Loss-of-function of vacuolar-type H+ pyrophosphatase gene lead to reduce in stomatal aperture and density

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Abstract. Transgenic plants over-expressing vacuolar H+ type pyrophosphatase (V-PPase) gene were reported to display drought resistance, reduced vacuolar pH and raised stomatal conductance. To further understand the role of V-PPase on stomatal regulation, loss- and gain-of-function approaches were combined for analysing relationship between stomatal aperture and V-PPase gene expression. Homozygous mutants of this gene were isolated by polymerase chain reaction (PCR) method. BCECF-AM fluorescence probe was used for detecting cellular pH. The result here indicated that Arabidopsis plant lines over-expressing of V-PPase gene displayed raised stomatal aperture. Both of stomatal aperture and density of homozygous vpp mutants were less than that of control plants. In addition, cellular pH of guard cells in vpp mutants was higher than control evidently. In general, our results suggested that V-PPase activity regulates stomatal aperture by changing guard cell pH.

1. Introduction
Pyrophosphatase (EC3.6.1.1) hydrolyses pyrophosphate (PPi) to phosphate ions. This enzyme is involved in hydrolysis of pyrophosphate formed in various metabolic pathways of proteins, carbohydrates, nucleic acids, etc. Based on their solubility and subcellular localizations, pyrophosphatases are classified into two isoforms: a soluble cytosolic inorganic pyrophosphate and an insoluble pyrophosphate, which tightly bound to membrane (V-PPase). Inorganic pyrophosphatase was first purified from yeast in 1950s. And plant V-PPase was first isolated from root of sugar beet [1]. Thereafter cloning of V-PPase cDNA from Arabidopsis thaliana, Iris lactea, Jatropha curcas, maize, pear, Suaeda corniculata, Suaeda salsa, wheat and tobacco has been obtained successively [2-11]. V-PPase not only hydrolyses pyrophosphate to two inorganic phosphorus molecules, but also pumps H+ from the cytoplasm into the membrane through the vacuole membrane, thus acting as a proton pump. Therefore, V-PPase should be involved in regulating cellular pH of guard cells. Indeed, transgenic plants over-expressing V-PPase gene with a lower cellular pH compare with wild-type plants [12]. However, it was found that Arabidopsis V-PPase also catalyse the reverse reaction for synthesizing PPi in vacuolar acidification and cytosolic PPi scavenging recently [13].

Research on V-PPase has been focused primarily at leaf development defects, auxin transport and stress resistance. Over-expression of V-PPase conferred biomass and yield increase, drought resistance, and salt resistance in plants [14-21]. Arabidopsis V-PPase loss-of function mutants displayed disruption of auxin transport inhibition [22], gluconeogenesis inhibition [23] and development defects [22-24].

The roles of sugar metabolism in regulation of stomatal aperture received increasing interest recently. The content of glucose-6-phosphate was demonstrated to be positively correlated with stomatal aperture [25]. Over-expression of hexokinase genes leads to decrease of stomatal aperture under highly light radiation, meanwhile promote stomatal opening under low light stress [26]. We have found that activity of vacuolar invertase in guard cells was associated stomatal aperture recently [27], and we suggested a
hypothesis that stomatal aperture was regulated by guard cell saccharase activity in a pH dependent manner [28]. Furthermore, sucrose synthase, another sucrose decomposition enzyme, was also reported to regulate stomatal aperture [29-31].

*Arabidopsis* line with ectopic expression of a *Sophora Alopecuroid V-PPase* gene was studied here for further understand the effect of cellular pH on stomatal movement. Since overexpressing V-PPase gene was shown to increase stomatal conductance and decreased vacuolar pH in plants [12]. Changes of stomatal conductance should be caused by stomatal aperture or density alteration normally. We found that the transgenic *Arabidopsis* plants, whose V-PPase genes were overexpressed, displayed increased stomatal aperture. This result prompted us to investigate whether this stomatal aperture increase was caused by cellular pH changed in V-PPase loss-of-function mutants. Here, we show that disruption of V-PPase in *Arabidopsis* leads to decrease stomatal aperture and cytosolic alkalinization in guard cell. Phytohormone abscisic acid (ABA) also resulted in concentration of indole acetic acid (IAA) increasing, stomatal aperture reduction and alkalinization cytosolic in guard cell [32]. Here we also show that treatment with auxin receptor antagonists resulted in cytosolic alkalinization in *Arabidopsis* guard cells.

2. Materials and methods

2.1. Plant materials

The plant materials used in this study includes *Arabidopsis thaliana* wild-type (Col-0 ), and T-DNA insertion line in At1g15690 (Gabi-kate line 005D04 and Gabi-kate line 596C07, hereafter vpp-1 and vpp-2). The seeds of aforementioned plants were surface-sterilized with 70% ethanol followed by 10% sodium hypochlorite. After 2 weeks of culturing on medium containing half MS formula with 30 mg•L⁻¹ sucrose in a culture room, the plantlets were transferred to soil and cultured in an artificial illumination incubator for two weeks, and in a greenhouse until flowering at 22 ℃. The artificial illumination incubator and the greenhouse provide 16-h light and 16-h light a day, respectively.

2.2. Chemicals

The fluorescein probe 2′,7′-bis(2-carboxyethyl)-5(6)-carboxy fluorescein-acetoxy methyl ester was purchased from Thermo Fisher. Auxin receptor antagonists, PEO-IAA and auxinole, were kindly provided by Dr. Ken-ichiro Hayashi [31][32]. Other chemicals were purchase from Sangon Biotech (www.sangon.com) and were of the highest analytical grade.

2.3. Measuring stomatal aperture and monitoring cellular pH

Epidermal strips of *Arabidopsis* leaves were prepared, and samples were observed with a Motic fluorescence microscope connected to a MTR3CCD06000KPA digital camera. The data was calculated by Image J (image analysis software). Stomata were opened by incubating the epidermal strips under an opening buffer. The value of width divided by length was calculated as stomatal aperture. The changes in cellular pH were measured by a pH monitor, BCECF-AM [33]. In detail, the epidermal strips were treated with ABA, IAA and auxin antagonist after stomata opening in light for 3h, respectively. And they were loaded with buffer containing BCECF-AM for 10 min at 25°C without light. Stomatal aperture measurements were repeated more than three times. About 100 aperture measurements were calculated per treatment.

3. Results

3.1. Over-expression of V-PPase gene induces increased stomatal aperture

Several research groups reported that transgenic plants over-expressing V-PPase genes confer drought resistance [12][14][36]. Meanwhile, transgenic plants, in which V-PPase gene were over-expressed, have higher stomatal conductance compared with control [12]. It seems that aforementioned phenomena opposed each other, since plants with higher stomatal conductance are not drought-resistant normally. The raise of stomatal conductance might attribute to stomatal aperture or stomatal density. To
further understand this, we used a transgenic Arabidopsis thaliana expressing V-PPase gene from Sophora Alopecuroid [36] for analyzing stomatal aperture. The present results showed that transgenic Arabidopsis plants confer foreign V-PPase gene have higher stomatal aperture compared with wild-type plants (Fig. 1).

![Fig. 1. Over-expression of V-PPase gene induces increased stomatal aperture. VPP-1 and VPP-8 were two independent lines of transgenic Arabidopsis thaliana expressing V-PPase gene from Sophora Alopecuroid controlled by 35S promoter. For stomatal aperture measurements, n = 50.](image)

3.2. vpp mutants display increased cellular pH, decreased stomatal aperture and density

Disruption of AtV-PPase leads to decrease stomatal aperture and cytosolic alkalinization in guard cell. To further examining the role of AtV-PPase in regulating stomatal aperture, two T-DNA insertion lines were obtained from GABI-Kat, hereafter referred to as vpp1-1 and vpp1-2, respectively (Fig. 2A). Homozygous vpp mutants were screened by PCR method with specific primers designed according to the AtV-PPase genomic close to DNA insertion sit on both sites and agrobacteria T-DNA sequence (Table 1). We found that homozygous vpp mutants have smaller stomatal aperture (Fig. 2B). In accordance with this, vpp mutants also have smaller stomatal density (Fig. 2C).
Fig. 2. Disruption of *AtV-PPase* leads to decrease stomatal aperture and cytosolic alkalinization in guard cell. (a) *AtV-PPase* gene structure and location of T-DNA insertions in the *vpp1-1* (Gabi-kate line 005D04) and *vpp1-2* (Gabi-kate line 596C07) mutant alleles. (b) Stomatal aperture of wild-type plants, *vpp1-1* and *vpp1-2* was compared. The width divided by the was calculated as stomatal aperture. (c) Stomatal density of *vpp* mutants and control plants. The stomatal density was calculated by number of stoma in a microscope field. (d) Cellular pH in guard cells in *vpp* mutants. The cellular pH in guard cells was monitored by BCECF-AM fluorescence probe. For stomatal aperture measurements, n = 50; for stomatal density measurements, n=30 and for cellular pH measurements, n=5.

| Table 1. Primer sequences |
|---------------------------|
| **Name of primers**      | **Sequences**          |
| vpp1-1F                  | CCTTTTGTTTTGTTTGGTC   |
| vpp1-1R                  | TGAACCTGCTGCGAACTT    |
| vpp1-2F                  | TCTATCTGGTTTCTTGAG    |
Transgenic plants over-expressing V-PPase gene not only decreased cellular pH, but also increased stomatal conductance compared to wild-type plants [12]. Therefore, we measured guard cells pH of vpp mutants using BCECF-AM. We found that vpp mutants guard cells were more alkaline than that in wild-type plants (Fig. 2D).

3.3. Treatment with auxin receptor antagonists results guard cells alkalinization

Exogenous application of ABA induces stomatal closure normally, and cellular vacuolar pH increases 0.5 units gradually 15 minutes during ABA application [37]. In addition, ABA treatment rapidly decreases indole acetic acid (IAA) concentrations in Arabidopsis leaf cells, while auxin receptor antagonist (PEO-IAA) also induced stomatal closure [32]. As stated above, ABA is likely to regulate cell cellular pH via IAA. In other words, auxin receptor antagonists might also lead to cytosolic alkalinization in guard cells. To confirm this, auxin receptor antagonists (PEO-IAA and auxinole) were added before cellular pH detection. We found that cellular pH of guard cells was increased after PEO-IAA or auxinole treatment (Fig. 3).

Fig. 3. Auxin receptor antagonists treatment results in cytosolic alkalinization in guard cells. Guard cell pH increased after auxin receptor antagonists (PEO-IAA and auxinole) treatment. The cellular pH in guard cells was monitored by BCECF-AM fluorescence probe. For cellular pH measurements, n=5.

4. Discussion

Here, we found that V-PPase over-expression plants displayed enhanced stomatal aperture (Fig. 1) and loss of function mutants displayed diminished stomatal aperture compared to wild-type plants (Fig. 2b). It suggested that expression level of AtV-PPase gene is positively correlated with stomatal aperture. The exact mechanism of this regulation remains to be elucidated. A possible explanation could be that V-PPase activity or V-PPase gene expression level regulates stomatal behavior via modulation of cellular pH or vacuolar pH in guard cells. Because transgenic plants with high level of V-PPase gene expression decreased cellular pH [12], and the present result also showed V-PPase mutation lead to increase cellular pH (Fig. 2). Low pH is known to promote stomatal opening [38], high pH is associated with stomatal aperture [37][39]. We have suggested that vacuolar invertase (VIN) regulates stomatal/aperture in a cellular pH dependent manner base on the finding that guard cell vacuolar invertase activity regulates stomatal aperture and it might be altered by cellular pH fleetly [28]. It is also possible that inorganic phosphate concentration in guard cells was mediated by V-PPase expression.

Pi inhibited sucrose phosphate synthase (SPS) activity via allosteric action[38][39] (Doehlert & Huber 1983; Volkert et al. 2014), and the accumulation of sucrose in plant cells is positively correlated with SPS activity [42]. Sucrose within guard cells was proposed to be an osmolytic substance in regulating stomatal movement [30]. Indeed, overexpressing sucrose catalytic enzyme genes leads to
stomatal closure [30][27]. Several research groups reported that transgenic plants over-expressing V-PPase genes confer drought resistance [2] [14] [36]. It seems that we came to conclusions opposed to theirs, because our results here showed that transgenic plants over-expressing V-PPase genes displayed increased stomatal aperture. However, the absorption ability of root and photosynthesis efficiency might play an important role in drought resistance [12].

Cellular pH is also regulated by phytohormone ABA. Here we show that cellular pH of guard cells was increased after treating by auxin receptor antagonists and decreased by IAA (Fig. 4). Due to that IAA level in guard cell decreased during exogenous ABA application [32], ABA might regulate cellular pH via IAA reduction. It will be interesting to understand if V-PPase activity depend on endogenous ABA or IAA concentration.

![Fig.4](image-url)

Fig.4. ABA and IAA treatment results in cytosolic alkalinization acidification and in guard cells, respectively. Guard cell pH increased after ABA treatment and decreased after IAA application. The guard cell pH monitored by BCECF-AM fluorescence was associated with the cellular pH, for cellular pH measurements, n=5.

5. Conclusions
In general, the results here indicated that V-PPase regulates stomatal aperture and drought resistance via cellular pH changes.

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