	CGTCTTCGCATTTGTTTATACCAC	TGAGATTGACAACTCCCAAGTTTAC
αVPE	ATCTTCGTCTCCGCCCTATATTA	CACTGTTTCTTTTGTCTGTGACATC
βVPE	TACAATACCTACTCAGGTGGCTCTC	GATACATATGCCAAAGGAAGAGAAG
δVPE	TGTACCAGAGACTTCTCATGTATGC	AGAATGTCTGTAATGCTCTGATCG
ACT2	TGCTTCTCCATTTGTTTGTTTC	GGCATCAATTCGATCACTCA
PP2AA3	CAGCAACGAATTGTGTTTGG	AAATACGCCCAACGAACAAA

Results

As different transcriptomic analyses revealed that the *γVPE* gene is highly expressed in guard cells (Bates *et al.* 2012, Bauer *et al.* 2013), we performed a RT-qPCR analysis on the four *VPE* genes in the whole leaves and guard cells. Among the *VPE* genes, the *γVPE* was highly expressed in both samples, particularly in the guard cells, followed by *βVPE* and then by *α*- and *δ*-*VPE* for which the transcription was nearly undetectable (Table 2). The *γVPE* transcription was transiently increased up to 4 h of the ABA treatment in both the guard cells and entire rosette leaves, and decreased at 8 h. The guard cell kinetics preceded the leaf one (Table 3). The results suggest that the *γVPE* expression was responsive to ABA,

Table 2. The RT-qPCR expression analysis of *VPE* genes in *Arabidopsis* whole leaves and guard cells. The expression level of *βVPE* in the leaves was used as calibrator for the relative expressions in each line. The *ACTIN2* and *PP2AA3* genes were used as controls. Data represent means ± SD of three biological replicates.

Gene	Leaves	Guard Cells
αVPE	0.10 ± 0.00	0.08 ± 0.01
βVPE	1.00 ± 0.09	3.82 ± 0.55
γVPE	26.45 ± 1.50	58.36 ± 7.10
δVPE	0.01 ± 0.00	0.00 ± 0.00

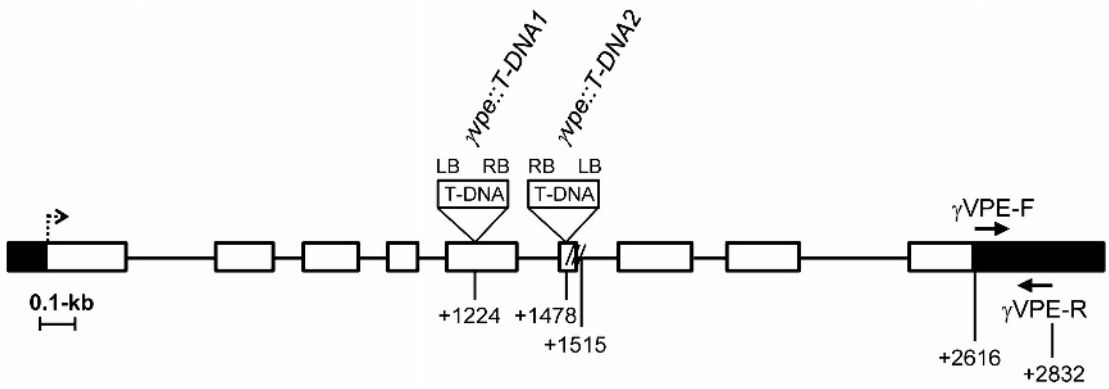

Fig. 1 A schematic representation of the *γVPE* gene. *Boxes* indicate exons (*white*) and the 5' and 3' UTR (*black*), *black lines* indicate introns. The translational start of the gene is represented by a *black arrow*. The positions of the T-DNA insertions in the *γvpe::T-DNA1* and *γvpe::T-DNA2* mutants are represented as *inverted triangles* and their orientations and positions from the ATG are shown. *Diagonal lines* indicate the lacked DNA sequence in the *γvpe::T-DNA2* mutant. Scale (0.1 kb) is marked.

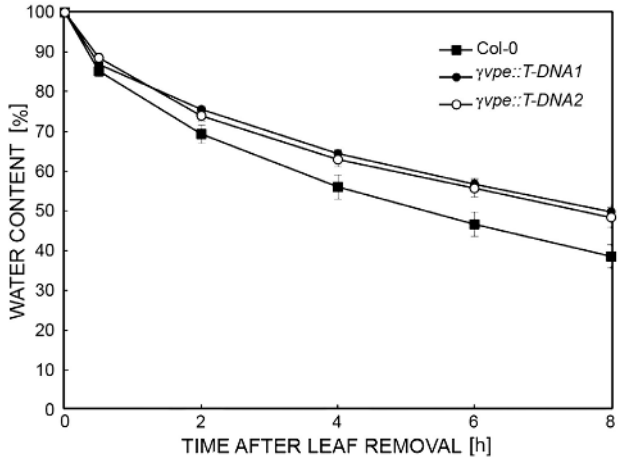

Fig. 2 Water loss measurements in detached leaves of *γvpe::T-DNA1, γvpe::T-DNA2*, and wild type, expressed as percentage of the initial fresh mass at indicated intervals. Means ± SE, $n = 12$ (3 leaves from 4 individual plants). Results shown are representatives of three independent experiments.

a well-known stress-response hormone (Raghavendra *et al*. 2010) and that *γVPE* may be involved in an ABA response in the guard cells as well as in other leaf tissues.

To investigate the potential role of γVPE in response to drought, we isolated two *γVPE* allele mutants: *γvpe::T-DNA1*, previously described (Gruis *et al*. 2004), and *γvpe::T-DNA2*, carrying a T-DNA insertion within exon 5 and exon 6, respectively. The T-DNA insertion sites were confirmed by sequencing the T-DNA/gene junctions (Fig. 1). In the *γvpe::T-DNA2* mutant, there was a deletion of the sequence comprised between positions +1478 bp and +1515 bp from the start codon, probably due to a rearrangement caused by the insertional element (Fig. 1). No *γVPE* transcript was detected in the two mutants by the RT-qPCR analysis, neither using primers designed in the 3'UTR (Fig. 1), the most specific region for *VPE* genes (Table 4), nor using primers flanking the T-DNA insertion (data not shown). No morphological or developmental abnormalities were observed in homozygous mutant plants compared with the wild type when grown under standard conditions (data not shown). To test the role of γVPE in response to desiccation, we measured the water loss in detached *γvpe* mutant rosette leaves. Throughout the duration of the experiment, the mutant leaves lost significantly less water than the wild type ($P < 0.05$, Fig. 2). To estimate a whole-plant transpiration rate under water scarcity, we determined the relative water content (RWC) of both the *γvpe::T-DNA1* and *γvpe::T-DNA2* mutants after suspension of irrigation. The RWC represents a useful indicator of water balance of plant as it considers interactions among all the factors involved in maintaining the flow of water through plant (Gonzalez and Gonzalez-Vilar 2001). The plants were properly irrigated for 24 d and then subjected to the drought stress by complete termination of irrigation. No significant differences between the wild type and mutant plants could be observed in the dry mass (data not shown). After 10 d of absence of irrigation, the RWC was more reduced in the wild type plants compared with the *γvpe* ones ($P < 0.001$, Table 5).

Since the water status in leaves is mainly controlled by stomatal opening and closure and the *γVPE* gene is highly expressed in guard cells (Table 2), we examined the stomatal density and index (Table 6). Except a very

Table 3. The RT-qPCR expression analysis of the *γVPE* gene in *Arabidopsis* leaves and guard cells in response to 100 μM ABA. The expression of *γVPE* in leaves at time 0 h was used as calibrator for the relative expression in each line. The *ACTIN2* and *PP2AA3* genes were used as controls. Data represent means ± SD of three biological replicates.

Time [h]	Leaves	Guard Cells
0	1.00 ± 0.03	2.61 ± 0.36
1	0.58 ± 0.09	5.18 ± 0.29
4	14.66 ± 1.25	11.30 ± 0.84
8	1.65 ± 0.29	1.52 ± 0.19

Table 4. The RT-qPCR expression analysis of *γVPE* gene in wild-type, *γvpe::T-DNA1*, and *γvpe::T-DNA2* plants. *γVPE* primers positions are shown in Fig. 1. The Col-0 wild-type sample was used as calibrator for the relative expression in each line. The *ACTIN2* and *PP2AA3* genes were used as controls. Means ± SD of three biological replicates.

Genotype	Relative *γVPE* expression
Col-0	1.00 ± 0.03
γvpe::T-DNA1	0.00 ± 0.00
γvpe::T-DNA2	0.00 ± 0.00

Table 5. Relative water content (RWC) [%] of wild-type, *γvpe::T-DNA1*, and *γvpe::T-DNA2* plants during drought. Means ± SD, n = 16. The Student's t-test was performed for statistical analysis, and different letters within a column indicate statistically significant differences between the mutant and Col-0 plants at $P < 0.001$.

Genotype	Control	Drought [10 d]
Col-0	84.79 ± 1.01a	59.18 ± 3.30a
γvpe::T-DNA1	82.20 ± 0.69a	72.93 ± 1.66b
γvpe::T-DNA2	83.39 ± 1.04a	78.12 ± 2.45b

small increase in the stomatal density observed only in the the *γvpe::T-DNA2* mutant compared to the wild type, no other differences were detected. Then we analysed the stomatal movements in epidermal peels of the *γvpe* mutants. Under irradiance, the stomata of *γvpe* were less open than those of the wild type ($P < 0.005$, Fig. 3*A*), whereas no significant differences in the stomatal aperture were detected between the mutants and the wild type after being moved to the darkness for 1 h (Fig. 3*A*). Stomatal movements are mainly controlled by ABA (Zimmerli *et al*. 2012) which is able to induce the *γVPE* expression (Table 3). However, the ABA-induced stomatal closure was similar in the *γvpe::T-DNA1* and *γvpe::T-DNA2* and wild type plants (Fig. 3*B*), indicating that a guard cell response to ABA is intact in the *γvpe* mutants. As ABA promotes stomatal closure in a dual way, *via* its well-known effect of ion transport in guard cells, and through a decrease in water permeability in leaf vascular tissues (Pantin *et al.* 2013), we investigated also a response to the ABA treatment of intact leaves. We did not observe any differences in the ABA response between the wild type and the mutant plant in stomatal pore area (Fig. 3*C*) confirming that ABA responses in leaves of mutants were not affected. We also did not observe any differences in stomatal area between the wild type and the mutant plants under the control conditions (Fig. 3*C*). These apparently contrasting data suggest that the incubation of the epidermal peels under irradiance triggered a response that limited stomatal opening in the mutant, whereas under the normal conditions, the presence of other tissues than only epidermis in intact leaves attenuated this response.

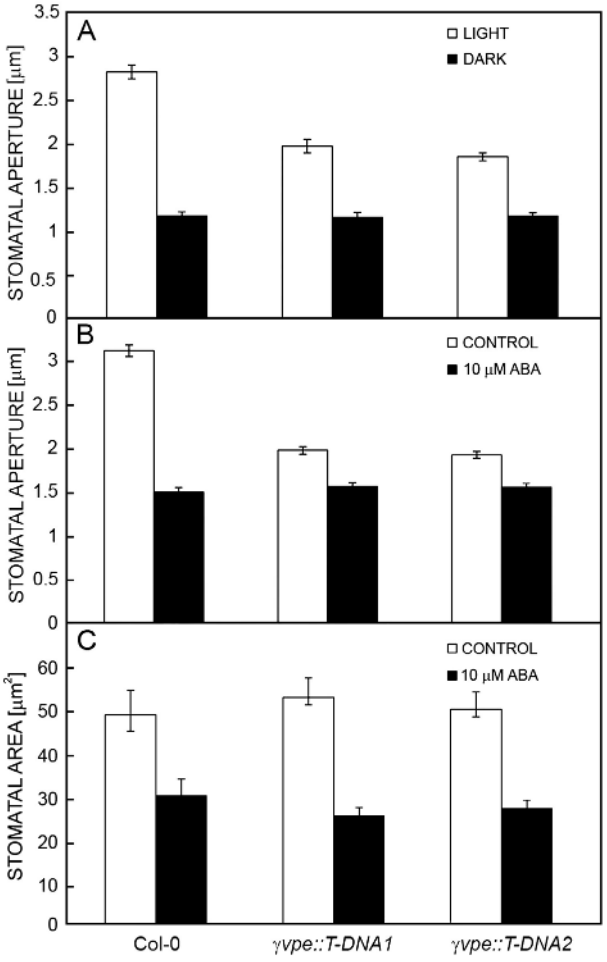

Fig. 3 Stomatal aperture in epidermal peels of Col-0, *γvpe::T-DNA1*, and *γvpe::T-DNA2* plants in dark and light conditions (*A*) and after a 2 h treatment with 10 μM ABA (*B*). Stomatal area in the epidermal peels of the Col-0, *γvpe::T-DNA1*, and *γvpe::T-DNA2* plants after a 2 h treatment with 10 μM ABA on the whole leaf (*C*). Means ± SD, n ~100. Results shown are representatives of three independent experiments.

Table 6. Stomatal density [mm^{-2}] and stomatal index of wild-type, *γvpe::T-DNA1*, and *γvpe::T-DNA2* plants. One rosette leaf from seven individual plants for each genotype were analysed. Means ± SD, n = 150. Different letters within a column indicate statistically significant differences between the mutant and Col-0 plants at $P < 0.001$ (the Student's t-test).

Genotype	Stomatal density	Stomatal index
Col-0	161.78 ± 8.32a	0.23 ± 0.01a
γvpe::T-DNA1	178.25 ± 9.56a	0.25 ± 0.01a
γvpe::T-DNA2	153.66 ± 10.27a	0.29 ± 0.01b

Discussion

This study is an attempt to understand the possible role of the *γVPE* gene in the response to water stress. The *γVPE* encodes for a vacuolar processing enzyme with a caspase-1 like activity, already characterised for its involvement in tissue senescence and pathogen-induced hypersensitive cell death (Yamada *et al*. 2005). Through a transcriptome analysis performed on *Arabidopsis* guard cells, a strong expression of the *γVPE* compared to the other three *VPE* genes present in its genome was observed (Bauer *et al*. 2013). We confirmed this result by the RT-qPCR analysis and found that the *γVPE* expression in the guard cells was higher than in the entire leaves (Table 2) suggesting that this gene could play a fundamental role in guard cells.

In *Arabidopsis*, the VPEs function in the maturation of seed storage proteins, leaf senescence, and plant cell death (Hara-Nishimura *et al*. 2005, Hatsugai *et al*. 2006, Hara-Nishimura and Hatsugai 2011), but their possible role and the molecular mechanism of their activity in regulating stomatal movements have not been described so far. There are only indications of a *VPE* role in guard cells in *Nicotiana tabacum*, where the suppression of *NbVPE1a* and *NbVPE1b*, the most abundant *VPE*s in tobacco, affects stomatal closure triggered by elicitors (Zhang *et al*. 2010). The phenotypic analysis on the *Arabidopsis γvpe* mutant plants showed a reduced sensitivity to the water stress compared to the wild type plants: the suppression of the *γVPE* gene significantly reduced the water loss from the plant both in the detached leaves and the whole plants (Fig. 2 and Table 5). Moreover, we observed an impaired aperture of mutant stomata in the epidermal peels after the incubation under irradiance (Fig. 3*A*). However, there were no differences between wild type and mutant stomatal pore areas when the peels from the plants grown under the control conditions were immediately analysed without the incubation (Fig. 3*C*). These quite unexpected data suggest that in the context of the intact leaves under the normal growth conditions mutant stomata behaved in a normal way, hiding a phenotypic alteration evident only through the analysis of epidermal peels consisting mainly of guard cells. It is not clear which mechanism is responsible for this opposite stomatal behaviour. It is possible to speculate that another *VPE* gene may be partially redundant with *γVPE* only in the intact leaves. The *γvpe* mutants did not show any alterations in the ABA-induced stomatal closure (Fig. 3*B*,*C*) either on the epidermal peels or on the intact leaves. Nevertheless, our data suggest that a *γVPE* transcript accumulation both in the purified guard cells and in the entire rosette leaves is induced by ABA (Table 3), a key endogenous messenger involved in stomatal closure by triggering K^+ efflux and release of anions with a consequent reduction in pressure potential (Sirichandra *et al*. 2009). ABA also modulates the expression of many stress-responsive genes (Agarwal and Jha 2010). From microarray analysis of plants overexpressing the AtMYBR1 transcription factor, it resulted that *γVPE* expression is regulated by AtMYBR1 (Jaradat et al. 2013). The *AtMYBR1* gene was previously characterised for its involvement in stomatal movements (Jung *et al.* 2008). Recently it was shown that the *AtMYBR1* gene expression was induced in response to ABA, but the *mybr1* knock-out mutant was more tolerant to drought (Jaradat *et al.* 2013). Jaradat and colleagues (2013) suggested that AtMYBR1 is a negative regulator of ABA and stress response. Under non-stressed conditions ABA treatment induces unnecessary stress responses. AtMYBR1 role consists in blocking and restoring normal pattern of expression of its target genes. On the other side, under water stress conditions, when the *AtMYBR1* gene is repressed or in the knock-out mutant, the absence of its activity allows the effects of ABA to be fully manifested. Also, *γVPE* is downregulated by drought in intact leaves (Winter *et al.* 2007). As *γVPE* is transcriptionally regulated by AtMYBR1, *γVPE* is then probably a part of the complex regulatory network demonstrated for AtMYBR1. Its molecular role in this context remains elusive, however, it is tempting to speculate about some hypotheses. *γVPE* is located in the vacuole, where it exhibits a proteolytic activity towards the carbonyl terminal of asparagine or aspartic acid residues (Hara-Nishimura *et al*. 2005). It activates downstream vacuolar hydrolytic enzymes, such as nucleases, lipases, and proteases (Yamada *et al*. 2005). Plant vacuoles play a universal role in the generation of Ca^{2+} signals, since they are the major calcium storages in cells, and can store a large amount of other solutes affecting the osmotic potential (Peiter 2011). Guard cell vacuoles have a fundamental role in controlling stomatal movements as the uptake or release of ions and organic metabolites lead to changes in the osmotic and pressure potentials (Blatt 2000). We can assume that *γVPE*, as vacuolar proteolytic enzyme, could activate some proteins involved in the Ca^{2+} storage or release, acting on the signal cascades that trigger stomatal opening. By now, only a few γVPE targets have been identified: the vacuolar carboxy-peptidase AtCPY, a protease enzyme directly activated by γVPE, and the vacuolar invertase AtFruct4, a sucrose cleavage enzyme, although AtFruct4 is probably not directly degraded by γVPE but rather by other proteases activated by γVPE (Rojo *et al*. 2004). Stomatal opening and closure are directly regulated by changes in the solute content of guard cells (Sirichandra *et al*. 2009) and the invertase irreversibly hydrolyzes sucrose to fructose and glucose (Ni 2012). Further analyses will be necessary to demonstrate and clarify this interaction and to identify other γVPE targets to better understand the complex mechanism that regulates stomatal opening and closure. This work provides evidence of a new role of γVPE in stomatal movements and consequently in water stress response.

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