AtPV42a and AtPV42b Redundantly Regulate Reproductive Development in Arabidopsis thaliana

Lei Fang, Xingliang Hou, Li Yen Candy Lee, Lu Liu, Xiaojing Yan, Hao Yu*

Department of Biological Sciences and Temasek Life Sciences Laboratory, National University of Singapore, Singapore, Republic of Singapore

Abstract

Background: The conserved SNF1/AMPK/SnRK1 complexes are global regulators of metabolic responses in eukaryotes and play a key role in the control of energy balance. Although β-type subunits of the SnRK1 complex have been characterized in several plant species, the biological function of β-type and γ-type subunits remains largely unknown. Here, we characterized AtPV42a and AtPV42b, the two homologous genes in Arabidopsis, which encode cystathionine-β-synthase (CBS) domain-containing proteins that belong to the PV42 class of γ-type subunits of the plant SnRK1 complexes.

Methodology/Principal Findings: Real-time polymerase chain reaction was performed to examine the expression of AtPV42a and AtPV42b in various tissues. Transgenic plants that expressed artificial microRNAs targeting these two genes were created. Reproductive organ development and fertilization in these plants were examined by various approaches, including histochemical analysis, scanning electron microscopy, transmission electron microscopy, and phenotypic analyses of reciprocal crosses between wild-type and transgenic plants. We found that AtPV42a and AtPV42b were expressed in various tissues during different developmental stages. Transgenic plants where AtPV42a and AtPV42b were simultaneously silenced developed shorter siliques and reduced seed sets. Such low fertility phenotype resulted from deregulation of late stamen development and impairment of pollen tube attraction conferred by the female gametophyte.

Conclusions: Our results demonstrate that AtPV42a and AtPV42b play redundant roles in regulating male gametogenesis and pollen tube guidance, indicating that the Arabidopsis SnRK1 complexes might be involved in the control of reproductive development.

Introduction

As sessile organisms, plants are exposed to a constantly changing environment. It is therefore essential for them to sense and integrate endogenous and environmental stimuli to generate suitable cell responses for optimizing growth and development [1]. The control of energy balance is one of the crucial factors for such adaptive processes in plants, which involves a group of plant protein kinases, the SNF1-Related Kinase 1 (SnRK1) family [2].

SnRK1 is a serine/threonine kinase that has a catalytic domain similar to that of Sucrose non-fermenting 1 (Snf1) from yeast and AMP-activated protein kinase (AMPK) from mammals [2,3]. In yeast, Snf1 is one of the main regulators of carbon metabolism and mediates the diauxic shift from fermentative to oxidative metabolism in response to glucose starvation [4,5]. AMPK, the mammalian counterpart of SnF1, is an energy sensor that regulates energy balance by activating the processes that produce energy, while inhibiting those that consume energy [6–8]. In plants, SnRK1-type kinases play an important role in the global regulation of metabolism, and are also involved in plant development and stress responses [1]. SnRK1s from different plant species can complement the yeast snf1Δ mutant phenotype, demonstrating an evolutionary conservation in their function [9–15].

SnF1, AMPK-α, and SnRK1 serve as the catalytic α-subunits that are associated with other two regulatory subunits (β-type and γ-type) in the conserved heterotrimeric kinase SnF1/AMPK/SnRK1 complexes found in fungi, mammals and plants [1]. Association of the three subunits in SnF1/AMPK/SnRK1 complexes is differentially regulated by various hormonal and environmental signals, cell and tissue types, and developmental stages. In yeast, β subunits anchor α and γ subunits, thus directing the kinase complexes into their targets or specific subcellular localizations, while γ subunits function in activating the kinase activity of α subunits [16–19].

Three γ-type subunits (AMPKγ1, AMPKγ2, AMPKγ3) in mammals have been identified as being homologous to the γ subunit of the SnF1 complex, SnF4, in yeast [20]. Furthermore, phylogenetic analysis of SnF4-like plant proteins has revealed three subgroups of γ-type subunits in plants: AKINβγ, AKINγγ, and PV42-type proteins [3,15,21–23]. While it has been shown that AKINβγ contributes to SnRK1 heterotrimeric complexes in Arabidopsis and is possibly involved in plant-pathogen interactions.
the biological function of AKINγ- and PV42-type proteins remains unclear.

The conservation among γ-type subunits in fungi, mammals and plants partly lies in the four cystathionine-β-synthase (CBS) domains found in these proteins [1,24]. The CBS domain was first discovered in the genome of the archaeabacterium Methanococcus jannaschii [24,25]. It is about 60 residues long, and composed of a sheet of three β strands packed with two α helices. CBS domains have been found to bind to metallic ions such as Mg2+ and adenosyl compounds such as AMP, ATP, and S-adenosyl-L-methionine, which may trigger a conformational change in the CBS domains, thus regulating the activity of associated enzymatic domains [26–28]. The CBS domain-containing proteins comprise a large family of evolutionarily conserved proteins that have been found in all kingdoms of life, among which the mammalian ones are so far the best characterized. In humans, CBS domain-containing proteins are highly diversified and have been found to undertake various biological roles, ranging from metabolic enzymes and transcriptional regulators to ion channels and transporters [29]. In contrast, very few information is available for the CBS domain-containing proteins in plants. So far, 48 Arabidopsis proteins have been designated as CBS domain-containing proteins [29], which include γ-type subunits of the SnRK1 complex.

In this study, we show that AtPV42a and AtPV42b, the two homologous genes in Arabidopsis, encode CBS domain-containing proteins that belong to the PV42 class of γ-type subunits of the plant SnRK1 complexes. They are expressed in different tissues throughout the developmental stages of Arabidopsis. Artificial microRNA-mediated silencing of both AtPV42a and AtPV42b exhibits the defects in late stamen development and pollen tube attraction conferred by the female gametophyte, which results in reduced seed sets. These results suggest that AtPV42a and AtPV42b play redundant roles in regulating male gametogenesis and pollen tube guidance in Arabidopsis.

Results

AtPV42a and AtPV42b are putative γ-type subunits of the plant SnRK1 complexes

AtPV42a (At1g13530) and AtPV42b (At1g00090) genes are two close homologues in Arabidopsis. The AtPV42a gene consists of 2 exons and 1 intron, while AtPV42b consists of 5 exons and 4 introns (Figure 1A). A BLAST search against the NCBI protein database revealed that AtPV42a and AtPV42b were two Arabidopsis proteins homologous to PV42 from Phaeosolus vulgaris, which is a founding member of the PV42 class of γ-type subunits of the plant SnRK1 complexes [3,23]. AtPV42a and AtPV42b shared 60% and 54% amino acid identity with PV42, respectively. Multiple sequence alignment and protein domain analysis revealed that like other γ-type subunits of the Snf1/AMPK/SnRK1 complexes, such as Snf4 from yeast, AtPV42a and AtPV42b contained four CBS domains (Figure 1B). These sequence analyses imply that both AtPV42a and AtPV42b are putative members in the PV42 class of γ-type subunits of the Arabidopsis SnRK1 complex.

Expression of AtPV42a and AtPV42b in Arabidopsis

To examine the spatial and temporal expression patterns of AtPV42a and AtPV42b in Arabidopsis, real-time PCR analyses were performed with gene-specific primers using total RNA extracted from various tissues in 28-day-old adult plants and from the whole seedlings at different developmental stages (3-, 8-, 14-day-old). Overall, AtPV42a and AtPV42b exhibited a similar spatial expression pattern in most of tissues examined in adult plants (Figure 2A). Their expression was relatively high in rosette leaves, cauline leaves, open flowers, and developing siliques, but low in stems and floral buds. The only discrepancy was that the relative expression of AtPV42b in roots as compared with other tissues was lower than that of AtPV42a. It is noteworthy that both AtPV42a and AtPV42b were expressed at the highest levels in dry seeds (Figure 2A). In the seedlings 3, 8, and 14 days after germination, the expression of both genes remained at stable levels, with a slight decrease in transcripts levels concomitant to an increase in seedling age (Figure 2B).

We further performed in situ hybridization to study the expression of these two genes in developing flowers. Both genes were detectable in the developing septum inside the gynoecia and microspore mother cells in the locules of stage 9 flowers (Figure 2C, F). Their expression levels were either low or absent in anther cells in the flowers at late stages (Figure S1), but persistent in developing ovules in stages 11 and 13 flowers. In stage 11 flowers in which integuments were just initiated on the ovules, the expression of both genes was detected in funiculi and ovules (Figure 2D, E, G, H). In stage 13 flowers in which integuments completely enveloped the nucellus, both genes were also expressed in whole ovules (Figure 2I, J).

High sequence similarity and comparable gene expression patterns between AtPV42a and AtPV42b indicate that they may play similar roles in Arabidopsis growth and development.

Artificial microRNA-mediated silencing of AtPV42a and AtPV42b

To investigate the biological roles of AtPV42a and AtPV42b in Arabidopsis, we first attempted to identity insertion mutants from public resources. While insertion mutants of AtPV42a were not available in all public resources searched, a SAIL line (CS823876) containing a T-DNA insertion at the last exon of the AtPV42a gene was obtained from Arabidopsis Biological Resource Center and named as atpv42a-1 (Figure 1A). The T-DNA insertion and the resulting disrupted transcription of AtPV42a in atpv42a-1 were confirmed by genotyping PCR and RT-PCR using the primers flanking the insertion site, respectively (Figure S2, Figure S3). atpv42a-1 did not exhibit visible phenotypes under normal growth conditions. This could be due to two reasons: (1) the incomplete AtPV42a transcript produced from the coding region preceding the T-DNA insertion site may still function; (2) functional redundancy between AtPV42a and AtPV42b may exist.

To create knockout lines for AtPV42a and AtPV42b, we generated amiR-atpv42a and amiR-atpv42b-1 transgenic plants that expressed artificial microRNAs specifically targeting these two respective genes. We obtained 17 and 20 independent transformants for amiR-atpv42a and amiR-atpv42b-1 at the T1 generation, respectively. None of the 17 amiR-atpv42a transgenic lines showed visible phenotype, whereas 9 amiR-atpv42b-1 lines developed shorter siliques and reduced seed sets (Figure 3A–C). Since AtPV42a and AtPV42b shared high sequence similarity, microRNAs designed for either of them could simultaneously affect the expression of the other in transgenic plants. Thus, the expression of both AtPV42a and AtPV42b was examined in rosette leaves of 7 and 10 selected amiR-atpv42a and amiR-atpv42b-1 T1 transformants, respectively. As expected, the expression levels of AtPV42a were dramatically decreased, whereas the levels of AtPV42b were not significantly changed in the 7 amiR-atpv42a transfectants (Figure 2K). However, in most of the 10 selected amiR-atpv42b-1 lines, the transcript levels of both AtPV42a and AtPV42b were significantly downregulated, and such downregulation was relevant to the low fertility phenotype exhibited by these transgenic
Figure 1. Sequence analysis of AtPV42a and AtPV42b. (A) Gene structures of AtPV42a and AtPV42b. A triangle indicates the T-DNA insertion site in atpv42b-1 (CS823876). Black boxes, grey boxes, and lines represent exons, introns, and untranslated regions, respectively. (B) Alignment of CBS-domain containing proteins from plants including Arabidopsis (At), Phaseolus vulgaris (Pv), and Medicago truncatula (Mt), and yeast (Saccharomyces cerevisiae). Identical residues are marked with asterisks. Conserved and semi-conserved substitutions are denoted by ':' and '.', respectively. The overlined CBS domains were predicted using Pfam in the following website (http://www.sanger.ac.uk/Users/agb/CBS/CBS.html). A triangle indicates the position of the T-DNA insertion in atpv42b-1.

doi:10.1371/journal.pone.0019033.g001
Figure 2. Expression of AtPV42a and AtPV42b in wild-type and transgenic plants. (A) Transcript levels of AtPV42a and AtPV42b in various tissues from 28-day-old adult plants and dry seeds of Col wild-type. R, root; RL, rosette leaf; St, stem; CL, cauline leaf; Bud, unopen floral bud; OF, open flower; Si, silique; DS, dry seed. (B) Transcript levels of AtPV42a and AtPV42b in the seedlings 3, 8, and 14 days after germination. (C-J) In situ hybridization of AtPV42a and AtPV42b in wild-type developing flowers. (C, F) Transverse section of a stage 9 flower hybridized with the antisense AtPV42a (C) or AtPV42b (F) probe. Arrows indicate the labelled septum inside the gynoecia, while arrowheads indicate some labelled microspore mother cells in the locules. Bars, 100 μm. (D, E, G, H) Transverse section of a gynoecium from a stage 11 flower hybridized with the antisense (D) or sense probe (E) of AtPV42a or the antisense (G) or sense probe (H) of AtPV42b. f, funiculus; o, ovule. Bars, 50 μm. (I, J) Longitudinal section of a gynoecium from a stage 13 flower hybridized with the antisense AtPV42a (I) or AtPV42b (J) probe. Bars, 70 μm. (K) Transcript levels of AtPV42a and AtPV42b in rosette leaves of 7 selected amiR-atpv42a independent transgenic lines at the T1 generation. (L) Transcript levels of AtPV42a and AtPV42b in rosette leaves of 10 selected amiR-atpv42b-1 independent transgenic lines at the T1 generation. Asterisks indicate the transgenic lines showing the low fertility phenotype as shown in Figure 3A. Transcript levels in (A, B, K, L) were determined by real-time PCR and are shown relative to TUB2 expression. Values are the mean ± standard deviation from three replicates.

doi:10.1371/journal.pone.0019033.g002
lines (Figure 2L, Table 1). These observations demonstrate that plants are defective in reproductive development only when the expression of both \( \text{AtPV42a} \) and \( \text{AtPV42b} \) is significantly compromised, suggesting that \( \text{AtPV42a} \) and \( \text{AtPV42b} \) function redundantly in controlling \( \text{Arabidopsis} \) reproductive development. Therefore, we chose the transgenic line 10 of \( \text{amiR-atpv42b-1} \) (hereafter referred to as \( \text{amiR-atpv42b-1} \)) (Figure 2L, Table 1), which displayed the strongest phenotypes, for further morphological and molecular characterization.

To confirm the functional redundancy of \( \text{AtPV42a} \) and \( \text{AtPV42b} \), the transgenic line 3 of \( \text{amiR-atpv42a} \) (hereafter referred to as \( \text{amiR-atpv42a} \)) (Figure 2K) was crossed with the T-DNA insertion mutant \( \text{atpv42b-1} \) (Figure 1A). The resulting homozygous progenies, where the expression of both \( \text{AtPV42a} \) and \( \text{AtPV42b} \) is disrupted (Figure S4), exhibited a similar low fertility phenotype to \( \text{amiR-atpv42b-1} \) (Table 1). This is in agreement with our suggestion that \( \text{AtPV42a} \) and \( \text{AtPV42b} \) function redundantly in reproductive development.

To further test whether a single knockdown of \( \text{AtPV42b} \) impairs the reproductive process, we generated \( \text{amiR-atpv42b-2} \) transgenic plants overexpressing another artificial microRNA targeting \( \text{AtPV42b} \). Real-time PCR assay showed that in 6 selected \( \text{amiR-} \)
Table 1. Phenotypic analysis of wild-type, amiR-atpv42b-1, and amiR-atpv42b-2 seeds.

|                | Normal | Unfertilized | Aborted |
|----------------|--------|--------------|---------|
| Col            | 467 (92.8%) | 29 (5.8%) | 7 (1.4%) |
| amiR-atpv42b-1 (line 2) | 203 (40.0%) | 297 (58.6%) | 7 (1.4%) |
| amiR-atpv42b-1 (line 3) | 191 (35.1%) | 331 (60.8%) | 22 (4.0%) |
| amiR-atpv42b-1 (line 10) | 74 (14.3%) | 443 (85.4%) | 2 (0.4%) |
| amiR-atpv42a atpv42b-1 | 259 (57.3%) | 187 (41.4%) | 6(1.3%) |
| amiR-atpv42b-2 (line 4) | 483 (91.7%) | 31 (5.9%) | 13 (2.5%) |

For each genotype, the phenotype was scored using fully grown siliques from at least 10 plants.

doi:10.1371/journal.pone.0019033.t001

amiR-atpv42b-1 is defective in late stamen development

To uncover the developmental events responsible for the reduced fertility in amiR-atpv42b-1, we compared the morphology of amiR-atpv42b-1 and wild-type plants at different developmental stages. amiR-atpv42b-1 appeared normal during the vegetative phase, floral transition, and early stages of flower development (data not shown). In amiR-atpv42b-1 floral buds at stage 12, floral organs were still morphologically normal (Figure 3D, E, H, I), whereas at the anthesis stage (flower stages 13 and 14), fewer pollen grains were produced (Figure 3F, G, J, K), whereas at the anthesis stage (flower stages 13 and 14), fewer pollen grains were produced (Figure 5), indicating that microspores in amiR-atpv42b-1 lost most of the cell contents (Figure 5), indicating that there is a continuous improper cellular osmotic homeostasis from another stage 9 to 12. These TEM results explain why the microspores at another stage 11 in amiR-atpv42b-1 demonstrate reduced amount of cell contents (Figure 4C).

Taken together, these observations suggest that downregulation of AtPV42a and AtPV42b in amiR-atpv42b-1 results in the production of some collapsed pollen grains particularly at late stages of anther development. This might partly contribute to the reduced fertility observed in amiR-atpv42b-1.

Female gametophytes of amiR-atpv42b-2 are defective in pollen tube reception

To determine whether the defective stamen development is the only cause of the low fertility found in amiR-atpv42b-1, reciprocal crosses between amiR-atpv42b-1 and wild-type plants were performed. After saturated pollination with either wild-type or amiR-atpv42b-1 pollen grains, the majority of wild-type ovules were normally fertilized (Table 2). However, more than 50% of amiR-atpv42b-1 ovules were unfertilized regardless of the father plants (Table 2). These results indicate that female tissues in amiR-atpv42b-1 are partly defective. As the morphology of amiR-atpv42b-1 carpels during flower development was almost normal (Figure 3H-K), we then examined the ovules inside the carpels before and after fertilization using SEM. amiR-atpv42b-1 ovules were morphologically comparable to wild-type ones prior to fertilization (Figure 6A, D); SEM of wild-type pistils two days after saturated pollination with either wild-type or amiR-atpv42b-1 pollen grains revealed that most of the ovules have been fertilized (Figure 6B, C), which is in agreement with the observation on seed sets in wild-type plants (Table 2). This implies that some healthy pollen grains produced in amiR-atpv42b-1 could function normally during fertilization. On the contrary, many amiR-atpv42b-1 ovules were not fertilized after saturated pollination regardless of the source of pollen grains (Figure 6E, F), demonstrating the fertilization defect in amiR-atpv42b-1 female gametophytes.

We further investigated the growth of pollen tubes inside the pistils after reciprocal crosses to explore the underlying mechanism of the fertilization defect in amiR-atpv42b-1. At 20 hours after saturated pollination, pistils were collected, fixed, cleared, stained with aniline blue, and observed under UV microscope (Figure 6G–J). Likewise, the pollen tube growth was independent of the source of pollen grains, but mainly relied on the maternal plants used. In wild-type pistils pollinated with either wild-type or amiR-atpv42b-1 pollen grains, pollen tubes elongated longitudinally through the stylar transmitting tract from the stigma, and then elongated laterally to the ovules for fertilization (Figure 6G, H). However, in amiR-atpv42b-1 pistils, although the longitudinal pollen tube growth through the transmitting tract was similar to that in wild-type pistils, the lateral pollen tube growth towards the ovules was largely abolished (Figure 6I, J). These results demonstrate that the fertilization defect in amiR-atpv42b-1 pistils is mainly due to abnormal pollen tube attraction by ovules.

As synergid cells are responsible for guiding pollen tubes to the embryo sac by secreting chemical attractants [31,32], we tested the expression of several synergid cell-specific genes in amiR-atpv42b-1 and found that the expression of LORELEI (LRE) was altered.
It has been reported that LRE is involved in proper pollen tube reception [33]. In lre mutants, pollen tubes reaching the embryo sac frequently continue to grow inside the embryo sac, resulting in the failure of fertilization. In amiR-atpv42b-1, where pollen tube attraction was blocked, the expression level of LRE was significantly increased in open flowers (Figure S6), indicating that disruption of pollen tube guidance to the embryo sac in amiR-atpv42b-1 might be relevant to the upregulation of LRE.

**Figure 4. Pollen grain development in wild-type and amiR-atpv42b-1.** (A) Scanning electron micrograph (SEM) of mature pollen grains collected from wild-type flowers at stages 13–14. (B) SEM of pollen grains collected from amiR-atpv42b-1 flowers at stages 13–14. The majority of the pollen grains are shrunken and exhibit a collapsed morphology (arrowheads). (C) Transverse sections of wild-type and amiR-atpv42b-1 anthers at anther stages 9, 10, 11, and 12. Arrowheads indicate defective pollen grains in amiR-atpv42b-1 anthers. Bars, 50 μm.

doi:10.1371/journal.pone.0019033.g004
Plant SnRK1-type kinases, which serve as the catalytic \(\alpha\)-subunits and are associated with non-catalytic \(\beta\)-type and \(\gamma\)-type subunits in the SnRK1 complexes, play important roles in the global regulation of metabolism in response to cellular and environmental signals [1]. Studies in a wide range of plant species have shown that SnRK1s are involved in the regulation of various developmental processes. For example, disruption of the SnRK1 kinase in transgenic barley plants results in abnormal pollen development and male sterility [34]. The rice SnRK1A protein kinase acts as an important intermediate in the sugar signaling cascade and plays a key role in regulating seed germination and seedling growth [35]. In the moss \textit{Physcomitrella patens}, downregulation of SnRK1 kinases shows pleiotropic phenotypes including developmental abnormalities [36]. Although these studies have shed light on the function of the catalytic \(\alpha\)-subunits in the SnRK1 complexes, the biological function of \(\gamma\)-type subunits and their effects on plant development are so far unknown.

In this study, we have characterized \textit{AtPV42a} and \textit{AtPV42b}, two close homologues encoding CBS domain-containing proteins in the PV42 class of \(\gamma\)-type subunits of the SnRK1 complexes in \textit{Arabidopsis}. Downregulation of \textit{AtPV42a} and \textit{AtPV42b} not only disturbs late stamen development, but also impairs pollen tube reception conferred by the female gametophyte, which eventually results in the low fertility. The function of \textit{AtPV42a} and \textit{AtPV42b} in the reproductive development is consistent with their expression in floral organs. Both genes are expressed in the developing septum inside the gynoecia and microspore mother cells in stage 9 flowers, and later in developing ovules. These results suggest that the non-catalytic \(\gamma\)-type subunits in the SnRK1 complexes play an important role in mediating plant reproductive growth. This is in accordance with a previous finding that shows the involvement of an \(\alpha\)-type subunit of the SnRK1 complexes in pollen development in barley [34].

In \textit{amiR-atpv42b-1} where \textit{AtPV42a} and \textit{AtPV42b} are downregulated, defective stamen development is mainly attributed to abnormal microgametogenesis. Histological analysis of anther transverse sections and SEM and TEM of pollen cells demonstrate that anther defects in \textit{amiR-atpv42b-1} mainly occur in microspores at the mitotic phase. Pollen development is divided into two phases, microsporogenesis and microgametogenesis [37–39]. During microsporogenesis, pollen mother cells undergo meiosis and form tetrads of microspores within the pollen sacs. During microgametogenesis, separate microspores undergo mitosis and differentiate into mature pollen grains. In this process, enzymes

![Figure 5. Transmission electron microscopy of wild-type and \textit{amiR-atpv42b-1} pollen grains.](image)

**Table 2. Phenotypic analysis of reciprocal crosses between wild-type and \textit{amiR-atpv42b-1} plants.**

| Ovule x Pollen | Normal | Unfertilized | Aborted |
|----------------|--------|--------------|---------|
| Col x Col      | 254 (92.4%) | 7 (2.5%) | 14 (5.1%) |
| Col x \textit{amiR-atpv42b-1} | 245 (85.4%) | 37 (12.9%) | 5 (1.7%) |
| \textit{amiR-atpv42b-1} x Col | 146 (46.8%) | 160 (51.3%) | 6 (1.9%) |
| \textit{amiR-atpv42b-1} x \textit{amiR-atpv42b-1} | 133 (43.8%) | 158 (52.0%) | 13 (4.3%) |

Flowers were emasculated and fertilized manually with pollen. The phenotype was examined one week after fertilization. doi:10.1371/journal.pone.0019033.t002
are abundant and metabolism is highly active in microspores, which obtain nutrients and water from degenerated tapetum and undergo an asymmetric mitotic division to generate a large vegetative cell and a small generative cell. The generative cell further undergoes a second mitosis to form two generative cells. It is noteworthy that active carbohydrate metabolism not only provides energy for cell division and differentiation during microgametogenesis, but also stores essential substances required for subsequent pollen germination and pollen tube growth. As the SnRK1 complex is the global regulator of carbohydrate metabolism [40], it may also be involved in microgametogenesis. This hypothesis is partly supported by our observation that disruption of AtPV42a/42b, which encode the γ-type subunits of the SnRK1 complexes, compromises microgametogenesis, thus resulting in abnormal pollen grains.

amiR-atpv42b-1 also exhibits impaired pollen tube attraction by the female gametophyte. In Arabidopsis, synergid cells accompanying the egg cell are primarily responsible for the pollen tube guidance to the female gametophyte and the release of sperm cells [31,32,41]. Intracellular metabolism is highly active in synergid cells, which uptake and transport metabolites into the embryo sac, and secrete some chemical substances into the filiform apparatus that is involved in pollen tube guidance and reception [42–45]. So far the molecular mechanisms underlying the function of synergid cells still remain largely unknown. Several genes specifically expressed in synergid cells, including MYB98, ZmEA1, and LRE, have been suggested as important regulators for pollen tube guidance [32,33,43]. Our results have shown that LRE is upregulated in amiR-atpv42b-1, which might be partially responsible for the defect in pollen tube attraction. LRE encodes a putative plant-specific GPI-anchor protein (GAP), a eukaryotic protein that functions as a lipid raft in cell–cell signalling. LRE is required for proper pollen tube guidance to the embryo sac in Arabidopsis and silencing of LRE results in continuous pollen tube growth inside the embryo sac [33].

Figure 6. Phenotypic analyses of reciprocal crosses between wild-type and amiR-atpv42b-1 plants. (A) SEM of wild-type ovules at flower stage 12 prior to fertilization. (B, C) SEMs of developing seeds 2 days after pollination from the following reciprocal crosses: Col × Col (B) and Col × amiR-atpv42b-1 (C). (D) SEM of amiR-atpv42b-1 ovules at flower stage 12 prior to fertilization. (E, F) SEMs of developing seeds 2 days after pollination from the following reciprocal crosses: amiR-atpv42b-1 × Col (E) and amiR-atpv42b-1 × amiR-atpv42b-1 (F). Arrows indicate the unfertilized ovules. (G–J) Aniline blue staining of pollen tube growth inside pistils collected 20 hours after pollination from the following reciprocal crosses: Col × Col (G), Col × amiR-atpv42b-1 (H), amiR-atpv42b-1 × Col (I), and amiR-atpv42b-1 × amiR-atpv42b-1 (J). Arrows indicate the lateral growth of pollen tubes. Bars in (A-F), 100 μm.

doi:10.1371/journal.pone.0019033.g006
possible that the SnRK1 complex plays a role in the intracellular metabolism of synergid cells. Thus, downregulation of \textit{AtPV42a} and \textit{AtPV42b} may affect normal metabolism of synergid cells, which is relevant to the altered expression of key regulators, such as \textit{LRE}. This eventually results in the impaired pollen tube attraction by the female gametophyte.

Taken together, our results suggest that \textit{AtPV42a} and \textit{AtPV42b} play redundant roles in regulating male gametogenesis and pollen tube guidance. It will be interesting to further investigate how they interact with other subunits of the SnRK1 complex to regulate metabolic responses and contribute to the regulation of \textit{Arabidopsis} development. In addition, the highest expression levels of \textit{AtPV42a} and \textit{AtPV42b} are detected in dry seeds (Figure 2A). It has been reported that disruption of the \textit{\alpha}-type subunit of the SnRK1 complex retards seed germination in rice [35]. Thus, besides the roles in regulating microgametogenesis and pollen tube guidance, \textit{AtPV42a} and \textit{AtPV42b} might also play a role in mediating the function of SnRK1 complexes in post-fertilization processes, such as seed development. This will be another intriguing topic to be further studied.

Materials and methods

Plasmid construction
The artificial microRNAs (amiRNAs) and the primers for subsequent cloning were designed according to the procedures and criteria on the website (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) [46] using the mir319a precursor-containing plasmid pRS300 as a template. Predicated mature miRNA sequences were 5'-UGAAACGCUACCUACACUCU-3' for \textit{amiR-atpv42a}, 5'-UAAUGUACUAUGUGUCAGG-3' for \textit{amiR-atpv42b-1}, and 5'-UUACUGUCCUAAUGGACCGAU-3' for \textit{amiR-atpv42b-2}. Primers used in the construction of \textit{amiR-atpv42a} and \textit{amiR-atpv42b-1} are listed in Table S2. The final PCR products were digested with EcoRI and BamHI, and then cloned into the corresponding sites of the pGreen0229-35S binary vector [47].

To construct 35S:\textit{AtPV42a}, the coding region of \textit{AtPV42a} was amplified using the primers 5'-ATAAGATTCAGTGCAGTA-GAACCTGATGCAAAG-3' and 5'-GAAGTCGTTAATTAGGAAAGAGTATGATCCTAGG-3'. Similarly, to construct 35S:\textit{AtPV42b}, the coding region of \textit{AtPV42b} was amplified using the primers 5'-GAGCTGAGCATGAAATGAA-TGGAAGATTCAGTGCAGTA-GAACCTGATGCAAAG-3' and 5'-AAATGGAGTTGCGGATTTAAAACAGAT-CC-3'. The PCR products were digested and cloned into the pGreen0229-35S binary vector [47].

Arabidopsis transformation
Agrobacterium \textit{tumeficiens} GV3101 was used for floral dipping according to the published method with minor modifications [48]. Transgenic plants were screened for herbicide resistance against Basta (300 mg/l).

Expression analysis
Total RNA from different organs was isolated using the FavorPrep™ Plant Total RNA Mini Kit (Favorgen) and reverse transcribed using the SuperScript™ RT-PCR System (Invitrogen). Real-time PCR was carried out using the Power SYBR® Green PCR Master mix (Applied Biosystems) as previously reported [49]. Primers used in real-time PCR amplifications are listed in Table S2. Non-radioactive in situ hybridization and synthesis of RNA probes were carried out as previously published [49].

Aniline blue staining of pollen tube growth
Aniline blue staining of pollen tube growth in pistils was carried out as previously described [50].

Histological analysis
Inflorescence apices of wild-type and \textit{amiR-atpv42b-1} plants were collected, fixed overnight in the fixative solution (2.5% (v/v) glutaraldehyde in 1×PBS), dehydrated using increasing concentrations of ethanol (30%, 40%, 50%, 60%, 70%, 85%, 95%, 4×100%), embedded in histowax, and transverse-sectioned (8 μm) using a microtome. The transverse sections were deparaffinised by histoclear and rehydrated with a graded ethanol series (95%, 80%, 60%, and 30%). After rinsing with water, the sections were stained in 1% toluidine blue for 1 min and observed under a compound microscope.

Scanning electron microscopy (SEM)
Mature pollen grains collected from wild-type and \textit{amiR-atpv42b-1} flowers after anther dehiscence were spread onto the surface of adhesive tapes, and directly observed under a JSM-6360LV scanning electron microscope (JEOL) at an accelerating voltage of 20 kV. Pistils were collected 1 to 2 days after pollination, and carefully opened with a sharp needle. SEM of pistils was performed as previously reported with some modifications [51]. Pistils were fixed with FAA (50% ethanol, 3.7% formaldehyde, and 5% acetic acid) for 2 h, and dehydrated through increasing concentrations of ethanol (30%, 50%, 70%, 80%, 90%, and 100%). After replacing absolute ethanol with the transfer liquid (amyl acetate), pistils were critical-point dried using CO2, mounted for sputter coating with gold palladium for 25 s, and observed under JSM-6360LV.

Transmission electron microscopy (TEM)
TEM was performed as previously reported [52]. Wild-type and \textit{amiR-atpv42b-1} inflorescences were collected, fixed overnight with 2.5% (v/v) glutaraldehyde in 1×PBS (0.1 M, pH 7.4), washed with 1×PBS for five times, and post-fixed in 1% OsO4 for 16 h at 4°C. The specimens were subsequently washed with 1×PBS for five times, dehydrated in a graded ethanol series, replaced by epoxy dimethylmethane, and embedded in Epon812 resin. Semi-thin sections 2–4 μm in thickness were obtained using glass knives, stained with 0.5% toluidine blue in 1×PBS, and then examined under a JEM-1230 transmission electron microscope (JEOL) at 90 kV.

Supporting Information
Figure S1 In situ hybridization of \textit{AtPV42a} and \textit{AtPV42b} in a stage 11 wild-type flower. A transverse section was hybridized with the antisense \textit{AtPV42a} (A) or \textit{AtPV42b} (B) probe. There are hybridization signals inside the gynoecia (arrows), while no signals are detectable in anther cells (arrowheads). Bars, 50 μm.


**Figure S2** Genotyping of atpv42b-1 mutants using PCR analysis. Lanes 1, 3: PCR products amplified with the left (CS823076_LP) and right (CS823076_RP) primers flanking the *AtPV42b* genomic region. Lanes 2, 4: PCR products amplified with the T-DNA left border primer (Lb2_SAIL) and the right primer for *AtPV42b* (CS823076_RP). We detected the amplification of a T-DNA fragment (lane 4), but not the *AtPV42b* genomic region (lane 3) in *atpv42b-1*, indicating that *atpv42b-1* is a homozygous T-DNA insertion mutant.

(TIF)

**Figure S3** Transcript levels of *AtPV42b* in rosette leaves (RL) and open flowers (OF) of wild-type and *atpv42b-1* plants. *AtPV42b* expression is undetectable in *atpv42b-1* as compared to that in wild-type plants. Transcript levels were determined by real-time PCR using a pair of primers flanking the T-DNA insertion site and are shown relative to *TUB2* expression. Values are the mean ± standard deviation from three replicates.

(TIF)

**Figure S4** Transcript levels of *AtPV42a* and *AtPV42b* in rosette leaves of wild-type and *amiR-atpv42a atpv42b-1* plants. The expression of both *AtPV42a* and *AtPV42b* is very low in *amiR-atpv42a atpv42b-1* as compared to that in wild-type plants. Transcript levels were determined by real-time PCR and are shown relative to *TUB2* expression. Values are the mean ± standard deviation from three replicates.

(TIF)

**Figure S5** Transcript levels of *AtPV42a* and *AtPV42b* in rosette leaves of 6 selected *amiR-atpv42b-2* independent transgenic lines at the T1 generation. *AtPV42a* expression is not significantly affected in these transgenic plants as compared to that in wild-type plants, whereas *AtPV42b* expression is greatly decreased. Transcript levels were determined by real-time PCR and are shown relative to *TUB2* expression. Values are the mean ± standard deviation from three replicates.

(TIF)

**Figure S6** Expression of *LRE* in open flowers of wild-type and *amiR-atpv42b-1* plants. *LRE* expression is much upregulated in three independent *amiR-atpv42b-1* lines than in wild-type plants. Transcript levels were determined by real-time PCR and are shown relative to *TUB2* expression. Values are the mean ± standard deviation from three replicates.

(TIF)

**Table S1** Phenotypic analysis of *amiR-atpv42b-1* pollen grains.

(DOC)

**Table S2** Primers used in this study.

(DOC)

**Acknowledgments**

We thank C. Liu and L. Shen for critical reading of the manuscript, and the Arabidopsis Biological Resource Centre for the seeds of *atpv42b-1* (SAIL_563_D10, CS823876).

**Author Contributions**

Conceived and designed the experiments: LF HY. Performed the experiments: LF XH LXL XY. Analyzed the data: LF HY. Contributed reagents/materials/analysis tools: LF XH LYCL. Wrote the paper: LF HY.

**References**

1. Polge C, Thomas M (2007) SNF1/AMPK/SnRK1 kinases, global regulators at the heart of energy control? Trends Plant Sci 12: 20–28.
2. Hallford NG, Hardie DG (1996) SNF1-related protein kinases: global regulators of carbon metabolism in plants. Plant Mol Biol 37: 735–748.
3. Slocombe SP, Laurie S, Bertini L, Beaudoin F, Dickinson JR, et al. (2002) Identification of Snf1, a novel protein that interacts with SNF1-related protein kinase (SnRK1). Plant Mol Biol 49: 31–44.
4. Celenza JL, Carlson M (1984) Structure and expression of the SNF1 gene of Saccharomyces cerevisiae. Mol Cell Biol 4: 54–60.
5. Celenza JL, Carlson M (1996) A yeast that is essential for release from glucose repression encodes a protein-kinase. Science 233: 1175–1180.
6. Hardie DG (2004) The AMP-activated protein kinase pathway - new players upstream and downstream. J Cell Sci 117: 5479–5487.
7. Carling D (2004) The AMP-activated protein kinase cascade - a unifying system for energy control. Trends Biochem Sci 29: 18–24.
8. Hardie DG, Sakamoto K (2006) AMPK: A key sensor of fuel and energy status in the heart of energy control? Trends Plant Sci 12: 20–28.
9. Ignoul S, Eggermont J (2005) CBS domains: structure, function, and pathology in human proteins. Am J Physiol-Cell Ph 289: C1369–C1378.
30. Sanders PM, Bui AQ, Weterings K, McIntire KN, Hsu YC, et al. (1999) Anther developmental defects in Arabidopsis thaliana male-sterile mutants. Sex Plant Reprod 11: 297–322.
31. Berger F, Hamamura Y, Ingouff M, Higashiyama T (2008) Double fertilization - caught in the act. Trends Plant Sci 13: 437–443.
32. Kasahara RD, Portereiko MF, Sandalklie-Nikolova L, Rabiger DS, Drews GN (2005) MYB88 is required for pollen tube guidance and synergid cell differentiation in Arabidopsis. Plant Cell 17: 2981–2992.
33. Capron A, Gourgues M, Neiva LS, Faure JE, Berger F, et al. (2008) Maternal Control of Male-Gamete Delivery in Arabidopsis Involves a Putative GPI-Anchored Protein Encoded by the LORELEI Gene. Plant Cell 20: 3038–3049.
34. Zhang YH, Shewry PR, Jones H, Barcelo P, Lazzeri PA, et al. (2001) Expression of antisense SnRK1 protein kinase sequence causes abnormal pollen development and male sterility in transgenic barley. Plant J 28: 431–441.
35. Lu CA, Lin CC, Lee KW, Chen JL, Huang LF, et al. (2007) The SnRK1A protein kinase plays a key role in sugar signaling during germination and seedling growth of rice. Plant Cell 19: 2484–2499.
36. Thelander M, Olsson T, Ronne H (2004) Snf1-related protein kinase 1 is needed for growth in a normal day-night light cycle. EMBO J 23: 1900–1910.
37. McCormick S (1993) Male gametophyte development. Plant Cell 5: 1265–1275.
38. Goldberg RB, Beals TP, Sanders PM (1993) Anther development - basic principles and practical applications. Plant Cell 5: 1265–1275.
39. Goldberg RB, Beals TP, Sanders PM (1993) Anther development - basic principles and practical applications. Plant Cell 5: 1265–1275.
40. Goldberg RB, Beals TP, Sanders PM (1993) Anther development - basic principles and practical applications. Plant Cell 5: 1265–1275.
41. Goldberg RB, Beals TP, Sanders PM (1993) Anther development - basic principles and practical applications. Plant Cell 5: 1265–1275.
42. Goldberg RB, Beals TP, Sanders PM (1993) Anther development - basic principles and practical applications. Plant Cell 5: 1265–1275.
43. Goldberg RB, Beals TP, Sanders PM (1993) Anther development - basic principles and practical applications. Plant Cell 5: 1265–1275.
44. Goldberg RB, Beals TP, Sanders PM (1993) Anther development - basic principles and practical applications. Plant Cell 5: 1265–1275.
45. Goldberg RB, Beals TP, Sanders PM (1993) Anther development - basic principles and practical applications. Plant Cell 5: 1265–1275.
46. Goldberg RB, Beals TP, Sanders PM (1993) Anther development - basic principles and practical applications. Plant Cell 5: 1265–1275.
47. Goldberg RB, Beals TP, Sanders PM (1993) Anther development - basic principles and practical applications. Plant Cell 5: 1265–1275.
48. Goldberg RB, Beals TP, Sanders PM (1993) Anther development - basic principles and practical applications. Plant Cell 5: 1265–1275.
49. Goldberg RB, Beals TP, Sanders PM (1993) Anther development - basic principles and practical applications. Plant Cell 5: 1265–1275.
50. Goldberg RB, Beals TP, Sanders PM (1993) Anther development - basic principles and practical applications. Plant Cell 5: 1265–1275.
51. Goldberg RB, Beals TP, Sanders PM (1993) Anther development - basic principles and practical applications. Plant Cell 5: 1265–1275.
52. Goldberg RB, Beals TP, Sanders PM (1993) Anther development - basic principles and practical applications. Plant Cell 5: 1265–1275.
53. Goldberg RB, Beals TP, Sanders PM (1993) Anther development - basic principles and practical applications. Plant Cell 5: 1265–1275.
54. Goldberg RB, Beals TP, Sanders PM (1993) Anther development - basic principles and practical applications. Plant Cell 5: 1265–1275.
55. Goldberg RB, Beals TP, Sanders PM (1993) Anther development - basic principles and practical applications. Plant Cell 5: 1265–1275.