GAMETOPHYTE DEFECTIVE 1, a Putative Subunit of RNases P/MRP, Is Essential for Female Gametogenesis and Male Competence in Arabidopsis

Si-Qi Wang, Dong-Qiao Shi*, Yan-Ping Long, Jie Liu, Wei-Cai Yang*
State Key Laboratory of Molecular Developmental Biology, National Centre for Plant Gene Research, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China

Abstract

RNA biogenesis, including biosynthesis and maturation of rRNA, tRNA and mRNA, is a fundamental process that is critical for cell growth, division and differentiation. Previous studies showed that mutations in components involved in RNA biogenesis resulted in abnormalities in gametophyte and leaf development in Arabidopsis. In eukaryotes, RNases P/MRP (RNase mitochondrial RNA processing) are important ribonucleases that are responsible for processing of tRNA, and transcription of small non-coding RNAs. Here we report that Gametophyte Defective 1 (GAF1), a gene encoding a predicted protein subunit of RNases P/MRP, AtRPP30, plays a role in female gametophyte development and male competence. Embryo sacs were arrested at stages ranging from FG1 to FG7 in gaf1 mutant, suggesting that the progression of the gametophytic division during female gametogenesis was impaired in gaf1 mutant. However, the fitness of the mutant pollen tube was weaker than that of the wild-type, leading to reduced transmission through the male gametes. GAF1 is featured as a typical RPP30 domain protein and interacts physically with AtPOP5, a homologue of RNases P/MRP subunit POP5 of yeast. Together, our data suggest that components of the RNases P/MRP family, such as RPP30, play important roles in gametophyte development and function in plants.

Introduction

RNA biogenesis is an essential biochemical process that not only plays a housekeeping role for cellular life, but also mediates many aspects of vegetative and reproductive development. Studies on genes encoding essential proteins in RNA biogenesis have attracted more and more attention since last decades, and many reports have argued that ribosomal proteins or components for ribosome biogenesis, are involved in the cellular process, organ and organism development [1,2,3]. Various studies also indicated that mutations in genes involved in these processes result in fertility failure in Arabidopsis [4,5]. However, most of the reports have shown that genes involved in RNA processing often affect female gametophyte development more severely than that of the male. Consistently, expression profiling of female gametophytic cells reveals that genes encoding PAZ, PIWI domain or DUF1785 protein family involved in tRNA, rRNA, or mRNA processing, also exhibit high level of expression in the embryo sac [6]. The mutations in SLOW WALKER genes, such as SWA1, SWA2 and SWA3/AtRH36, impair the mitotic cell cycle progression during female gametogenesis, causing female sterility in Arabidopsis [7,8,9,10]. SWA1 is homologous to the yeast UTP15 and plays a role in 18S pre-rRNA processing [7]. SWA2 is most likely involved in export of pre-ribosomes from nucleus to cytoplasm [8]. SWA3, or AtRH36, encoding a putative RNA helicase, is also required for 18S pre-rRNA processing [9,10]. Similarly, NUCLEOLAR FACTOR 1 (NOF1), which is involved in nuclear functions and rRNA expression, is required for embryogenesis and female gametogenesis [11]. Besides the mitotic progression during embryo sac development, genes involved in RNA biogenesis are also reported to play a role in gametophytic cell fate, polar nuclei fusion, and female control of pollen tube attraction. For example, MAGA-TAMA3 (MAA3), a gene involved in many aspects of RNA metabolism, is essential for the polar nuclei fusion and pollen tube guidance [12]. These data suggest that the regulation of rRNA processing and ribosome biogenesis is essential for female gametophyte development and function. In addition, mutations in genes coding for proteins involved in pre-mRNA splicing, such as LACHESIS (LIS), GAMETOPHYTIC FACTOR1 (GEA1)/CLOTHO (CLO) and ATROPUS (ATO), also disrupt female gametophyte development. Intriguingly, these genes seem to play a yet unknown role in cell fate specification of the embryo sac [4,5,6,13,14,15,16,17]. In lis mutant, the synergid and central cell adopted egg cell fate, leading to supernumerary egg cells [14]. gha1 embryo sacs produce incorrect number of nuclei, aberrant cellular structure, and delayed maturation, as well as defects in embryo and pollen development [9,13]. Mutation in ATO also causes abnormal specification of the gametic cell fate [15].

As one of the most important RNA metabolism components, Ribonuclease P (RNase P) has been under extensive research since 2007.
its discovery. RNases P and MRP are essential site-specific endoribonucleases that are involved in processing the 5’ end of pre-rRNAs. RNase MRP was evolved from nuclear RNase P, and is responsible for pre-rRNA processing [18,19,20]. They share most of the protein subunits and structural features of their RNA subunits [21,22,23,24,25,26]. The structure and sequence of the RNA and protein components are highly conserved in eukaryotic kingdom [27]. Besides some common components, they also have specific RNAs and proteins individually. Recently, additional roles of eukaryotic RNases P/MRP have been revealed. It was shown that RNase P may play a role in stress response [28] and transcription by regulating RNA polymerase III as a transcription factor [29,30]. In yeasts, RNase MRP is involved in cell cycle regulation by cleaving CLB2 (cyclin B2) mRNA [27,31,32]. Loss of function of these two ribonucleases would cause serious consequences. In yeast, for example, rpp1 and rpp2 mutants are inviable [33,34]. Reduced expression of the RNA component of MRP causes the multifaceted human Cartilage-Hair Hypoplasia (CHH) disease [35,36], Pin Schmid metaphyseal chondrodysplasia (MDWH) disease [37,38], or anauxetic dysplasia disease [39]. These suggest a unique role of RNases P/MRP in both yeast and animal cells.

In plants, however, little is known about RNases P/MRP. Two MRP RNAs have been found in Arabidopsis and one in rice, respectively. However, no P RNA has been discovered in RNase P, which implies that P RNA genes in plants may be very different with the known genes in the nuclear genome [40]. Nevertheless, the function and composition of RNases P and MRP remain to be elucidated in plants. Here we report the detailed genetic and molecular characterization of a mutant, gametophyte defective 1 (gaf1), in which a gene encoding a predicted Arabidopsis RNases P/MRP protein subunit RPP30 is disrupted. Female gametophytic development and male competence were compromised in gaf1. Furthermore, we also showed that GAF1 could interact with another predicted Arabidopsis RNases P/MRP protein subunit AtPOP5 in yeast cells. All these data indicated that GAF1 is a subunit of RNases P/MRP that plays a role in gametophyte development and function in plants.

**Results**

**Isolation of gametophytic mutants**

Gametogenesis is a fine-tuned developmental process. During female gametogenesis, one of the meiotic products—the functional megaspore—undergoes three rounds of mitosis that generates an eight-nucleate coenocyte, subsequently, two polar nuclei migrate and fuse, thereafter simultaneous cellularization of the coenocyte occurs to produce the mature seven-celled female gametophyte [41,42,43]. During male gametogenesis, microspore undergoes two consecutive rounds of mitosis, namely PMI (pollen mitosis I) and PMII, to generate a tricellular male gametophyte, the pollen [44,45,46]. To dissect the function of RNases P/MRP in gametogenesis and plant development, gametophytic mutants showing segregation distortion and reduced seed set were isolated from the Ds insertion pool and their target genes were identified [7,47]. gaf1 mutant in which a gene encoding a putative RNases P/MRP component, was identified. Molecular analysis showed that a single copy of Ds element was present in gaf1. Instead of the typical 3:1 segregation ratio for sporophytic recessive mutation, self-pollinated gaf1 progenies showed a 0.63:1 (213:338) segregation ratio of kanamycin resistant (KanR) to kanamycin sensitive (KanS), which is typical for mutants defective in both male and female gametophytes. Consistently, the siliques of heterozygous gaf1 mutant contained 48.5% (324 in 668 ovules) aborted ovules (Figure 1).

In order to further confirm the effects of the mutation on both male and female gametophytes, reciprocal crosses between heterozygous gaf1 and the wild-type plants were conducted. When gaf1 was used as the pollen donor, about 34.4% of the F1 progeny (n = 1201) were KanR, indicating that the male gametophyte development or function was defective. When gaf1 was used as the egg donor, only about 0.46% of the F1 progeny (n = 658) showed KanR, suggesting that the female gametophyte development or function was severely impaired (Table 1). Consistently, no homozygote was recovered. We use gaf1 to represent gaf1/GAF1 heterozygous mutant in this report. Taken together, gaf1 is a gametophytic mutation which affects both male and female gametophytes with a stronger effect on the female than the male.

**The mutation impairs developmental progression of the female gametophyte**

Female gametogenesis in Arabidopsis is divided into seven sequential stages [48], namely female gametophyte 1 (FG1) to FG7 (Figure 2). The megaspore mother cell (MMC) undergoes meiosis to form four haploid megaspores. Shortly after the meiosis, female gametogenesis, one of the meiotic products—the functional megaspore—undergoes meiosis to form four haploid megaspores. Shortly after the meiosis, three megaspores towards the micropylar pole degenerate manifested as strong autofluorescence under confocal. The chalazal-most megaspore survives to form the functional megaspore that continues to development, entering FG1 phase (Figure 2A). The functional megaspore undergoes one round of

| Transmission | Cross | **Kan**<sup>R</sup> | **Kan**<sup>S</sup> | **Kan**<sup>R</sup>(expected) | **TE** |
|--------------|-------|---------------------|---------------------|-----------------------------|--------|
| Male         | +/+ X Ds/+ | 413                 | 788                 | 34.4% (50%)                 | 52.4%  |
| Female       | Ds/+ X +/+ | 3                   | 655                 | 0.46% (50%)                 | 0.46%  |
| Self         | Ds/+ X Ds/+ | 213                 | 338                 | 38.7% (75%)                 |        |

**Figure 1. Reduced seed set in gaf1 mutant siliques.** A. A gaf1 silique showing aborted ovules (white arrows). B. A wild-type silique with full seed set. Bar = 500 μm.
mitosis to produce two nuclei, one of which locates at the chalazal pole and the other at the micropylar pole (FG2, Figure 2B). Then, the two nuclei are separated by a central vacuole, entering stage FG3 (Figure 2C). Both nuclei undergo a second round of mitosis to give rise to four nuclei in embryo sac (FG4, Figure 2D). A third round mitosis results in an eight-nucleate embryo sac, with four at each pole (early FG5, Figure 2E). Two polar nuclei migrate along the chalazal–micropylar axis toward each other, and meet at the micropylar half of the embryo sac (late FG5, Figure 2F). Then, cellularization takes place simultaneously to produce a seven-celled embryo sac, and the embryo sac enters FG6 upon the two polar nuclei fusion (Figure 2G). Finally, the three antipodal cells degenerate, the remaining four cells, namely the central cell, egg and two synergids, form a female germ unit (FG7, Figure 2H). The female gametophyte is then ready for fertilization.

To investigate developmental defects in the mutant, flowers were emasculated at floral stage 12c [48] and observed 24 hours post emasculation. At this flower stage, most ovules in wild-type pistils were at stage FG6 or FG7. In gaf1 mutant pistils at the same flower stage, however, only about half of the ovules reached the stage FG6 or FG7 (Figure 3A), and the rest of the ovules were arrested at different stages ranging from FG1 to FG5 (Figure 3B–F). To further check at which stages the ovule development was arrested, a detailed CLSM observation was then carried out in both the mutant and the wild-type pistils. All the buds in the same inflorescence were dissected sequentially, and the developmental stage of each ovule was recorded (Table 2 and Table 3). In wild-type plants, ovules in each pistil predominantly take one or two adjacent developmental stages (Table 2), a phenomenon called developmental synchrony [7,49]. In contrast to this synchronous development, ovules in gaf1 pistil span several stages, seven at most in a pistil (Table 3). The mutant embryo sacs stop at any stage, indicating that the synchrony of FG development is seriously impaired.

To further investigate whether the retarded mutant embryo sacs have the potential to develop into functional female gametophyte, we performed delayed pollination experiment [7]. Pistils from gaf1 were emasculated at floral stage 12c, and pollinated with wild-type pollen at 24 h, 48 h, or 72 h post emasculation. The ratio of KanR progeny was increased significantly in delayed pollination (see Table 4), indicating that a small portion of the mutant ovules have the potential to develop into functional FGs despite the developmental retardation.

Taken together, gaf1 mutation affects the normal developmental progression and disrupts the synchrony of female gametophyte development.

The mutation affects slightly pollen competence

Given the low penetration of the mutation via the male gametophyte, experiment was carried out to investigate the developmental or functional defect of the male gametophyte. Mature pollen grains were primarily stained with Alexander’s solution [50] and 4′,6-diamino-2-phenylindole (DAPI) staining for their viability and development, respectively. When stained with Alexander’s solution, 96.3% of the pollen grains from gaf1/GAF1 plants showed positive staining (n = 1044), compared to 97.7% (n = 768) in the wild-type, indicating that gaf1 did not affect the viability of pollen grains (Figure 4A and 4C). As for DAPI staining, similar to the wild-type pollen grains (98.3%, n = 473), most of the plants showed positive staining (98.0%, n = 633) from the mutant plants display normal morphology with three nuclei, two small and compact sperm nuclei and one large less condensed vegetative nucleus, suggesting the mitotic progression in gaf1 pollen development proceeded as the wild-type (Figure 4B and 4D). Furthermore, pollen germina-
tion was carried out either in vitro or in vivo. For the in vitro germination test, germination rates for pollen grains from the wild-type and gaf1 plants are quite similar, and the ratios are 91.99% (n = 1582) and 89.63% (n = 1855), respectively (Figure 4E–G). The in vivo germination were checked with pollen from plants of GAF1/GAF1 qrt/qrt and gaf1/GAF1 qrt/qrt, the result showed similar germination ratio of pollen grains from plants of the above two genotypes, 65.25% (n = 400) for GAF1/GAF1 qrt/qrt, and 65.44% (n = 408) for gaf1/GAF1 qrt/qrt (Figure 4H).

Since gaf1 pollen grains could germinate in vivo normally, it suggests that the defective transmission most likely occurs after germination.

| Pistil Number | FG1 | FG2 | FG3 | FG4 | FG5 | FG6 | FG7 | Total FGs |
|---------------|-----|-----|-----|-----|-----|-----|-----|-----------|
| 1             | 40  | 1   |     |     |     |     |     | 41        |
| 2             | 23  | 25  | 2   |     |     |     |     | 50        |
| 3             | 8   | 14  | 16  | 8   |     |     |     | 46        |
| 4             | 6   | 12  | 33  | 11  |     |     |     | 62        |
| 5             | 3   | 6   | 16  | 31  | 2   |     |     | 58        |
| 6             | 1   | 9   | 43  |     |     |     |     | 53        |
| 7             |     |     |     |     | 27  | 8   | 10  | 45        |
| 8             |     |     |     |     | 3   | 32  | 2   | 37        |
| 9             |     |     |     |     | 8   | 13  | 32  | 53        |

Since the gaf1 pollen grains could germinate in vivo normally, it suggests that the defective transmission most likely occurs after germination. The in vivo fitness of gaf1 pollen was studied. Anthers from wild-type flowers at 12c stage were removed, and the emasculated wild-type pistils were pollinated with pollen grains from gaf1 after 24 h. Every resulting silique from three independent plants was chopped into three equal parts transversely upon maturation, and seeds were collected into three corresponding groups. Seeds of the first group are from that part of silique just next to the stigma, seeds of the second group are from the mid-one-third of the silique, and the seeds from the last part of the silique connecting to the stem were collected as the third group. Curiously, the KanR segregation ratio of these seedlings varies in these 3 groups. The KanR:KanS ratio of the first, second and third group was 0.775 (n = 1142), 0.521 (n = 996), and 0.483 (n = 749), respectively (Figure 5A and 5B). It is obvious that the mutation transmission from pollen grains varies according to the location of the seeds in silique, which means that the ovules close to the stigma have more possibility to be fertilized with mutant gaf1.
pollen, than those ovules further to the stigma, or closer to the stem. It implies that the mutant pollen tubes may be not so competent or elongate so fast as the wild-type tubes, so that the ovules locating further to stigma have less chance to be reached and fertilized by mutant pollen tubes. To elucidate the hypothesis, sparse pollination test was conducted. The pollen grains from gaf1 plants were dusted on wild-type stigma, and the pollen grains were fewer than 40 for each pistil, to exclude the competition of pollen tubes and make sure that each tube is able to reach an ovule in pistil. Then the seeds were counted for KanR. The data showed that the transmission efficiency through male gametophyte was raised to nearly 100% (KanR:KanS = 1.018, n = 1329) when the fitness of mutant pollen tubes is somehow weaker than those of pollen tubes can function normally during pollination, however, the number of ovules was in excess. We concluded that the mutant pollen tubes may be not so competent or elongate so fast as the wild-type tubes, so that the ovules locating further to stigma have less chance to be reached and fertilized by mutant pollen tubes.

GAF1 encodes an Arabidopsis homologue of yeast RPP1 and human RPP30

In order to identify the GAF1 gene, Ds flanking sequences were cloned with TAIL-PCR [51]. Sequence analysis revealed that the Ds element was reversely integrated at position of 1752 bp downstream the stop codon, and disturbed the third exon of At5g59980 (www.arabidopsis.org). The insertion resulted in an 8 bp duplication at the insertion site (Figure 6A).

The linkage between the Ds element and the gaf1 phenotype was verified. gaf1 was outcrossed with wild-type plants, and linkage analysis of more than 300 F2 plants showed that the kanamycin segregation distortion and gametophyte defect are closely linked with the Ds insertion.

Table 3. Asynchrony of Female Gametophyte Development in gaf1.

| Pistil Number | Number of Female Gametophytes at Developmental Stages |
|---------------|------------------------------------------------------|
|               | FG1 | FG2 | FG3 | FG4 | FG5 | FG6 | FG7 | Total FGs |
| 1             | 43  | 10  | 2   |     |     |     |     | 55        |
| 2             | 14  | 20  | 12  | 13  |     |     |     | 59        |
| 3             | 15  | 10  | 13  | 14  |     |     |     | 52        |
| 4             | 8   | 7   | 11  | 13  | 10  |     |     | 49        |
| 5             | 15  | 8   | 11  | 4   | 16  | 3   |     | 57        |
| 6             | 14  | 8   | 5   | 8   | 13  | 6   | 4   | 58        |
| 7             | 2   | 2   | 10  | 11  | 13  | 13  | 3   | 54        |
| 8             | 1   | 4   | 5   | 12  | 6   | 18  | 3   | 49        |
| 9             | 4   | 2   | 6   | 6   | 9   | 4   | 28  | 59        |

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Sequence analysis showed that At5g59980 is a single copy gene in the genome, however, it is predicted that two transcripts are produced from this locus. Transcript At5g59980.1 is 1986 bp and its genomic sequence (2521 bp) is composed of 7 exons and 6 introns, there are 1746 bp in its open reading frame (ORF), which encodes a peptide of 581 aa. Meanwhile, At5g59980.2 genomic fragment (2577 bp) contains 6 exons and 5 introns, the cDNA of At5g59980.2 is 2118 bp, with an ORF of 2118 bp as well, and encoding a peptide of 705 aa. The difference of the two transcripts comes from the 5th exon, which is interrupted with a short inton of 76 bp in At5g59980.1 and resulting in the shift of the reading frame and an in-frame stop codon in the 6th exon. The predicted At5g59980.1 and At5g59980.2 share identical amino acid sequence in fragment 1–568 aa at the N-terminus (www.arabidopsis.org). According to the prediction, primers were designed and RT-PCR was carried out. Results showed that both of At5g59980.1 and At5g59980.2 transcripts were present (data not shown). Therefore, we conducted complementation experiment to identify GAF1 gene.

First of all, complementation experiment was performed to confirm whether the Ds insertion was responsible for gaf1 phenotype. A 5.1 kb genomic fragment containing 0.6 kb upstream of ATG start codon, 4.5 kb of gene genomic fragments that covering all the exons and introns of both transcripts and sequence downstream the stop codon, was cloned into pCAMBIA1300 and introduced into gaf1 plants by Agrobacterium tumefaciens-mediated infiltration [32]. Seven independent transgenic lines were obtained after selection with hygromycin and kanamycin. Seed sets of these T1 plants were restored to a higher degree (from 73.8% to 90.6% in individual plants), compared with 51.5% in gaf1 plants. In T2 seeds from these seven lines, the KanR:KanS ratio was raised up to 2.62:1, indicating the 5.3 kb fragment can complement the semisterile phenotype in the gaf1 background. Furthermore, in 96 transgenic T2 plants, 23 gaf1/gaf1 homozygous plants were obtained. It confirmed that Ds insertion caused the phenotype of the gaf1 mutant. To further investigate the function of At5g59980.1 and At5g59980.2, we constructed two plasmids, pGAF1::GUS-EGFP and pEGFP::GAF1-EGFP, in which GUS (β-glucuronidase) and EGFP (enhanced green fluorescent protein) genes are inserted before the stop codon TAG of the genomic fragment to yield an in-frame fusion with At5g59980.1, respectively (Figure 6A). The plasmids were used for Arabidopsis transformation and the offspring of transgenic plants were scored for KanR, the results showed that both constructs could rescue the phenotype, with the highest ratio of KanR:KanS reached to 2.92:1. It means that At5g59980.1 is fully functional and sufficient.

Table 4. Delayed Pollination Test.

| Hours After Emasculation | Progeny | KanR: KanS | Std |
|--------------------------|---------|------------|-----|
| 24                       | KanR: KanS: 1501 | 0.0031 | 0.002241 |
| 48                       | KanR: KanS: 2357 | 0.0154 | 0.002721 |
| 72                       | KanR: KanS: 1481 | 0.0158 | 0.004448 |

gaf1 pistils were pollinated with wild type pollen at 24 h, 48 h, and 72 h after emasculation. At least 2 independent tests were carried out for each group, and the standard deviation is shown in the table.

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GAF1 Is Required for Gametogenesis in Plants

A

B

C

D

E

F

G

H

91.99% 89.63%

Ler gaf1

GAF1 qrt1 gaf1 qrt1

65.2% 65.4%

in vitro Germination Ratio

in vivo Germination Ratio
for gametophyte development. At5g59980.2, on the other hand, may function redundantly in gametogenesis. So we focused on At5g59980.1/GAF1 in our further study.

GAF1 is a peptide of 581 aa, with a calculated pI of 5.68 and a molecular weight of 64,080 Da (www.arabidopsis.org). The full length CDS (coding sequence) was amplified and sequenced, it revealed that a conserved RNase P subunit p30 (RPP30) domain was present in GAF1, from the 98th to the 248th aa (http://pfam.sanger.ac.uk/) (Figure 6C). The proteins with RPP30 domain are featured as a shared subunit of RNase P and MRP that are conserved in eukaryotes. Both of RNase P and MRP are consisted of an RNA subunit and one or more protein subunits, they play roles in RNA metabolism [20,53]. Phylogenetic analysis and blast results show that CAO21745 from Vitis vinifera, which also has an RPP30 domain, shares most homology with GAF1, with 64% identity and 81% similarity (Figure 6B, D). Alignment reveals that yeast RPP1 shares 26% identity and 54% similarity with GAF1, with higher homology at the N terminal with RPP30 domain than the C terminal (Figure 6D) among the homologues. RPP1 from yeast has been well studied, which is reported to participate in pre-tRNA and pre-rRNA processing [33].

**GAF1 is ubiquitously expressed in planta**

To analyze the expression pattern of GAF1, we checked GAF1 mRNA levels in different tissues with real-time RT-PCR. The results (Figure 7) indicate that GAF1 mRNA is present in all the tissues tested, and predominantly in inflorescence.

GUS reporter gene was also used to visualize the expression pattern of GAF1 in detail. The fusion protein of GAF1 and GUS reporter gene were expressed under the GAF1 promoter in transgenic plants from construct pGAF1::GAF1-GUS. GUS staining results were showed in Figure 8. GUS signal were detected in the young leaves (Figure 8A, arrow), root tip (Figure 8A, arrow and Figure 8B), and lateral primordia (Figure 8C) of 14-day seedlings. In reproductive organs, GUS signal were detected in the inflorescence (Figure 8D), especially in pollen grains (Figure 8E), nucellar cells and embryo sacs of different stages (Figure 8F–J). Localization of GAF1 protein was also observed. In the Arabidopsis root cells checked (Figure 8B, inset), GUS signal was specifically detected in the nucleus. While in other tissues, GAF1-GUS stain displayed a diffused pattern, suggesting it is also in the cytoplasm (Figure 8). Meanwhile, we also found that GAF1-GFP localized in nucleus and mitochondrion in tobacco leaf cells (data

![Figure 5. Seed set analysis of gaf1 mutant plants. A. A pistil was divided into three sections according to the distance to stem, namely distal, middle and proximal, from the stigma to stem, respectively. B. Kan:\ Kan ratio of F1 progeny varies in different sections of gaf1 silique. C. Kan:\ Kan ratio of F1 generation in abundant (1) and limited (2) pollination. doi:10.1371/journal.pone.0033595.g005](https://www.plosone.org/doi/10.1371/journal.pone.0033595)
Figure 6. Molecular characterization of GAF1. A. A scheme showing GAF1 gene structure and the Ds insertion. Black boxes show the ORFs of GAF1 (AT5G59980.1, upper panel) and AT5G56680.2 (lower panel), white boxes show the non-coding region of cDNA and the interval lines depict introns. The numbers of nucleotides are indicated in the figure. B. Phylogenetic tree of GAF1 and its homologues from representative organisms. C.
Amino acid sequence of GAF1. The underlined red sequence is the predicted RPP30 domain. D. Alignment of GAF1 and its homologues. Identical amino acids are shown as white letters in red boxes, and similar amino acids are shown as black letters in yellow boxes. GAF1 in Arabidopsis; CAO21745, homologue in Vitis vinifera; OS12G0175900 and OS12G01060, homologues in Oryza sativa (cv japonica); RPP1_Yeast, homologue in Saccharomyces cerevisiae; RPP30_Human, homologue in Homo Sapiens. doi:10.1371/journal.pone.0033595.g006

not shown). As the same construction could complement the gaf1 mutant, the GUS or GFP signal reflects the expression pattern and the subcellular localization of GAF1. This data suggest that GAF1 is localized in both nucleus and mitochondrion.

GAF1 interacts with Arabidopsis homologue of POP5 in yeast

RPP30 is a conserved protein subunit shared by RNase P and MRP, which can be found in all eukaryotic species as well as in Archaea [53]. As GAF1 encodes the only homologue of RPP30 in Arabidopsis, it is likely a protein subunit of RNases P/MP, and functions in processing pre-tRNA and pre-rRNA. GAF1 homologue RPP1 in yeast is essential for processing pre-rRNA and 35S pre-rRNA, and interacts with POP1, POP3, POP4, POP5, POP6, POP7, POP8, RPR2 (a unique subunit of RNase P), RMP1 and SNM1 (RMP1 and SNM1 are unique subunit of RNase MRP) in pairwise interaction assays [54]. In human, RPP30 was also identified as a scleroderma autoimune antigen [55], and interacts with RPP14, RPP40, RPP21 (homology to yeast RPP2), POP1, RPP38, RPP29 (hPOP4) and RPP30 itself [56,57].

As little is known about RNases P/MPR in plants, we searched for homologues of these protein subunits in Arabidopsis. Besides GAF1/ARPP30, five other homologues of the RNases P/MPR complexes were found: At2G47300 (AtPOP1), At2G43190 (AtPOP4), AT1G04635 (AtPOP5), At5G41270 (AtRPR2), and At1G50910 (AtRMP1). In Arabidopsis, none of the homologues has been studied. To investigate whether these homologues have similar interaction in Arabidopsis as in yeast and human, we checked the protein-protein interaction with yeast two-hybrid system. The full-length CDS of the 6 homologues were cloned and inserted into pGADT7 and pGBKTK7 (see Materials and Methods), and then the constructs were co-transformed into yeast cell in pairs. Results showed that GAF1/AtRPP30 can only interact with AtPOP5 (Figure 9), and no interaction were detected between GAF1/AtRPP30 with any other proteins tested. This specific interaction further indicates that GAF1 is a protein subunit of RNases P/MP. However, interactions between other protein subunits or the unknown protein subunits of Arabidopsis RNases P/MP in plant are not excluded. Our data suggest that RNases P/MP are critical for female gametogenesis and fitness of male gametophyte in Arabidopsis.

Discussion

Here we showed that the insertion of Ds element into At5g39980 leads to aberrant development of embryo sac and reduced fitness of pollen in gaf1 mutant. Mutant ovules are defective in the mitotic progression of the embryo sac which leads to developmental arrest, meanwhile, the gaf1 pollen are less competent than that of the wild-type and cause partial male fertility. These suggest that GAF1 is essential for female gametogenesis and male fitness.

Structural analysis indicated that GAF1 contains a RPP30 domain at its N terminus. It is the only RPP30 homologous protein in Arabidopsis. RPP30 is one of the four conserved RNases P/MP protein subunits in eukaryotes [53]. In yeast, the homologue of RPP30, RPP1, is localized in nucleus [58] and required for pre-tRNA processing and 35S pre-rRNA processing [33]. Meanwhile, RPP1 is essential for yeast viability. Similarly, homozygous gaf1/ gaf1 mutant has never been obtained, which emphasizes an essential role of GAF1. RPP1 was proved to have interaction with POP1, POP3, POP4 and RPP1 itself in RNase MRP. A recent report indicates that POP5/RPP1 heterodimer exists in RNase MRP [59]. Consistently, GAF1 can interact with AtPOP5 in yeast cells. As well as GAF1, AtPOP5 is the Arabidopsis homologue of an eukaryotic conserved RNases P/MP subunit [19,20,60]. All these data suggest that GAF1 is a shared and conserved protein subunit of RNases P/MP. In yeast and human, both of the GAF1 homologues could interact with more than one protein subunits, and the interacting protein subunits are not exactly the same. This may explain why only the interaction between GAF1 and AtPOP5 was detected. Alternatively, protein-protein interactions are different in different organisms. In addition, the interaction between GAF1 and other proteins might require the presence of a third protein subunit, which might be the conserved ones or specific protein subunit in Arabidopsis. Furthermore, obtaining the structure of the two holoenzymes in vivo will unveil the truth. Above data indicate that, GAF1, a novel RPP30 homologue in Arabidopsis, may function as one of RNases P/MP subunits through interaction with a proposed subunit AtPOP5.

RNase P and MRP are essential endoribonucleases, they are structurally and functionally related, although they are physically separate complexes [61,62]. Both of RNase P and MRP are site-specific endoribonuclease. The well-known function of RNase P is the processing of the 5'-end of the pre-tRNA. In addition to the ubiquitous activity of pre-tRNA cleavage, RNase P is also reported to have variety of substrates resembling tRNA, such as 4.5S pre-rRNA [63,64], operon mRNAs [65,66,67], box C/D small nucleolar RNAs. However, only the activity of processing pre-
tRNA is the conserved function of RNase P in archael, bacterial and eukaryotic kingdoms [68]. In human cells, RNase P is also suggested to play roles in transcription of RNA polymerases I and III, and depletion of RNase P caused reduction of tRNATyr, tRNAMet, 5S rRNA, 7SL RNA and U6 snRNA in HeLa cells [29,62]. Compared with RNase P, RNase MRP is found involved in maturation of 5.8S rRNA in yeast [69,70,71,72]. Via special cleavage of 5'-UTR of CLB2, RNase MRP regulates the degradation of CLB2 mRNA and consequently cell cycle in yeast [27,31,32]. As an RPP30 subunit, GAF1 interacts with AtPOP5, another protein unit of RNases P/MRP, and it may possess the ubiquitous function of RNases P/MRP in processing of tRNA and rRNA. However, it is not clear that if plant RNases P/MRP have activity in mRNA degradation, transcription of RNA polymerase, or non-coding RNA generation [73] as in yeast and human cells, since function of plant RNases P/MRP is still poorly understood. Anyway, it is significant that GAF1 may attribute to biogenesis of pre-tRNA and pre-rRNA, just as other components of RNases P/MRP have activity in mRNA degradation, transcription of RNA polymerase, or non-coding RNA generation [73] as in yeast and human cells, since function of plant RNases P/MRP is still poorly understood. Anyway, it is significant that GAF1 may attribute to biogenesis of pre-tRNA and pre-rRNA, just as other components of RNases P/MRP have activity in mRNA degradation, transcription of RNA polymerase, or non-coding RNA generation [73] as in yeast and human cells, since function of plant RNases P/MRP is still poorly understood. Anyway, it is significant that GAF1 may attribute to biogenesis of pre-tRNA and pre-rRNA, just as other components of RNases P/MRP have activity in mRNA degradation, transcription of RNA polymerase, or non-coding RNA generation [73] as in yeast and human cells, since function of plant RNases P/MRP is still poorly understood. 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16-h light/8-h dark cycle. The mutant was isolated from a Ds gene trap population as reported previously [74]. Seeds were sterilized in 20% bleach for 10 min, and rinsed 5 times with sterilized water, then germinated on MS medium. Antibiotic was added as required: 50 mg/L kanamycin for kanamycin selection; 20 mg/L hygromycin for hygromycin selection. Plant transformation was performed by Agrobacterium tumefaciens-mediated infiltration [52].

Phenotype analysis

Plant pollination and confocal observation of the embryo sacs of gaf1 and wild-type plants were performed as described previously [7]. Inflorescences or emasculated pistils were fixed in 4% glutaraldehyde in 12.5 mM cacodylate (pH 6.9), and the plant tissues were dehydrated through a conventional ethanol series. Pistils were dissected and mounted in immersion oil before observation. The samples were observed with Zeiss LSM510 META laser scanning microscope (Zeiss, Jena, Germany) with a 63x objective and a fluorescence filter set. The samples were observed with Zeiss LSM510 META laser scanning microscope (Zeiss, Jena, Germany) with a 63x objective and a fluorescence filter set.

Molecular cloning and complementation

The sequence information comes from The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org). A 5049 bp genomic fragment, including 575 bp promoter region and 2288 bp downstream of the TGA stop codon, was amplified with primer 286-f-Kpn and 286-g-Sac (the primers used in this research are listed in Table 5). The fragment was subcloned into pCAMBIA1300 at KpnI and SacI sites. Positive construct was introduced into gaf1 mutant plants by Agrobacterium-mediated infiltration [52].

The GUS coding sequence was amplified from pWM101 [77] with primers GUSF-Kpn and GUSRV-Pst. The GUS fragment was then subcloned into pCAMBIA1300 at KpnI and SacI sites to produce p1300-GUS. A genomic fragment containing 575 bp upstream of the ATG start codon and the genomic sequence of At5g59980.1 and 2288 bp downstream of the TGA stop codon was amplified with primer combinations 286promoterF-HindIII/286gnsR-Hind and 286-3utrF-Sac/286-gR-Sac, respectively. Two fragments were then subcloned into p1300-GUS at HindIII and SacI, respectively, to generate construct pGAF1::GUS. The GUS CDS in pGAF1::GUS was replaced by EGFP, and pGAF1::GUS was constructed. All constructs were confirmed by sequencing.

Phylogenetic analysis and sequence alignment

The RPP30 homologous protein sequences of different organisms were obtained from National Center for Biotechnology Information (NCBI) using Blastp (http://www.ncbi.nlm.nih.gov). The amino acid sequences of the conserved RPP30 domain of the homologous proteins were used to generate a neighbor joining phylogenetic tree with Phylib3.68 software. Multiple protein sequence alignments were performed by Clustal at NPS@ Clustal [http://www.ebi.ac.uk/Tools/clustalw2/index.html].

RT-PCR analysis

Total RNA was isolated from wild-type Landsberg erecta with TRizol (Invitrogen, USA), and digested with RQ1 DNase I (Promega, USA). cDNA was synthesized by SuperScriptII (Invitrogen, USA). Specific primers At5g59980cDNA-F/At5g59980.2cDNA-R were used for amplification of At5g59980 cDNA, At5g59980cDNA-F/At5g59980.1cDNA-R, and At5g59980cDNA-F/At5g59980.1cDNA-R2 were used for amplification of At5g59980.1 cDNA. Real-time PCR was performed using SYBR Green Master mix (Applied Biosystems) and ABI 7900 sequence detection system. For GAF1 amplification, primer 286-Q1F and primer 286-Q1R were used, and amplification of Actin2/8 mRNA was used as an internal control.

GUS staining

GUS staining of plants transformed with pGAF1::GUS was performed and observed as described previously [13,76]. Tissues were incubated in GUS staining solution containing 1 mg/mL 5-bromo-4-chloro-3-indoxyl-beta-D-glucuronide cyclohexylammonium salt (X-Gluc), 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, 10 mM EDTA, 0.1% Triton X-100, and 100 mg/mL chloramphenicol in 50 mM sodium phosphate.
buffer, pH 7.0) for 3 to 5 days at 37°C after an initial 30 min vacuum. The stained samples were cleared in 20% lactic acid/20% glycerol solution, and observed by a Zeiss Axioskop II microscope.

Yeast two hybrid assay

The sequences of Arabidopsis homologues of RNases P/MRP protein subunits were obtained from NCBI and TAIR (as mentioned above). CDSs of AtPOP1, AtPOP4, AtROP5, AtRPR2, AtRPP30/GAF1, and AtRMP1 were amplified with following primers: POP1-F-R; POP1-R-Nde; POP4-F-EcoR; POP4-R-EcoR; POP5-F-EcoR and POP5-R-EcoR; RPR2-F-EcoR and RPR2-R-EcoR; RPP30-F-Nde and RPP30-R-Nde; RPM1-F-EcoR and RPM1-R-EcoR. CDS fragments were cloned into both pGADT7 and pGBK T7 at EcoR I or Nde I. Constructs were confirmed with sequencing and transformed into AH109 as described in Yeast Protocol Handbook (Clontech, USA). The transformed cells were adjusted to OD$_{600}$ = 0.3 and grown on SD/-Trp-Leu plates for 3 days and on SD/-Trp-Leu-His-Ade plates for 5 or 12 days at 28°C.

Author Contributions

Conceived and designed the experiments: WCY SQW DQS. Performed the experiments: SQW YPL. Analyzed the data: WCY SQW DQS. Contributed reagents/materials/analysis tools: JL. Wrote the paper: SQW DQS WCY.

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