Both male and female gametogenesis require a fully functional protein S-acyl transferase 21 in Arabidopsis thaliana

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SUMMARY

S-Acylation is a reversible post-translational lipid modification in which a long chain fatty acid covalently attaches to specific cysteine(s) of proteins via a thioester bond. It enhances the hydrophobicity of proteins, contributes to their membrane association and plays roles in protein trafficking, stability and signalling. A family of Protein S-Acyl Transferases (PATs) is responsible for this reaction. PATs are multi-pass transmembrane proteins that possess a catalytic Asp□His□His□Cys cysteine-rich domain (DHHC-CRD). In Arabidopsis, there are currently 24 such PATs, five having been characterized, revealing their important roles in growth, development, senescence and stress responses. Here, we report the functional characterization of another PAT, AtPAT21, demonstrating the roles it plays in Arabidopsis sexual reproduction. Loss-of-function mutation by T-DNA insertion in AtPAT21 results in the complete failure of seed production. Detailed studies revealed that the sterility of the mutant is caused by defects in both male and female sporogenesis and gametogenesis. To determine if the sterility observed in atpat21-1 was caused by upstream defects in meiosis, we assessed meiotic progression in pollen mother cells and found massive chromosome fragmentation and the absence of synapsis in the initial stages of meiosis. Interestingly, the fragmentation phenotype was substantially reduced in atpat21-1 spo11-1 double mutants, indicating that AtPAT21 is required for repair, but not for the formation, of SPO11-induced meiotic DNA double-stranded breaks (DSBs) in Arabidopsis. Our data highlight the importance of protein S-acylation in the early meiotic stages that lead to the development of male and female sporophytic reproductive structures and associated gametophytes in Arabidopsis.

Keywords: protein S-acyl transferase, S-acylation, palmitoylation, gametogenesis, sterility, meiosis, doublestranded breaks (DSBs), Arabidopsis thaliana.

INTRODUCTION

Cells respond to and communicate with the environment through proteins that localize on the plasma membrane, such as G protein receptors (GPRs), that can transmit signals from the extracellular to intracellular domain, or vice versa (Casey, 1995). Not all proteins attached to the membranes are transmembrane proteins. Many soluble proteins can also associate with membranes after specific lipid modification, such as N-myristoylation, prenylation and S-acylation. S-acylation, also known as palmitoylation, is a post-translational lipidation of proteins. It allows for high affinity interaction with membranes that are around 10 times stronger than myristoylation and 100 times stronger than farnesylation (Silvius and l'Heureux, 1994; Hemsley, 2009). In contrast to myristoylation and prenylation, S-acylation is reversible with palmitate (C16:0), or other long chain fatty acids, being attached to specific cysteine residue(s) of a target protein via a thioester bond (Resh, 2006; Greaves and Chamberlain, 2011). S-acylation is oftencoupled with myristoylation or prenylation, facilitating membrane attachment and trafficking of otherwise soluble proteins. Integral transmembrane proteins can also be palmitoylated (Bijlmakers and Marsh, 2003; Blaskovic et al., 2013). In this case, palmitoylation can alter the structure of the transmembrane domains, regulate the association with lipid rafts and affect interactions with other proteins or other lipid modifications (Blaskovic et al., 2013). Another function of palmitoylation is to protect proteins from ubiquitination and subsequent degradation (Valdez-Taubas and Pelham, 2005; Blaskovic et al., 2013).

Protein S-acylation is catalyzed by Protein S-Acyl Transferases (PATs). PATs are transmembrane proteins containing 4–6 transmembrane domains and an approximately 50 amino acid long, highly conserved Asp□His□His□Cys cysteine-rich domain (DHHC-CRD), in which the enzyme activity is believed to reside. A large scale genomic survey of 31 fully sequenced plant genomes found varying numbers of DHHC-CRD-containing sequences per plant genome and a total of 804 putative PATs were identified (Yuan et al., 2013). At present, only five plant PATs have been studied in some detail – AtPAT24 (TIP1) (Hemsley et al., 2005), AtPAT10 (Qi et al., 2013; Zhou et al., 2013), AtPAT13 (Lai et al., 2015), AtPAT14 (Lai et al., 2015; Li et al., 2015; 3 Zhao et al., 2016) and AtPAT4 (Wan et al., 2017). They are all from the model plant Arabidopsis thaliana in which at least 24 putative PATs are found (Hemsley et al., 2005; Batisti□c, 2012). These studies have shown that PATs play important roles in growth, development, senescence and stress responses in Arabidopsis.

Here, we report the functional characterization of a largely unknown PAT from Arabidopsis, AtPAT21. We first show that AtPAT21 has PAT activity using in vitro biochemical assays and in vivo complementation studies in yeast and in Arabidopsis. To study the biological function of AtPAT21, we isolated a T-DNA insertion transcriptional null mutant line, named atpat21-1 and found that it has growth defects and is sterile. To understand the sterility exhibited by the atpat21-1 mutant, we studied both developmental and pollination related aspects of the phenotype. We found that both male and female sporophytes and gametophytes of the mutant are defective and together these lead to failure in seed production. Our results clearly demonstrate that AtPAT21-mediated protein S-acylation plays a crucial role in both male and female gametogenesis and perhaps other aspects of reproduction in Arabidopsis.

RESULTS

AtPAT21 shares highly conserved sequence motifs with other known DHHC-CRD PATs

AtPAT21 (At2g33640) encodes a 61 kDa protein making it the fifth largest (after AtPAT19, AtPAT20, AtPAT24, and AtPAT22) of the 24 PATs identified in Arabidopsis. The most noticeable feature of AtPAT21 is that it has an extreme C-terminal extension (Batisti□c, 2012). AtPAT21 is predicted (by TMHMM v2.0) to possess four transmembrane domains (TMD) that are located between the 12–34th, 44–66th, 189–211st and 240–262nd amino acid positions, respectively. The DHHC-CRD domain, which is the core catalytic S-acyl transferase functional domain, is located between the second and the third TMDs and is predicted to be cytosolic. A short N-terminal region and the extended C-terminal domain are also predicted to be cytosolic (Figure S1a; Batisti□c, 2012).

Protein sequence alignment shows that AtPAT21 shares high homology within the DHHC-CRD regions to other known DHHC-CRD PATs from Arabidopsis, yeast and mammals. Outside of this region, only very low homology was found (Figure S1b). Other conserved regions/motifs, such as the DPG and TTxE (Mitchell et al., 2006), are also found in the AtPAT21 sequence (Batisti□c, 2012).

PAT21 is an S-acyl transferase

Complementation assays using the yeast PAT AKR1 knockout mutant akr1 have been used previously to test the enzyme activity of three plant PATs (Hemsley et al., 2005; Qi et al., 2013; Li et al., 2015). Although the restoration of temperature sensitivity of akr1 requires both the ankyrin repeats and S-acyltransferase function of the AKR1 protein (Hemsley and Grierson, 2011) both AtPAt10 and AtPAT14, which lack the N-terminal ankyrin repeats, can nevertheless partially rescue the growth defect of akr1, and this was largely dependent on its S-acyltransferase function (Qi et al., 2013; Li et al., 2015). To determine if AtPAT21 (again, lacks the ankyrin repeats) also has S-acyl transferase activity, we transformed pYES-AtPAT21 into akr1 yeast cells. We observed the cell phenotype of transgenic akr1 mutants compared with wild-type (WT) at the non-permissive temperature of 37°C. As shown in Figure 1(a), WT yeast cells appeared round with a single nucleus. However, most akr1 cells were elongated with multiple nuclei that grew poorly compared with WT. Introducing a transgene carrying AtPAT21 partially rescued these growth defects of akr1. The transgenic cells grew much better and were rounder compared with the akr1 cells, and they contained only one nucleus, as in the WT (Figure 1a). Therefore, AtPAT21 partially rescues the growth defects of akr1. To determine if the S-acyl transferase activity of AtPAT21 relies on its DHHC domain, we changed the cysteine residue in the DHHC domain to serine and transformed the mutant GAL1-AtPAT21DHHC174S into the akr1 cells. AtPAT21DHHC174S transformed akr1 cells remained elongated, contained multiple nuclei and grew as poorly as akr1 cells (Figure 1a), showing that a functional DHHC domain is necessary for AtPAT21 function, at least in yeast. This verifies the result from a different yeast complementation assay that showed AtPAT21 can act as a PAT to target Vac8 to tonoplast in yeast (Batisti□c, 2012).

Next, we carried out a biochemical assay to determine if AtPAT21 is auto-acylated, a characteristic of all DHHC-CRD PATs characterized to date. To do this, we utilized a pulldown acylation assay to capture S-acylated proteins from total cell lysates derived from transgenic akr1 cells expressing either AtPAT21 or its point mutation variant AtPAT21DHHC174S. We detected the presence of AtPAT21 and AtPAT21DHHC174S by western blotting by virtue of V5 epitope tags. As shown in

Figure 1(b), while AtPAT21 could be captured by the beads and subsequently detected, demonstrating auto-acylation, AtPAT21DHHC174S could not, hence it is not able to attach a fatty acid, i.e. is not auto-acylated.

These combined results demonstrate that AtPAT21 has PAT activity and its PAT activity is dependent on the cysteine residue located in the DHHC domain.

AtPAT21 is expressed ubiquitously

Understanding the spatial and temporal expression patterns of a gene can provide important clues as to its function. Therefore, we carried out RT-PCR with GAPc as the internal control to monitor the expression levels of AtPAT21 in roots, stems, leaves, flowers and siliques of mature WT Col-0 plants. We detected AtPAT21 transcripts in all of these tissues, but they reached their highest levels in flowers (Figure S2a). This is consistent with eFP gene expression data available through The Arabidopsis Information Resource (TAIR) website. To further confirm the AtPAT21 expression data, we transformed WT Arabidopsis with an AtPAT21promoter:GUS reporter construct. Consistent with the RT-PCR data, the AtPAT21 promoter drove expression in all tissues, but again with especially strong expression in flowers (Figure S2b). Therefore, we reasoned that AtPAT21 function might be particularly important during reproductive development.

AtPAT21 is predominantly localized to the plasma membrane (PM)

We next examined the subcellular localization of AtPAT21 in primary roots of 7-day-old transgenic seedlings expressing an AtPAT21–YFP fusion protein under the control of the CaMV35S promoter (35S:AtPAT21–YFP). For co-localization analysis, AtPAT21–YFP-containing plant lines were crossed with mCherry-tagged endomembrane Wave marker lines (Geldner et al., 2009). We found that AtPAT21– YFP largely co-localizes (Figure 2) with the mCherry plasma membrane marker, R138 (PIP1;4, Boursiac et al., 2005), which is consistent with a previous study that demonstrated a plasma membrane localization of transiently expressed AtPAT21 in tobacco leaves (Batisti□c, 2012).

Identification and characterization of AtPAT21 loss-offunction mutant

To understand the biological roles of AtPAT21 in Arabidopsis growth and development, we obtained a T-DNA insertion line, SALK_016521, from the Arabidopsis Biological Resource Center (ABRC). We identified homozygous T-DNA insertion plants by PCR-based genotyping. By sequencing PCR products amplified by T-DNA left border (LB) primer LBb1 and gene-specific primer SALK_016521RP1, we showed that the T-DNA was inserted at nt1748 within the 7th exon of the gene sequence, downstream of the DHHC domain which is located between nt 1385–1396 in the 5th exon (Figure 3a). We carried out RT-PCR using total RNA isolated from leaf tissue of WT plants and those homozygous for the T-DNA insertion (Figure 3b). AtPAT21 transcripts were successfully detected in WT plants, but not from the T-DNA line confirming this to be a null knockout mutant line, which we named atpat21-1. We backcrossed this line with the WT (Col-0) for three generations to remove any possible second site insertions. The fact that we could still recover the same mutant demonstrated that the mutant phenotype is indeed caused by the disruption of PAT21 due to the T-DNA insertion.

Phenotypic assessment of atpat21-1 plants revealed that they display a range of abnormalities that affect both vegetative and reproductive growth and development. Under long days, atpat21-1 mutant plants are semi-dwarf. Their leaves are smaller than WT and also have a rough surface (Figure 3c–e). Generally, the epidermal cells of the mutant rosette leaves are much smaller than those of WT (Figure S3). Strikingly, the mature siliques of atpat21-1 are very short and do not

contain any seeds, and have only what 4 appear to be shrivelled ovules (Figure S4). Therefore, atpat21-1 is completely sterile. The mutant has a prolonged growth period (>2 months), which may in part result from being sterile. In addition, the mutant plants are more branched than WT (Table S1).

To confirm that AtPAT21 loss-of-function caused the growth defects of atpat21-1, we transformed heterozygous mutant plants with 35S:AtPAT21–YFP. Through PCR-based genotyping, we identified 35S:AtPAT21 transgenic plants that were homozygous for the atpat21-1 mutation and these plants were indistinguishable from WT. However, when we used a construct carrying a cysteine to serine mutation in the DHHC domain (35S: AtPAT21DHHC174S– YFP), the atpat21-1 phenotype was not rescued (Figure 3f). These results clearly demonstrated that the aberrant phenotype of atpat21-1 is caused by loss of AtPAT21 and that the Cys residue in the DHHC domain essential for its PAT activity as we demonstrated above, is also essential for its function within the plant.

AtPAT21 loss-of-function causes male sporophytic and gametophytic defects

To find out the cause of the sterility of atpat21-1 mutant plants, we first observed the anthers and pollen grains from mature flowers of WT and atpat21-1 mutant plants (Figure 4). In WT flowers, the stigma is encircled by anthers and covered by pollen grains (Figure 4a,b), whereas stigmas of the atpat21-1 mutant were devoid of pollen grains and the relative positioning of the anthers and stigma was different, such that anthers were shorter and held below the level of the stigma. Importantly, mutant anthers failed to dehisce and release pollen grains (Figure 4d,e) suggesting sterility arises from earlier defects in meiosis and/or pollen development, rather than purely from positional defects. Analysis of the pollen from atpat21-1 anthers revealed markedly reduced size compared with WT pollen, and with nuclei being generally absent (16.1 1.5 lM in mutant compared with 32.5 1.5 lM in WT, see Figure 4c,f). The small proportion of mutant pollen grains that did contain two sperm cell nuclei was also much smaller than WT pollen grains.

To determine at what point pollen development is perturbed in atpat21-1, we embedded floral material covering all stages of anther development in plastic, sectioned and imaged with WT as the control (Figure 5). We found that the mutant microsporocytes were able to complete meiosis but normal ‘tetrads’ were not formed, rather, asymmetrical polyads containing 2–6 microspores made up the majority of meiotic products. Microspores were successfully released from polyads (Figure 5g); however, the sizes of released mutant pollen grains were not identical and the majority degenerated soon thereafter (Figure 5h). Direct observation of the anthers at this stage also confirmed these findings (Figure S5). These may explain the large proportion of the shrivelled pollen grains that we observed in atpat21-1 mutant anthers (Figure 4e).

AtPAT21 loss-of-function causes female gametophytic defects

To determine whether atpat21-1 is also defective in female sporophytic and/or gametophytic development, we next observed and compared the pistils from fully opened flowers in the mutant and WT plants. The mutant had smaller ovules than WT (40.0 3.6 lM in mutant compared with 76.36 5.6 lM in WT, Figure 6a top panel). To check whether ovules in mutant plants were functional, we carried out manual pollinations using pollen from WT plants. Following pollination, we observed the development of mutant and WT ovules at 1, 2 and 15 days after pollination (DAP) (Figure 6a). At 1 and 2 DAP WT ovules became enlarged and a globular embryo was clearly visible (arrows in Figure 6a, the second and third panels on the left). However, atpat21-1 ovules lacked an embryo although they did show an increase in overall size by 2 DAP (Figure 6a, the second and third panels on the right). By 3 DAP, WT ovules continued to enlarge while mutant ovules appeared to abort and were

completely shrivelled. On maturity at 15 DAP WT siliques had a full set of ~55 seeds, whereas no seeds were present in atpat21-1 siliques (Figure 6a bottom panel). This suggests that atpat21-1 has defects in either supporting the growth or guidance of WT pollen tubes and/or that atpat21-1 ovules failed to produce a normal female gametophyte capable of double fertilization and subsequent seed development.

To determine the cause of female sterility, we observed pollen tube growth in the stigmas and styles of both WT and atpat21-1 plants at 15 h after pollination with WT pollen. The WT pollen grains had germinated and pollen tubes had penetrated both the atpat21-1 and WT stigmas (arrowheads in the left pictures, Figure 6b). At this stage, there was no observable difference between WT pollen tubes (arrows in the left pictures, Figure 6b) transmitting through the styles of atpat21-1 or WT plants, clearly indicating that mutant stigmas were capable of supporting pollen tube growth. However, although pollen tubes (arrows) could be seen entering the WT ovule via the micropyle (arrowhead) no pollen tubes were observed to enter atpat21-1 ovules (Figure 6b, right panel). Therefore, mutant atpat21-1 ovules most likely fail to provide the appropriate guidance cues to WT pollen tubes. These data further support the proposition that mutant ovules fail to produce pollen tube guidance cues, suggesting that appropriate development of ovules may be perturbed in atpat21-1 plants. Therefore, these combined results clearly demonstrated that atpat21-1 has catastrophic female gametophytic defects, resulting in its sterility.

Both male and female gametophytic defects are partially independent of their sporophytic defects in atpat21-1

Gametophytic defects can be caused either by the effects of a mutation in diploid sporophytic tissues such as in stamens or pistils, or independently of the sporophyte if the disruption appears after meiosis (McCormick, 2004). For sporophyte independent gametophytic defects these must be maintained in heterozygotes (McCormick, 2004). To determine if the observed gametophytic defects were present in plants heterozygous for atpat21-1, we genotyped progenies derived from self-pollinated atpat21-1(+/□) plants (♀Het 9 ♂Het, selfed), and from heterozygous atpat21-1 (+/□) plants reciprocally crossed to WT (♀WT 9 ♂Het or ♀Het 9 ♂WT) (Table 1 and Figure S6). In the progeny of self-pollinated heterozygotes, the number of WT-phenotype (WT and heterozygous) plants to homozygous atpat21-1 mutant plants was 5.2:1 which was higher than the expected 3:1 (Table 1). This points to a gametophytic defect existing in either male or female gametes, or both. To address this, we next carried out reciprocal crossing between atpat21(+/□) plants and WT and found that when the heterozygote acted as the pollen donor (♀WT 9 ♂Het), the ratio of WT to heterozygotes from the F1 progeny was 1.3:1. This is higher than the expected 1:1 ratio (Table 1) and demonstrates that not all mutant pollen grains produced by atpat21-1(+/□) plants are viable. Around 23% of the mutant pollen grains failed to fertilize WT ovules that would have subsequently developed into heterozygous seeds, leading to a higher number of WT pollen grains to fertilize WT ovules and hence more WT seeds produced. Therefore it seems that a considerable proportion of male gametophytes derived from atpat21-1(+/□) plants were defective. Conversely, when the reciprocal cross of ♀ Het 9 ♂WT was carried out, the number of the heterozygous plants recovered in the next generation was only about half that of WT plants (AtPAT21(+/+):atpat21-1 (+/□) = 1.9:1 instead of the expected 1:1 ratio) (Table 1). This indicates that there is also a female gametophytic defect(s), which is much more severe than the male defects, observable in atpat21-1 heterozygous plants. Consistent with these results, we also found some unfertilized ovules from these crosses (arrowheads, Figure S6). In contrast with that observed in plants homozygous for atpat21-1, both male and female gametophytes are not completely sterile in the heterozygote. Therefore, the gametophytic effect of

atpat21-1 on sterility is not fully penetrant but is enhanced by the effects of atpat21-1(□/□) on sporophytic tissue.

To further dissect the male gametophytic defects, we analyzed pollen grains derived from atpat21-1(+/□) plants. While the majority of these pollen grains exhibited a similar size and appearance to WT, around 15% of them appeared much smaller (red arrows, Figure 7a, bottom left). In vitro pollen germination assays showed that, while 87.3% of WT pollen grains (n = 100) germinated, only 47.8% (n = 100) of pollen derived from atpat21-1(+/□) plants produced tubes. Interestingly, none of the small pollen grains germinated (red arrow, Figure 7a, bottom right). These abnormal small pollen grains are unable to effect fertilization in vivo and therefore probably contributed to the lower numbers of homozygous or heterozygous seedlings recovered when atpat21-1(+/□) was used as the pollen donor in the self- and reciprocal crosses detailed above (Table 1). The fact that the percentage of small pollen grains was much lower than 50% demonstrates that a large proportion of atpat21-1 mutant pollen grains appeared phenotypically similar to WT and that these 'normal' grains are capable of effecting double fertilization and subsequent seed production. Consistent with this, 43.6% of seedlings recovered from "♀WT 9 ♂Het" crosses were atpat21-1 (+/□), but still a 6.4% loss of fertility was caused by defects in pollen carrying an atpat21-1 allele (Table 1).

In summary, the gametophytic defects observed in the AtPAT21 loss-of-function mutant are partially related to sporophytic effects of the mutation.

We next phenotyped the female gametophytes in atpat21-1(+/□) plants. Stage 12c flowers derived from WT and heterozygous plants were emasculated and fixed 24 h later permitting the female gametophyte to reach maturity (FG7, four-celled stage, Figure 7b-1). Of the 221 ovules examined from atpat21-1(+/□) plants, 55% contained a mature female gametophyte similar to that of WT, suggesting that these ovules represent predominantly WT ovules. The remaining 45%, having an abnormal phenotype, likely corresponding to those harbouring a mutant female gametophyte. The mutant female gametophytes at this stage displayed a range of abnormal phenotypes with some containing no discernible nuclei, being reduced in size and having an accumulation of small vacuoles or the presence of a larger vacuole with mis-positioned nuclei (Figure 7b-2, -3). This result indicated that development of the mutant female gametophyte may be perturbed at an early stage.

To determine at which stage the lesion occurs, we analyzed female gametophytes in pistils from heterozygous plants (n = 67) at the FG1 to FG2 developmental stage. At FG1-2, 46% of ovules exhibited normal megaspore specification and the first nuclear division (Figure 7b-4 and 5), while 54% of ovules displayed several defects. These included gametophytes having an irregular nucleus with higher auto-fluorescence in the cytosol (Figure 7b-6), abnormalities in the subsequent nuclear division where a central vacuole was formed but no nuclei were observed, indicating degeneration of nuclei (Figure 7b-7), and a smaller megaspore with no apparent nucleus (Figures 7b8, -9).

These combined results clearly demonstrated that atpat21-1 female gametophytes had a defect that exerted its effects during megaspore specification, preventing progression into a functional 8-celled embryo sac.

AtPAT21 loss-of-function causes defects in meiotic doublestrand break repair

To determine if the sterility observed in atpat21-1 mutants was caused at least in part by upstream errors in meiosis, we assessed meiotic progression cytologically in DAPIstained chromosomal spreads from both atpat21-1 and WT Col-0 plants. In WT meiosis, homologous chromosomes synapse during prophase I via the formation of the synaptonemal complex which is required for stable crossover formation (Figure 8a). As well as generating genetic diversity, crossovers provide

points of physical connection between homologues and promote their even segregation during anaphase I (Figure 8b–d). Sister chromatids then separate during anaphase II to generate four daughter cells, each with half the original diploid chromosomal complement (Figure 8e).

Compared with WT, many meiotic errors were observed in atpat21-1 mutants. Firstly, during prophase I homologues in the mutants completely fail to synapse and fragments of chromatin fibres were observed in some cells (Figure 8f). We also observed chromosomal fragmentation during diakinesis and metaphase I in all atpat21-1 cells (Figure 8g,h), with chromosomes regularly clumping together in metaphase I to form a tangled-mess that contrasted with the five clear bivalents that are observed in WT meiosis. We also observed abnormalities in all atpat21-1 dyad and tetrad cells (Figure 8i,j), which represent the products of anaphase I and anaphase II, respectively. In both stages, chromosome fragments were observed and missegregated chromosomes led the formation of unbalanced daughter cells and micronuclei.

The meiotic phenotype observed in atpat21-1 was reminiscent of similar phenotypes previously observed in meiotic double-stranded break (DSB) repair mutants in A. thaliana (e.g. Abe et al., 2005; Bleuyard et al., 2004; Puizina et al., 2004). To determine if the meiotic errors observed in atpat21-1 arose from failures in DSB repair, we crossed atpat21-1 plants with atspo11-1 plants to generate atpat21-1/atspo11-1 double mutants. SPO11 is required for the initial formation of DSBs early in meiosis (Grelon et al., 2001). atspo11-1 single mutants failed to form meiotic DSBs, leading to homologue asynapsis during prophase I (Figure 8k) and a complete failure in crossover formation, producing 10 univalent chromosomes during diakinesis and metaphase I (Figure 8l,m). The univalent chromosomes readily missegregate during anaphase I leading to the formation of unbalanced dyads and tetrads later in meiosis (Figure 8n,o). In the atpat21-1/atspo11-1 double mutants the fragmentation phenotype observed in the atpat21-1 single mutant was absent, showing that the presence of fragments requires DSBs produced by SPO11. Homologue asynapsis, univalent formation and chromosome missegregation were still apparent in the double mutant, as in the atspo11-1 single mutant phenotype (Figure 8p–t). Taken together, this indicates that the chromosomal fragmentation phenotype observed in the atpat21-1 mutant arises from a failure in the repair of SPO11 induced meiotic DSBs.

AtPAT21 loss-of-function leads to somatic genome instability

Many proteins known to function in meiotic DSB repair are also required for the repair of somatic DSBs via homologous recombination (Puizina et al., 2004). To determine if PAT21 also plays a role in the maintenance of somatic genome integrity, which could help explain the somatic phenotype of atpat21-1 mutants, we cytologically screened mitotic anaphase cells for irregularities in both WT Col-0 and atpat21-1 plants. In atpat21-1 anaphase bridges or lagging chromosomes were observed in 17% (17/100) of cells (Figure 8v). Anaphase bridges and lagging chromosomes result from the formation of dicentric and acentric chromosomes, respectively, which can be generated by the misrepair of somatic DSBs. No anaphase bridges or lagging chromosomes were observed in any mitotic cells from WT Col-0 (0/100) (Figure 8u). Thus, there was a significant increase in the occurrence of lagging chromosomes in the atpat21-1 background (P = 7.3 9 10□6 , two-tailed Fisher's exact test). It is therefore likely that somatic genome instability contributes to the defects observed in the vegetative growth of atpat21-1 plants.

As AtPAT21 functions through its PAT enzyme activity (Figures 1 andS4) and SPO11 has not been shown experimentally to be palmitoylated, we analyzed its potential palmitoylation site(s) using the clustering and scoring strategy software for the prediction of palmitoylation sites (CSS-Palm 4.0) (Zhou et al., 2006; Ren et al., 2008). This gave negative results, i.e. no such sites are predicted in the

SPO11 sequence. Therefore, using the same software, we analyzed other protein sequences of over 80 genes that have been reported to have roles in the process of meiosis in plants (reviewed by Mercier et al., 2015). In general, loss-of-function mutants of these genes have defects in both male and female fertility. It is possible that AtPAT21 palmitoylates one or more such proteins that are involved in the repair of SPO11-mediated DSBs during meiosis. Among these protein sequences a few scored very high and the higher the score is for a particular cysteine residue (s) within the predicted protein sequence the higher the possibility would be for this cysteine(s) to be palmitoylated. For instance, the cysteine residues at position 3 (C3) and 6 (C6) of HEI10 (Enhancer of cell invasion 10) were scored at 20 and 40, respectively; the ninth cysteine (C9) of PRD1 (Putative recombination initiation defect 1) was 34 and the score of the 18th cysteine (C18) of MRE11 was 26, indicating that these proteins are likely palmitoylated at these specific cysteine residues.

DISCUSSION

We report here the characterization of a protein S-acyltransferase, AtPAT21, from Arabidopsis. We showed that disruption of AtPAT21 by T-DNA insertion results in sterility and other growth and development defects in the mutant plants (Figures 3 and 4). We also showed that AtPAT21, which contains the characteristic DHHC-CRD domain (Figure S1 5), is indeed a functional S-acyl transferase. Importantly, we showed that Cys to Ser mutation in the DHHC catalytic domain of AtPAT21 destroys its ability to restore the growth defect of yeast akr1 lines and to auto-acylate (Figure 1). We further studied the biological function of AtPAT21 using a T-DNA knockout mutant allele, atpat21-1 (Figure 3). The mutant plants had stunted growth and altered development, and were sterile.

The effects of mutating AtPAT21 are diverse, but are clearly particularly damaging for reproduction. First, we found some evidence that AtPAT21 might be involved in anther dehiscence, though whether this is a direct effect is not yet clear. Anthers of atpat21-1 mutant plants fail to dehisce and as a result no pollen grains are released. We showed by scanning electron microscopy that the stomium of WT anthers was completely broken down at dehiscence, leading to the exposure and release of the anther contents, whereas the stomium in atpat21-1 anthers remained largely intact (Figure 4). Pressure generated by swelling of a full complement of pollen grains in the anther is required to split the stomium. Indeed, anthers of the lap5-1 lap6-1 (LESS ADHESIVE POLLEN) double mutant failed to dehisce due to the shrivelled nature of the pollen grains (Dobritsa et al., 2010). This may also be the case for atpat21-1 anthers as they contain largely degenerated pollen grains which would exert much less pressure within the anther. However, anthers of the male sterile mutant acos5 (AcylCoA Synthetase 5) are still able to dehisce despite the fact that they contain shrivelled and inviable pollen grains (De Azevedo Souza et al., 2009). This indicates that dehiscence of the Arabidopsis anther is more complex and does not depend on its contents alone. The fact that loss-of-function of AtPAT21 exhibits disruption in anther dehiscence demonstrates that protein S-acylation mediated by AtPAT21 may also be involved in this important process.

We also found that male and female sterility in the AtPAT21 loss-of-function mutant is caused by severe meiotic defects. Failure to produce pollen or the production of abnormal pollen can be caused by either meiotic defects, defects in subsequent mitoses, or abnormalities in the cell layers surrounding the locules (Sanders et al., 1999). In the atpat21-1 mutant anthers all cell layers were present and seemed to develop properly. However, the production of tetrads was abnormal with asymmetrical polyads containing 2–6 microspores being frequently observed (Figure S5). Therefore,

the defect caused by loss of AtPAT21 could have affected the meiotic stage of pollen development leading to deviations from the typical number of four meiotic products found in tetrads of WT anthers. Ovule development in atpat21-1 also showed severe defects that similarly seem to act at the meiotic stage of development.

Indeed, observation of DAPI-stained chromosomal spreads prepared from fixed buds containing male meiocytes revealed many errors throughout meiosis in atpat21- 1 plants, leading to chromosome fragmentation, homologue missegregation and the formation of unbalanced daughter cells and micronuclei (Figure 8). Further analysis of the double mutant atpat21-1 atspo11-1 confirmed that the meiotic defects in atpat21-1 were due to the failure in repair of early meiotic DSBs induced by SPO11.

Our data clearly demonstrated that Arabidopsis PAT21 is involved in both male and female microsporogenesis and megasporogenesis through the repair of SPO11 induced DSBs during early stages of meiosis. Further analysis of potential palmitoylation sites in meiosis proteins suggests HEI10, PRD1 and MRE11 as especially good candidates as targets of AtPAT21. Consistent with this, the transcriptional null mutant of HEI10 also has asymmetric tetrads or polyads containing more than four microspores (Chelysheva et al., 2012); the loss-of-function mutant for PRD1 has very short siliques that contain very few seeds (2.62 seeds/silique) and this is caused by both male and female gametogenesis or/and sporogenesis defects (De Muyt et al., 2007); and the mre11-3 mutant plants are dwarfed with shorter roots in addition to a lack of pollen grains and sterility (Bundock and Hooykaas, 2002). All of these defects have also been observed in atpat21-1, suggesting that some, or all, these proteins may be the palmitoylating targets of AtPAT21 in Arabidopsis. Loss-of-function of AtPAT21 would lead to a failure in the palmitoylation of these proteins and loss of appropriate membrane localization and function resulting in the defects observed in these mutants. However, it is puzzling how AtPAT21 exerts its role as an S-acyltransferase since these putative substrate proteins are localized in the nucleus while AtPAT21 is localized in plasma membrane. In fact, no DHHC-PATs have been reported to localize within the nucleus. Future research will be required to determine if and how these putative palmitoylated proteins interact with AtPAT21 to shed new light on the roles played by palmitoylation in reproductive biology, especially during meiosis in Arabidopsis.

It is noteworthy that a new study has been published linking RIF1 S-acylation by the DHHC family palmitoyl acyltransferase Pfa4 to DSB repair in yeast (Fontana et al., 2019). As no RIF1 orthologue exists in plants the PAT21 mediated process must be mechanistically different. Therefore, our finding of the involvement of PAT21 in DSB repair in Arabidopsis is both relevant and timely. This may open a new area of investigation in examining how S-acylation affects nuclear/DNA events in general with a special emphasis on plants; something that has hitherto largely been ignored in any eukaryote so far. In summary, we have shown that Protein S-Acyltransferase 21 is involved in reproduction in Arabidopsis. We identify AtPAT21 as a positive regulator of fertility and hence seed production in Arabidopsis that acts by modulating both male and female microsporogenesis and megasporogenesis through the regulation of meiosis and mitosis.

CONCLUSIONS

In this study, we demonstrated that the knockout mutant of a single gene, AtPAT21 leads to defects ranging from vegetative growth to reproductive development. This is perhaps not surprising as

AtPAT21 is ubiquitously expressed (Figure S2 and Batistič, 2012). Mutant atpat21-1 plants have reduced stature, smaller and uneven leaves and more branching of inflorescences compared to the WT Col-0 Arabidopsis plants (Figure 3 and Table S1), indicating that AtPAT21 is essential for normal vegetative growth in Arabidopsis. Perhaps most strikingly, AtPAT21 loss-offunction causes both male and female sterility and the mutant plants do not produce seeds. We confirmed both in vivo and in vitro that AtPAT21 is an S-acyl transferase. Therefore, it may function through S-acylation of one or multiple target proteins that are involved in a range of processes in the plant. We focused our study on the roles of AtPAT21 in reproduction. This led to the conclusion that successful male and female microsporogenesis and megasporogenesis relies on S-acylation of proteins that participate in the regulation of meiosis and mitosis in both male and female reproductive tissues in Arabidopsis.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Wild-type and the T-DNA insertion line SALK_016521 of Arabidopsis thaliana in the background of Columbia-0 (Col-0) were obtained from the ABRC, http://www.arabidopsis.org/abrc/). Seeds were surface sterilized, germinated and plants were grown under long days (LD) as described previously (Qi et al., 2013).

RT-PCR and GUS staining

To detect the transcript levels of AtPAT21 in seedlings and different parts of Arabidopsis plants total RNA was extracted from WT seedlings and leaves, stems, flowers and siliques of mature plants. First-strand cDNA was synthesized and PCR was carried out using the AtPAT21 gene-specific pair of primers (Table S2).

To make the promotor-GUS expression construct about 800 bp upstream of the start code of AtPAT21 was used and amplified with a pair of primers pPAT21attB1/pPAT21attB2 (Table S2). This DNA fragment was recombined into the pMDC162 vector (Curtis and Grossniklaus, 2003) via Gateway cloning technology to make the promotor-GUS fusion. Tissues from proAtPAT21:GUS transgenic plants were stained in the staining buffer (100 mM sodium 6 phosphate buffer, pH7.0; 10 mM EDTA; 0.1% Triton X-100; 1 mM K3Fe(CN)6;2mM X-Gluc) at 37°C for overnight. The samples were cleared with 100% alcohol for 12 h and repeated several times before being observed and photographed (Jefferson, 1987).

Identification of the AtPAT21 T-DNA insertion mutant

Homozygous T-DNA insertion mutant Arabidopsis plants were isolated and characterized according to Qi et al. (2013) and Li et al. (2015) using primers listed in Table S2.

Complementation in yeast and Arabidopsis

To determine the PAT activity of AtPAT21, complementation assays in yeast and Arabidopsis were carried out. Briefly, the coding region of AtPAT21 was PCR-amplified from first-strand cDNA without the stop codon and cloned into the Gateway pDONR/Zeo vector (Invitrogen). To change the cysteine residue to serine in the DHHC motif a first round PCR was carried out using two pairs of primers, DHHCtoS For + End and Beg + DHHCtoS Rev (Table S2), followed by a second round of PCR using primer pair Beg and End to assemble the full length DHHS-AtPAT21. This product was again cloned into pDONR/Zeo. The WT-PAT21 and DHHS-PAT21 were recombined into pYES-DEST52 (C-terminal V5 fusion) (Invitrogen) and pEarleyGate 101 (C-terminal YFP fusion) (Earley et al., 2006) for expression in yeast and Arabidopsis, respectively. Transformation of akr1 yeast cells and Arabidopsis

plants and subsequent growth conditions were carried out as described previously (Qi et al., 2013; Li et al., 2015).

Auto-acylation assays in yeast

Here, 20 ml of transgenic yeast cells was grown at 25°C in selective minimal medium containing 2% galactose to induce protein expression. After 2 days, the cells were collected by centrifugation. Cells were resuspended in 0.5 ml of lysis buffer (100 mM HEPES, 1.0 mM EDTA, 19 Roche complete protease inhibitor, pH 7.5) and broken open by adding 0.5 g of acid-washed sand (400– 600 lM, Sigma 7) and vortexing for 1 min. This was repeated four more times with cooling on ice between each vortexing step. This was followed by blocking the free –SH groups and capturing the S-acylated proteins utilizing the Acyl-RAC method (Forrester et al., 2011). The proteins were separated via 10% SDS-PAGE and PAT21/DHHS-PAT21 were detected by western blotting with an anti-V5 antibody (mouse monoclonal antibody, KWBio, China) and ECL as described previously (Li et al., 2015).

Subcellular localization of AtPAT21

For determining subcellular localization atpat21-1 mutant plants complemented by the 35S:AtPAT21–YFP construct were crossed with the mCherry-tagged marker Wavelines (Geldner et al., 2009). Primary roots of 7-day-old seedlings from the crossed F1 progeny were observed and imaged according to Qi et al. (2013).

Pistils, after being pollinated for 5 and 15 h, were excised and submerged in fixing solution (10% v/v acetic acid, 30% v/v chloroform, 60% v/v ethanol) for 16 h. The fixative was then removed and the pistils were rinsed twice for 5 min in 50 mM potassium phosphate buffer (pH7.0) twice. The pistils were softened in 4 M NaOH at room temperature for 15 min. They were rinsed twice in 50 mM potassium phosphate buffer before being stained in aniline blue (0.1% w/v Aniline Blue in 0.1 M K3PO4, pH 11) for 1 h followed by washing in 50 mM potassium phosphate as before (modified from Kho and Baer, 1968). After removing the potassium phosphate buffer a drop of mounting medium (50% glycerol in 50 mM potassium phosphate pH 7.0) was applied and the pistils were transferred onto a glass slide and covered with a coverslip. The pistils were gently squashed to expose the pollen tubes and visualized under ultraviolet (UV) light using a Nikon Eclipse 90i Eclipse epifluorescence microscope equipped with a Nikon Digital Sight DSU1 camera for image capture.

In vitro pollen germination

Mature pollen grains from WT and heterozygous AtPAT21/atpat21-1 flowers were collected and placed on freshly made pollen tube growth medium as described above and incubated at 24°C overnight. The germination of pollen grains was checked under a dissecting microscope. The number of germinated pollen grains were counted and calculated as percentage of total pollen grains placed on the media.

Microscopy of anthers, ovules and pollen grains

For scanning electron microscopy (SEM) of pollen grains freshly opened flowers were frozen in liquid nitrogen and freeze-dried overnight. Several anthers were taken and coated with gold. Observation and image capture was carried out using a scanning electron microscope (JSM-6480-LV).

For observation of mature ovules both unfertilized and fertilized ovules were submerged in clearing solution (Chloral hydrate (g): Glycerol (ml):ddH2O (ml) = 8:1:3) for several hours (vary depending on the age of samples observed) prior to imaging using a Nikon Eclipse 90i microscope, as described

above. For visualization of pollen grains freshly collected samples were suspended in 10% glycerol and observed directly or stained for 10 min in DAPI solution (0.1 M sodium phosphate, pH 7.0, 1 mM EDTA, 0.1% Triton X-100, and 0.5 lg ml□1 DAPI) and observed under UV light using the Nikon Eclipse 90i microscope.

For observation of pollen and ovule development inflorescences were fixed in 4% glutaraldehyde in 12.5 mM cacodylate buffer, pH 6.9, and dehydrated through a conventional ethanol series and subsequently cleared in 2:1 benzyl benzoate:benzyl alcohol. The dissection of ovules at different developmental stages was according to Chen et al. (2007). For sectioning floral buds were embedded in Historesin and semi-thin (0.5 lm) sections were obtained using an ultra-microtome (Leica Microsystems, Nussloch, Germany). Sections were stained with 0.05% of Toluidine Blue O for 40–60 sec, and photographed under the microscope as described previously (Chen et al., 2007).

Cytological analysis of mitotic and meiotic chromosomes

Both meiotic and mitotic DAPI-stained chromosome spreads were prepared as described previously (Caryl et al., 2000). In brief, inflorescences were fixed in 3:1 ethanol:acetic acid for at least 24 h and fixed buds containing male meiocytes were then dissected from the main inflorescence, washed in 0.01 M citrate buffer and incubated in digestion medium (0.33% pectolyase (Sigma P5936), 0.33% cellulase (Sigma C1794) dissolved in 0.01 M citrate buffer) for 90 min at 37°C. Digested buds were macerated with a brass rod in a drop of 65% acetic acid on a glass slide before adding another 14 ll 65% acetic acid and placing the slide on a hot plate at 45°C for 1 min while stirring with a mounted needle. Cells were then fixed to the slide by the addition of 400 ll 3:1 fixative before drying the slide and then mounting a coverslip with 7 ll 10 lg ml□1 DAPI in Vectashield (Vector Labs 8). Slides were imaged using a Zeiss Axio Imager Z2 epifluorescence microscope.

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DATA AVAILABILITY STATEMENT

All data referred to are included in the manuscript or supplementary materials.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTIONS

BQ conceived the study and designed experiments. YL, HL, CM and BQ performed the experiments. BQ and YL wrote the manuscript with contributions from all authors.

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