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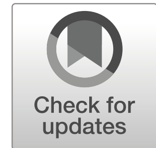
A rice GDSL esterase/lipase protein (GELP) is required for anther and pollen development



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Abstract Male reproductive development is vital for crop seed production. However, the mechanism underlying remains largely unknown. Here, we isolated a male sterile mutant *osgelp34-1* in rice. Cytological analysis indicated that the mutant exhibited delayed degradation of anther wall layers and abnormal pollen development, which resulted in the production of shrunk and non-viable pollen grains. MutMap and CRISPR/Cas9 analysis confirmed that a point mutation in *OsGELP34* is responsible for the male sterile phenotype. *OsGELP34* is highly expressed in reproductive tissues and encodes a putative GDSL lipase. OsGELP34 protein is located to the endoplasmic reticulum (ER) and conserved in land plants. Collectively, our findings elaborated that *OsGELP34* plays a vital role in rice male reproduction and has potential applications in rice hybrid breeding.

Keywords Rice · Pollen · Anther · OsGELP34 · Male sterile

Guoqiang Yuan and Ting Zou contributed equally to this work.

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Introduction

Hybrid breeding has contributed to enhancing the grain yield of rice. Male sterile lines, especially cytoplasmic male sterile (CMS) lines and environment-sensitive genic male sterile (EGMS) lines, are wildly used in hybrid seed production by providing means for eliminating the expensive and labor-intensive practice of manual emasculation. The most common cause of male sterility is pollen abortion. Pollen development occurs in anther and is initiated by the differentiation of microspore mother cells (MMCs), which undergo meiosis to form tetrads thereafter (Scott et al. 1991). Then, the microspores are released from tetrads to form the pollen wall and go through two-step mitosis to develop into mature pollen grains eventually (Scott et al. 1991).

Pollen development is accompanied by the differentiation, development, and degradation of the surrounding anther somatic cell layers such as tapetum, which provides nutrients for pollen formation (Gómez et al. 2015; Lei and Liu 2019; Shi et al. 2015; Wilson and Zhang 2009). These processes are finely regulated by several key genes (Lei and Liu 2019; Shi et al. 2015). Genes encoding transcription factors (TFs) in *Arabidopsis* such as *DYSFUNCTIONAL TAPETUM1* (*DYT1*) (Feng et al.

2012; Zhang et al. 2006), *TAPETAL DEVELOPMENT AND FUNCTION 1* (*TDF1*) (Gu et al. 2014; Zhu et al. 2008), *ABORTED MICROSPORES* (*AMS*) (Sorensen et al. 2003; Xu et al. 2014; Xu et al. 2010), and their homologs *UNDEVELOPED TAPETUM1*(*UDT1*) (Jung et al. 2005), *OsTDF1* (Cai et al. 2015), and *TAPETUM DEGENERATION RETARDATION* (*TDR*) (Li et al. 2006) in rice, respectively, were found to regulate the development of the tapetum. Other TFs also function in controlling tapetum degradation. ETERNAL TAPETUM 1 (EAT1), a rice bHLH transcription factor, promotes tapetal cell death and ensures the normal degradation of tapetum cells (Ji et al. 2013; Niu et al. 2013b). A PHD-Finger Protein, PERSISTENT TAPETAL CELL 1 (PTC1), is reported to regulate tapetal cell death (Li et al. 2011). The tapetum cells in *ptc1* mutant did not degrade regularly and remained on the inner anther wall even at the mature pollen stage, resulting in shriveled pollen grains. Besides, other functional proteins also have roles in the programmed cell death (PCD) of the tapetum. For example, an ATP citrate lyase, EARLIER DEGRADED TAPETUM 1 (EDT1), is required for normal tapetum PCD, and the *edt1* mutant exhibited pre-degraded tapetal cells (Bai et al. 2019).

Pollen development also requires the metabolism of substances in tapetal cells, and several related enzymes have been reported. For example, CYP703A3 (a cytochrome P450 hydroxylase) protein catalyzes the intracyclic hydroxylation of cinnamic acid to produce the 7OH-C12 fatty acid, which acts as the substance for pollen wall formation (Yang et al. 2014). Other enzymes such as rice acyl-CoA synthetase (OsACOS12, an ortholog of *Arabidopsis* ACOS5) (Li et al. 2016), rice POLYKETIDE SYNTHASE 1/LESS ADHESIVE POLLEN 6 (OsPKS1/OsLAP6) (Shi et al. 2018; Zou et al. 2017), and OsPKS2 (Zhu et al. 2017; Zou et al. 2018) also convert the substrates for pollen wall formation. The mutants of these genes also had defects in pollen development and showed complete male sterility. In addition to the synthesis and metabolism, the transport of materials from tapetum to pollen is also important for pollen development. In *post-meiotic deficient anther 1* (*pda1*) mutants, pollen abortion was caused by the absence of pollen exine and anther cuticle. *PDA1* encodes OsABCG15, a member of rice ABC transporter G subfamily, and is responsible for the transport of metabolites from tapetum to pollen surface (Niu et al. 2013a; Qin et al. 2012; Wu et al. 2014; Zhu et al. 2013). Besides, OsABCG3, another member of rice ABC transporter G subfamily, also plays an important role in the development of pollen intine and cytoplasmic contents (Chang et al. 2018; Luo et al. 2019).

The development of anther wall layers and pollen has a great influence on male fertility in rice. Identifications of genes involved in these processes are therefore beneficial to understand the mechanism of male reproduction. In this study, we isolated a completely male sterile mutant, which showed delayed degradation of anther wall layers and produced abnormal pollen grains. The causal gene of this mutant encodes an ER-localized GELP, OsGELP34. Using the CRISPR/Cas9 technology, we also developed several male sterile mutants in genetic background of different rice cultivars. Our results suggested that *OsGELP34* is important for both anther and pollen development, and can be applied in rice hybrid breeding.

Materials and methods

Plant material and growth condition

The *osgelp34-1* mutant was derived from the mutant library by ethyl methyl sulfonate (EMS)-induced mutation of an *indica* cultivar 9311. The loss of function mutant lines in a *japonica* cultivar ZH11 and an *indica* cultivar B48 were created via the CRISPR/Cas9 genomic editing system (Miao et al. 2013). All plants were grown in the paddy field in Chengdu (Sichuan, China) or in Lingshui (Hainan, China) under normal cultivation conditions.

Phenotypic characterization

The phenotypes of the whole plants and floral organs were photographed with a Nikon D5300 digital camera (Tokyo, Japan). Observations of mature pollen grains were conducted with an Axio Lab. A1 microscope (Zeiss, Oberkochen, Germany). Examination of semi-thin sections and scanning electronic microscopy (SEM) were carried out as described previously (Zou et al. 2018).

Causal gene mapping

The *osgelp34-1* mutant was backcrossed with its paternal wild type (WT) 9311 to generate BC_1F_2 progenies. MutMap strategy as described by Abe et al. (2012) was

utilized for gene mapping. In brief, forty male sterile plants isolated from the BC_1F_2 generation were mixed equally for DNA extraction, which were further sequenced with a HiSeq 2500 platform (Illumina, San Diego, CA, USA). Co-segregation analysis was carried out by phenotyping and genotyping of individuals in the BC_1F_2 population.

Expression pattern analysis

The RNA extracted from root, stem, leaf, and developing florets of rice plants at heading stage were used for expression pattern analysis. Spin Column Plant Total RNA Purification Kits (Sangon, Shanghai, China) were used for RNA extraction. The following RNA reverse transcription and quantitative real-time PCR (qPCR) were conducted with HiScript Q-RT SuperMix Kit and AceQ qPCR SYBR Green Master Mix Kit (Vazyme, Nanjing, China), respectively. The qPCR reactions were performed on a qTOWER 2.0 machine (Analytik Jena, Jena, Germany). The primers used in this study were listed in Table S1.

Protein subcellular localization

For subcellular localization, full-length *OsGELP34* coding sequence (CDS) was amplified from the WT and *osgelp34-1* mutant, and the CDS of *OsGELP34* without signal peptides (SP) and SP was amplified from the WT. These fragments were cloned into the pCAMBIA2300-GFP vector, respectively, for fusing with the GFP. ER marker was constructed by fusing mCherry with the HDEL ER retention signal (De Caroli et al. 2011). Different combinations of plasmids were transiently co-expressed in tobacco (*Nicotiana benthamiana*) leaf epidermal cells, and the signals of GFP and mCherry were observed as described previously (Zou et al. 2018).

Phylogenetic analysis

Full length of OsGELP34 amino acid sequence was used as the query for Basic Local Alignment Search Tool (BLAST) search in National Center for Biotechnology Information (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi). The peptides of retrieved OsGELP34 orthologs were further aligned with ClustalW program. Based on the result of alignments, a neighbor-joining phylogenetic tree was constructed using MEGA-X program with 1000 bootstrap replications.

Results

Morphological and cytological identification of *osgelp34-1* mutant

By screening the EMS-induced mutant library, we identified a male sterile mutant, namely *osgelp34-1* (see below). Compared with the WT, *osgelp34-1* had no obvious difference in vegetative growth (Fig. 1a) and floral organs morphogenesis (Fig. 1b). However, the mutant anthers are smaller and pale yellow than those of the WT (Fig. 1c). All the pollen grains produced by the mutant were dyed blue with Alexander staining (Fig. 1d), and could not be stained with 1% I_2-KI solution (Fig. 1e, f), indicating this mutant is completely male sterile. After pollination with the WT pollen, the *osgelp34-1* mutant showed a normal seed setting rate, suggesting the female part of the mutant was intact.

To investigate the cytological differences between the WT and *osgelp34-1* mutant, we compared the cross-sections of developing anther from stage 7 to stage 13, according to the cellular features reported by Zhang et al. (2011). From the stage 7 to stage 9, no obvious difference between the WT (Fig. 2a–d) and *osgelp34-1* (Fig. 2e–h) was found. At the stage 10, the middle layer (ML) of the WT had degraded (Fig. 2i). However, in *osgelp34-1*, the ML was still present (Fig. 2m). Besides, at this stage, compared with the concentrated and degrading tapetal cells in the WT (Fig. 2i), *osgelp34-1* exhibited swollen tapetum (ST) with less cytoplasm contents (Fig. 2m). Meanwhile, the mutant microspores seemed to display a lower vacuolation level (Fig. 2m) than those of the WT (Fig. 2i). Subsequently, the WT tapetum underwent PCD further (Fig. 2j) at stage 11, and completely degenerated during later stages (Fig. 2k, l), whereas the ST in *osgelp34-1* remained (Fig. 2n–p). In addition, the ML of the mutant anther always presented until stage 13 (Fig. 2m–p). At stage 13, the WT produced spherical pollen grains (Fig. 2l). In contrast, the pollen grains of *osgelp34-1* showed shrunk shape and were surrounded with tapetal remnants (TR) (Fig. 2p).

To further observe the detailed defects of *osgelp34-1* mutant, we examined the anther and pollen surfaces of the WT and *osgelp34-1* at stage 12 by SEM. The results showed that, in *osgelp34-1*, the size of whole anther was smaller (Fig. 3d), and the cuticle surface of epidermis was more compact (Fig. 3e) than which of the WT (Fig. 3a, b). Although the number of pollen grains, the Ubisch bodies along the inner surface

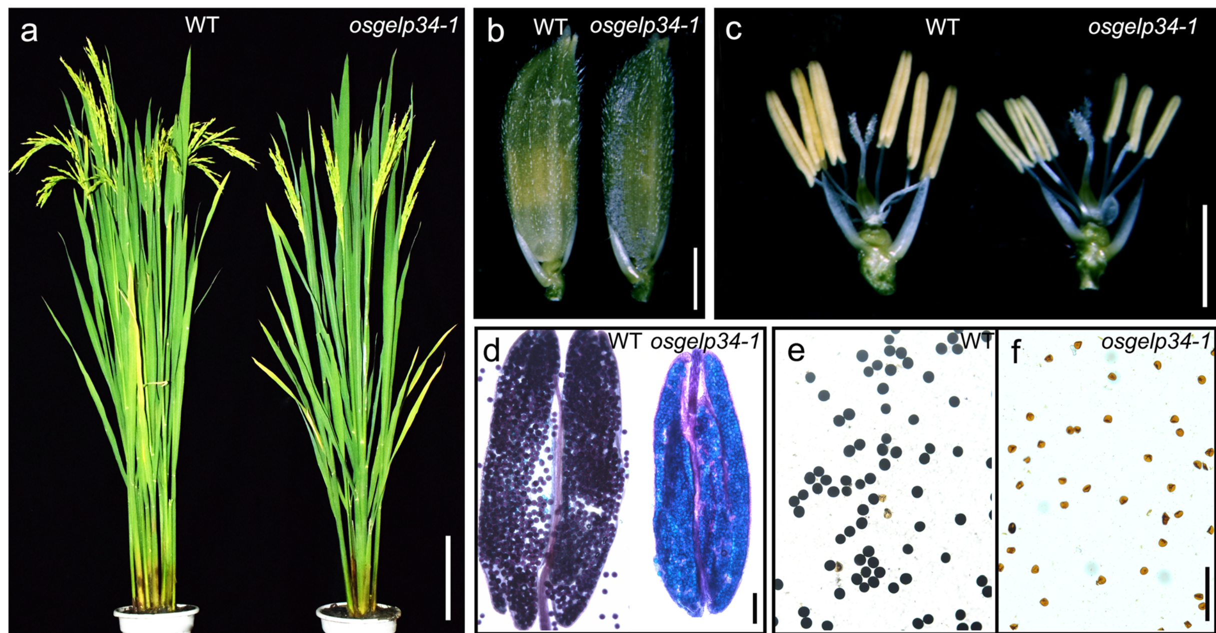

Fig. 1 Phenotypic characterization of *osgelp34-1* mutant. **a** The plants of the WT and *osgelp34-1* mutant at grain filling stage. **b** The floret of the WT and *osgelp34-1* mutant at heading stage. **c** The floret of the WT and the *osgelp34-1* mutant at heading stage after removal of lemma and palea. **d** Alexander staining of the anther from the WT and *osgelp34-1* mutant. **e**, **f** I_2-KI staining of the mature pollen grains from the WT and *osgelp34-1* mutant. Bar = 20 cm (**a**); 0.2 mm (**b**, **c**); 30 μm (**d**, **e**, **f**)

of the tapetum, and the tectum on the exine surface were comparable between the WT (Fig. 3c–j) and *osgelp34-1* (Fig. 3f–n), the mature pollen grains of the mutant had a shriveled morphology (Fig. 3l), which was consistent with the observations of light microscope. Additionally, the WT pollen had a typical germination aperture surrounded with the annulus protuberance structure (Fig. 3h, i); however, the germination aperture of the mutant pollen displayed an abnormal shape (Fig. 3m).

MutMap cloning of *OsGELP34* gene

Individual plants of the BC_1F_2 population showed an approximate 3:1 ratio between normal fertility plants and the male sterile mutants ($\chi 2 = 0.1667$; $P > 0.5$) (Table S2). This suggested that the male sterile phenotype of *osgelp34-1* is controlled by a single recessive gene. For mapping the causal gene, we utilized the MutMap approach (Abe et al. 2012). By analyzing the bulk sequencing results, a cluster of 18 single nucleotide polymorphisms (SNPs) with high index was identified in between 10.86-Mbp and 17.46-Mbp on chromosome 2 (Fig. 4a). Among these SNPs, only one SNP (C290T in *LOC_Os02g18870*) was located in the coding region (Table S3). Moreover, the results of subsequent segregation analysis showed that this SNP was co-segregated with the mutant phenotype (Fig. 4b, c). *LOC_Os02g18870* encodes OsGELP34, which is a member of rice GELP superfamily (Chepyshko et al. 2012). The protein domain predictions of OsGELP34 with Pfam (http://pfam.xfam.org/) and SignalP-5.0 (http://www.cbs.dtu.dk/services/SignalP/) indicated this protein contains a SP in N-terminal and a lipase GDSL domain (Fig. 4d). According to the report from Akoh et al. (2004), OsGELP34 has five conserved GDSL blocks in the lipase GDSL domain (Fig. S1). The amino acid substitution (Ser97Phe) caused by the mutation was located between blocks I and II (Fig. S1). These findings suggested that *OsGELP34* is the candidate gene for this locus. We thus named this mutant *osgelp34-1*.

Functional validation of *OsGELP34*

To verify the function of *OsGELP34* in male reproduction, we used the CRISPR/Cas9 genomic editing tool to knockout this gene in ZH11 with two independent target sites (Fig. 5a). Target 1 was located between the GDSL blocks I and II, while target 2 was overlapped with the GDSL block II (Fig. S2). We obtained a total of five positive transgenic plants, which harbored homozygous

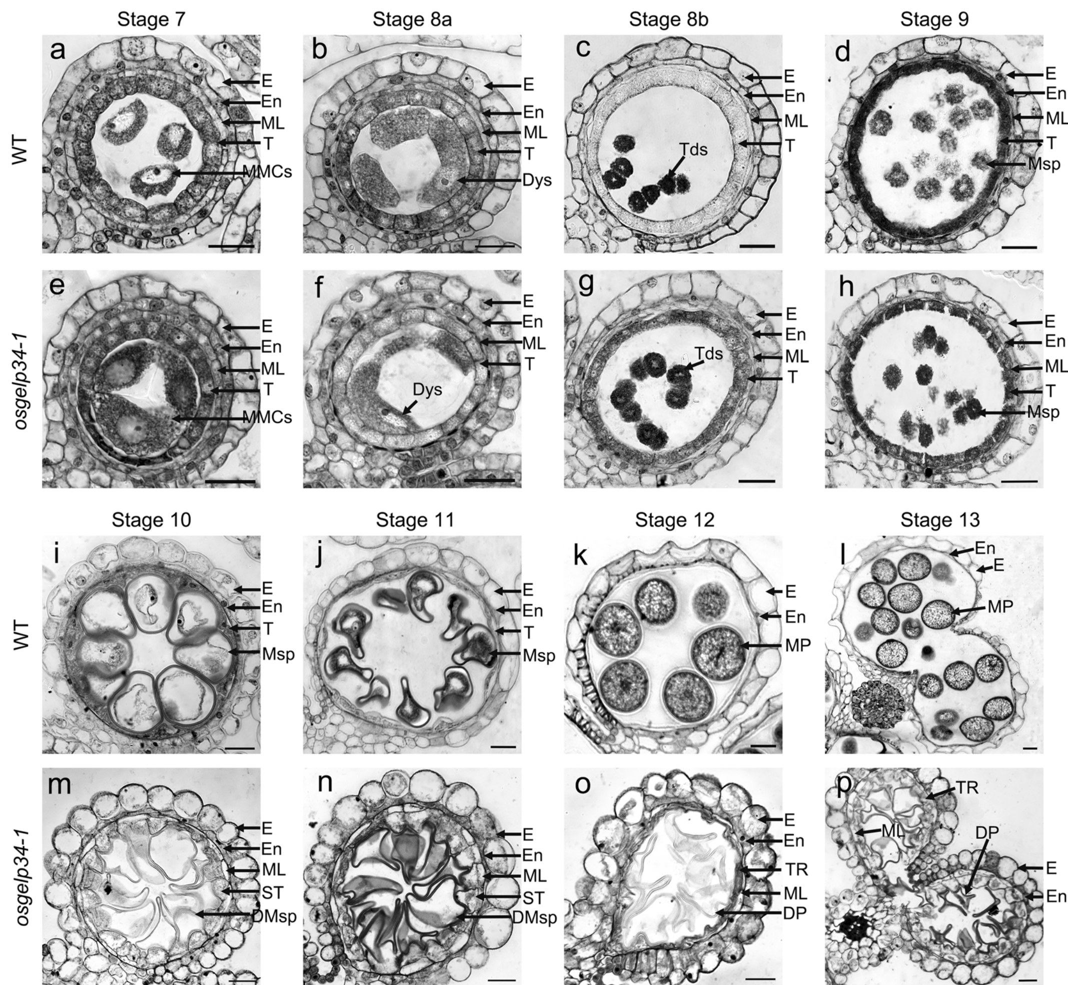

Fig. 2 Comparison of anther developmental differences between the WT and *osgelp34-1* mutant. **a**, **e** Stage 7. **b**, **f** Stage 8a. **c**, **g** Stage 8b. **d**, **h** Stage 9. **i**, **m** Stage 10. **j**, **n** Stage 11. **k**, **o** Stage 12. **l**, **p** Stage 13. E, epidermis; En, endothecium; ML, middle layer; T, tapetum; MMCs, microspore mother cells; Dys, dyad cells; Tds, tetrads; Msp, microspore; DMsp, deformed microspore; MP, mature pollen; DP deformed pollen; ST, swollen tapetum; TR, tapetal remnants. Bars = 20 μm (**a**–**p**)

insertions or deletions of base pairs (Fig. 5b), indicating a successful genetic knocking-out (KO) of *OsGELP34*. Among these transgenic plants, the mutations of *ko-1-2*, *ko-2-2*, and *ko-2-3* caused premature truncations of OsGELP34 protein, while that in *ko-1-1* and *ko-2-1* had only one amino acid deletion (Fig. 5c, Fig. S2). Further phenotypic identifications showed that the vegetative growth and floret morphology of all these mutants were similar to that of ZH11 (Fig. 5d, e); however, their anthers are lighter in color and smaller in size than those of ZH11 (Fig. 5f), and their pollen grains were non-viable (Fig. 5g–l), mimicking the phenotype of *osgelp34-1*. These results demonstrated that *OsGELP34* plays a critical role in rice male gamete development. Consistently, other two recent works have also independently confirmed the key function of *OsGELP34* during rice pollen development (Zhang et al. 2020; Zhao et al. 2020).

Expression analysis of *OsGELP34*

To further understand the role of *OsGELP34* in rice development, we analyzed its expression patterns in different rice tissues. Our qPCR analysis indicated that *OsGELP34* mRNA accumulated highly in developing florets, rather than in other tissues (Fig. 6a). The strongest transcription of *OsGELP34* was detected in florets with anthers during stages 8 to 9 (Fig. 6a), overlapping

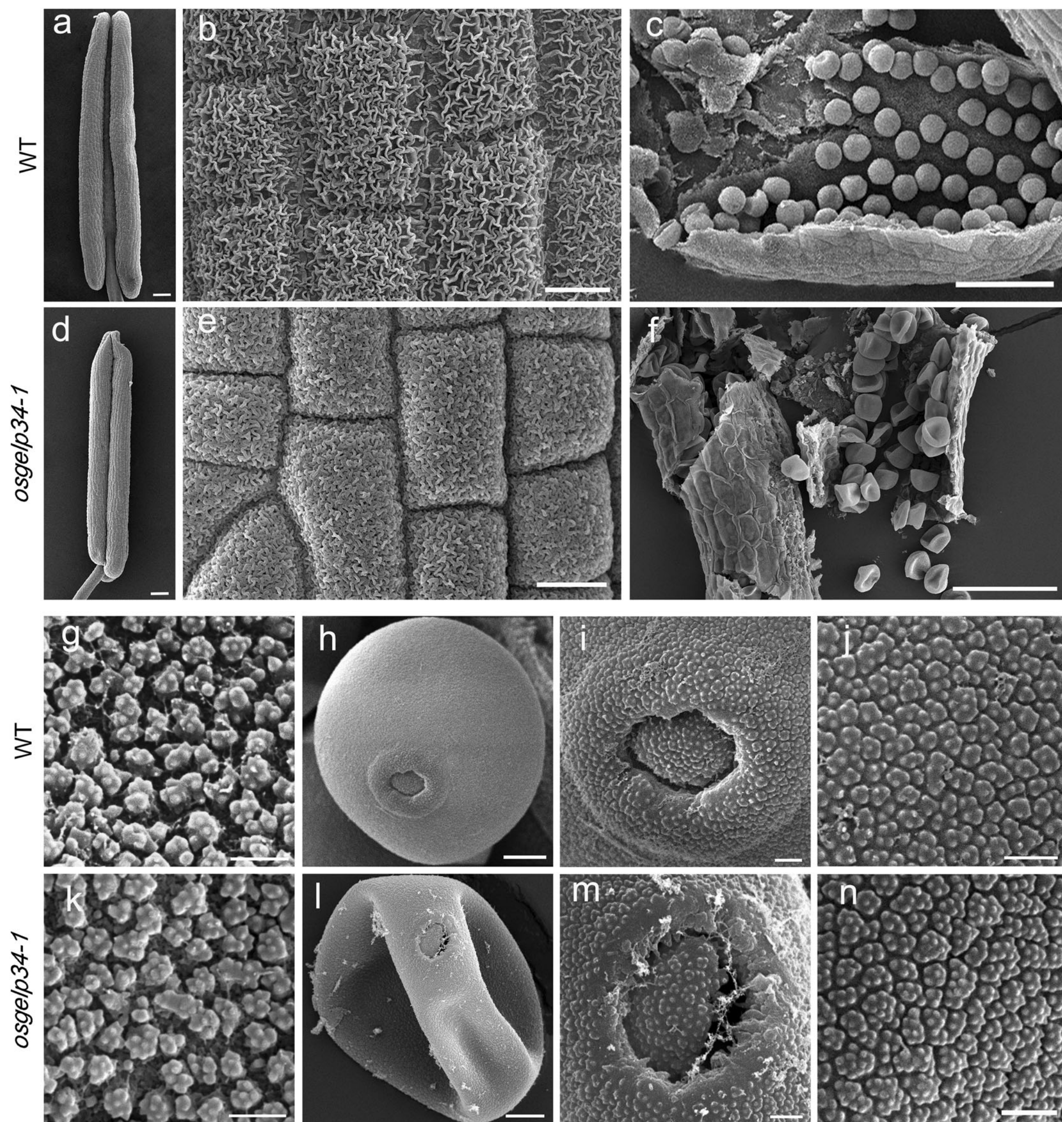

Fig. 3 SEM examination of anther and pollen grain in the WT and *osgelp34-1* mutant at stage 12. **a**, **d** Whole anther of the WT and *osgelp34-1* mutant. **b**, **e** Epidermal surface of the WT and *osgelp34-1* mutant anther. **c**, **f** Pollen grains in the WT and *osgelp34-1* mutant. **g**, **k** Ubisch bodies along the inner surface of tapetum in the WT and *osgelp34-1* mutant anther. **h**, **l** Mature pollen grains of the WT and *osgelp34-1* mutant. **i**, **m** The germination aperture of the WT and *osgelp34-1* mutant pollen grain. **j**, **n** Pollen grain surface of the WT and *osgelp34-1* mutant. Bars = 200 μm (**a**, **c**, **d**, **f**); 2 μm (**b**, **e**, **g**–**n**)

with the processes of microspore release and pollen wall initiation. Interestingly, the expression level of *OsGELP34* was upregulated in *osgelp34-1* at late anther developmental stages (Fig. 6b), suggesting a possible feedback regulation of this gene.

To investigate the potential function of OsGELP34 protein, we generated a series of constructs, including the full-length OsGELP34 of WT (OsGELP34-FL) and *osgelp34-1* mutant (osgelp34-1-FL), the SP of OsGELP34 alone (SP), and the truncated OsGELP34 without SP (ΔSP) (Fig. 6c), for subcellular localization analysis. When these constructs were co-expressed with ER marker (mCherry-HDEL) in tobacco leaves, we found that the GFP signals of OsGELP34-FL or osgelp34-1-FL were overlapped with mCherry signals (Fig. 6d, e). These results indicated that OsGELP34 protein is located to ER, and the *osgelp34-1* mutation may not affect the subcellular location pattern.

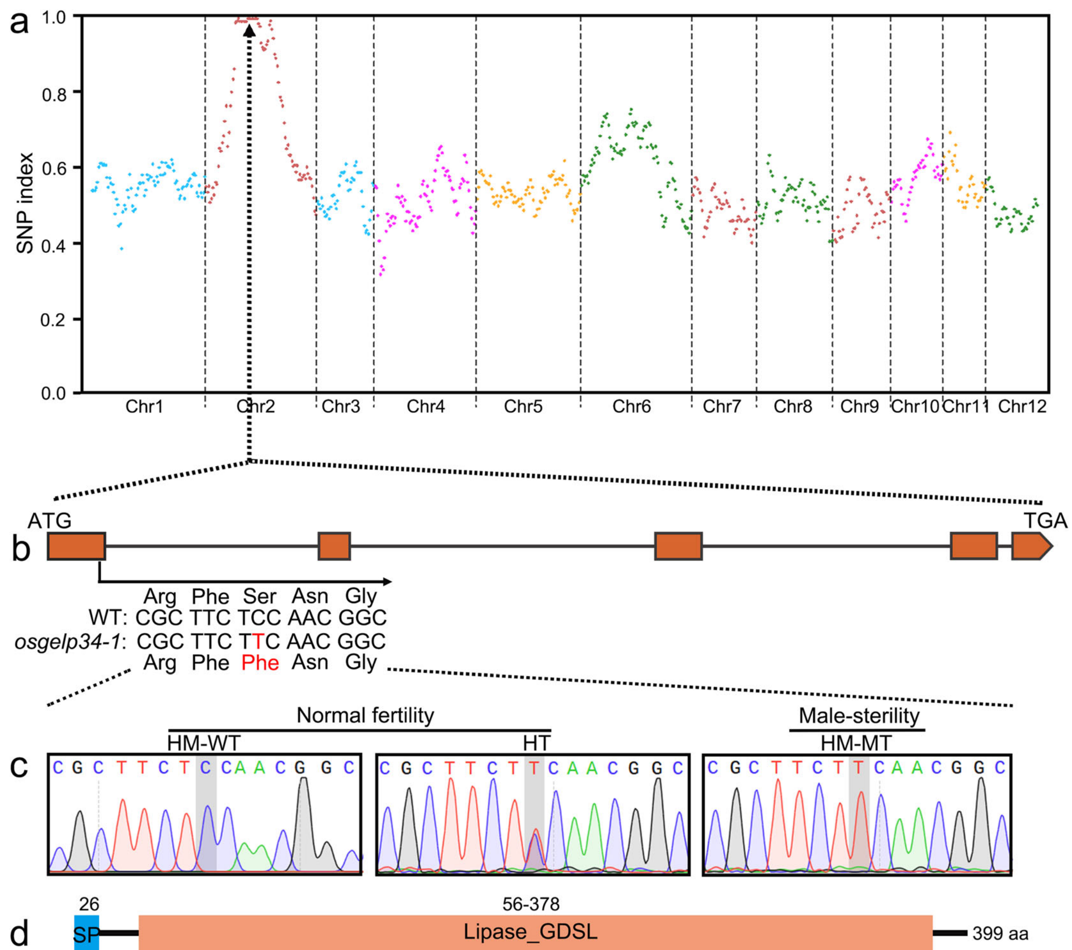

Fig. 4 Cloning of *OsGELP34*. **a** Distribution and index of SNPs along chromosomes. **b** Schematic structure of *OsGELP34* gene. The rectangle indicates the direction of the sequence. **c** Co-segregation analysis of the *osgelp34-1* mutation in BC_1F_2 generation. HM-WT, homozygous wild-type genotype; HT, heterozygous genotype; HM-MT, homozygous mutant. **d** The SP in N-terminal and the Lipase_GDSL domain of OsGELP34. The numbers represent the starting and the ending position of the amino acids in the domain

Intriguingly, the SP was also merged with ER marker (Fig. 6f), whereas ΔSP displayed a non-preferential distribution (Fig. 6g, h), similar to that of free GFP control (Fig. 6i). These results suggested that the SP of OsGELP34 protein is essential for its subcellular distribution.

OsGELP34's orthologs in land plants

To obtain evolutionary information of OsGELP34, we submitted the full-length amino acid sequence of OsGELP34 to BLAST tool in NCBI for searching its orthologs from other plant species. Twenty-one closest relatives were retrieved from different species (Table S4). Peptide alignment results indicated that all these proteins shared similar lipase GDSL domain and GDSL blocks (Fig. S3), suggesting that the orthologs of OsGELP34 might have conserved function among various plant species. Furthermore, a phylogenetic tree was constructed and clustered these OsGELP34 relatives into three clades, including monocots, dicots, and lower plants (Fig. 7a).

Notably, the ortholog of OsGELP34 in *Arabidopsis*, which shared ~ 53.01% identities with OsGELP34 (Fig. 7a), was predicted to be specifically expressed in the florets (Fig. S4), which was similar to the expression pattern of *OsGELP34*. Coincidently, a recent study termed this ortholog as reversible male sterile (RVMS), and revealed that mutation of *RVMS* resulted in a reduction of enzyme activity, which led to a temperature-

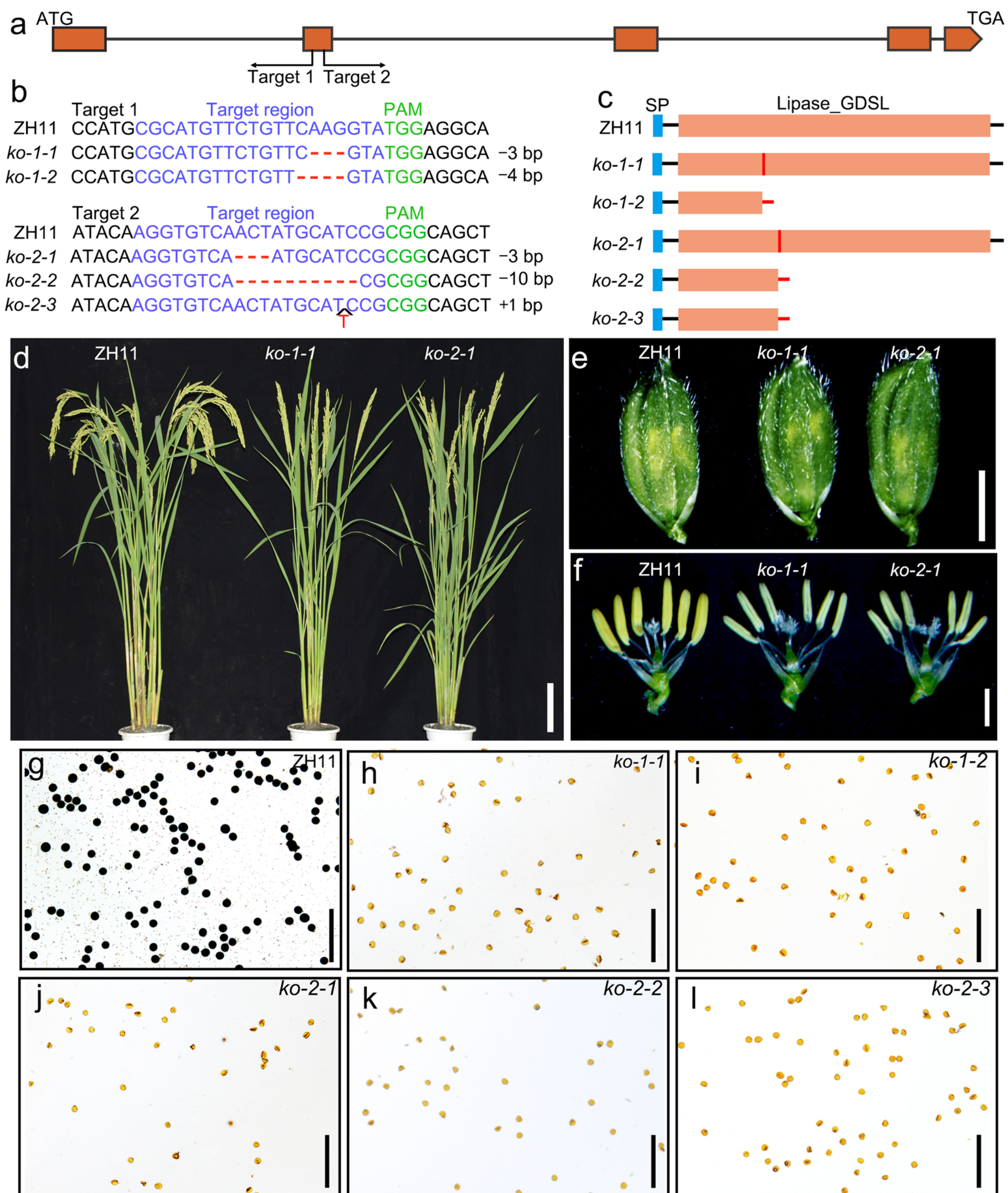

Fig. 5 Knocking-out (KO) of *OsGELP34* in ZH11 by CRISPR/Cas9 genomic editing system. **a** Schematic representation of target site position. The rectangles indicate the directions of two independent targets. **b** Sequencing analysis of target regions of transgenic plants. **c** Amino acid changes in transgenic plants. **d** The whole plant of ZH11 and the KO mutants. **e** The floret of ZH11 and the KO mutants at heading stage. **f** The floret of ZH11 and the KO mutants at heading stage after removal of lemma and palea. **g**–**l** Mature pollen grains of ZH11 and the KO mutants stained by I_2-KI solution. Bars = 15 cm (**d**); 2 mm (**e**); 1 mm (**f**); and 200 μm (**g**–**l**)

sensitive male sterile (TGMS) phenotype (Zhu et al. 2020). However, our temperature treatment results indicated that the *osgelp34-1* mutant showed complete male sterility at both high and low temperature (Fig. 7b). As a control, the *thermo-sensitive genic male-sterile 10* (*tms10*) mutant was male sterile at high temperature but fertile at low temperature (Fig. 7b) (Yu et al. 2017). These results suggested that the pollen viability of *osgelp34-1* may not be sensitive to temperature changes.

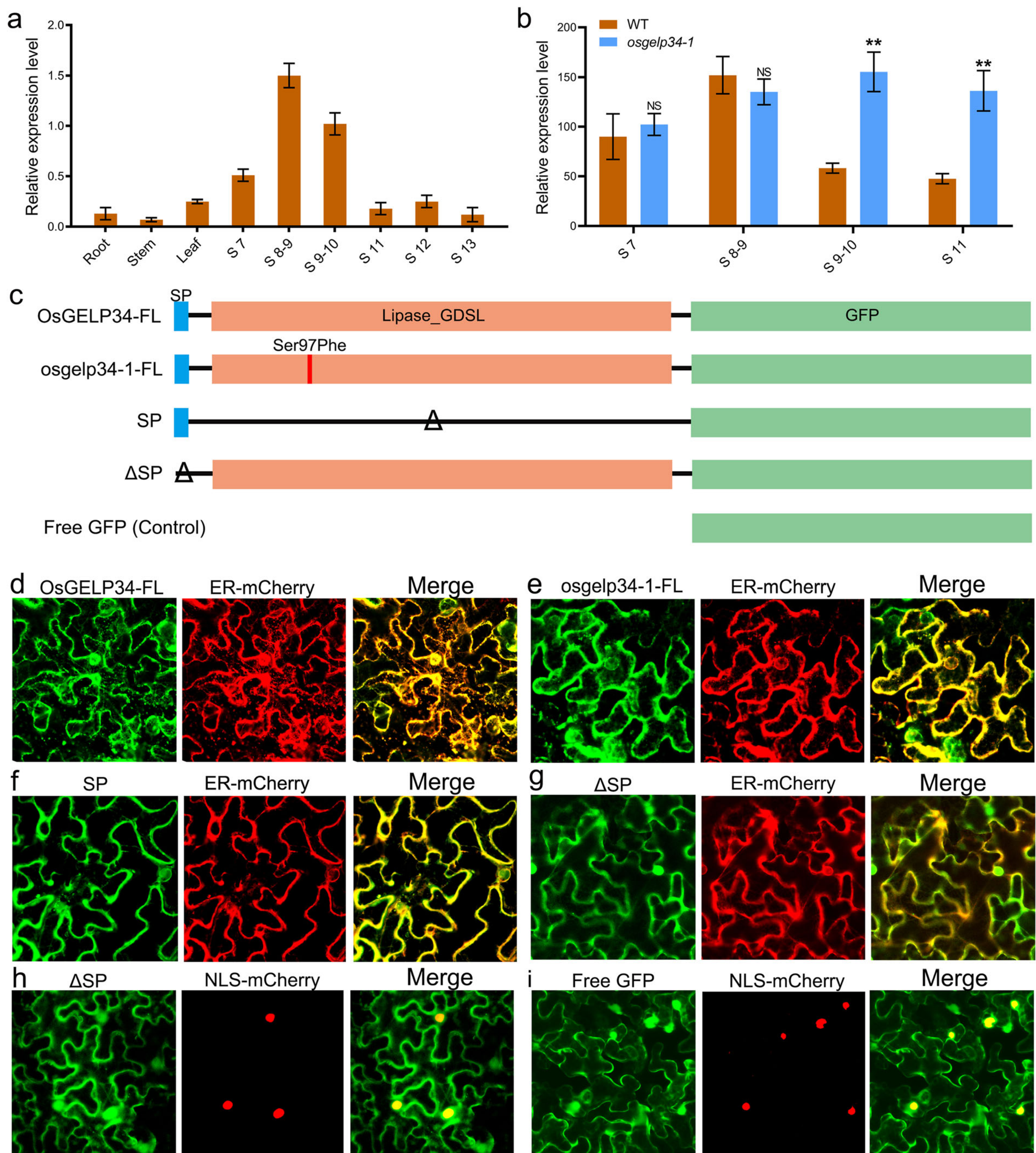

Fig. 6 Expression pattern and subcellular localization of OsGELP34. **a** Expression pattern of *OsGELP34* in various rice tissues. S, developing florets with anthers at different stages. **b** Expression profiles of *OsGELP34* in developing florets with anthers at different stages (S) from the WT and *osgelp34-1*. **c** Schematic representation of the vectors used for subcellular localization. **d–i** Subcellular localization of full-length OsGELP34 (OsGELP34-FL, **d**) in WT, full-length OsGELP34 in osgelp34-1 mutant (osgelp34-1-FL, **e**), the SP of OsGELP34 in WT (**f**), truncated OsGELP34 without SP (ΔSP) in WT (**g**, **h**), and free GFP control (**i**) in epidermal leaf cells of tobacco. Bars = 20 μm (**d**–**i**)

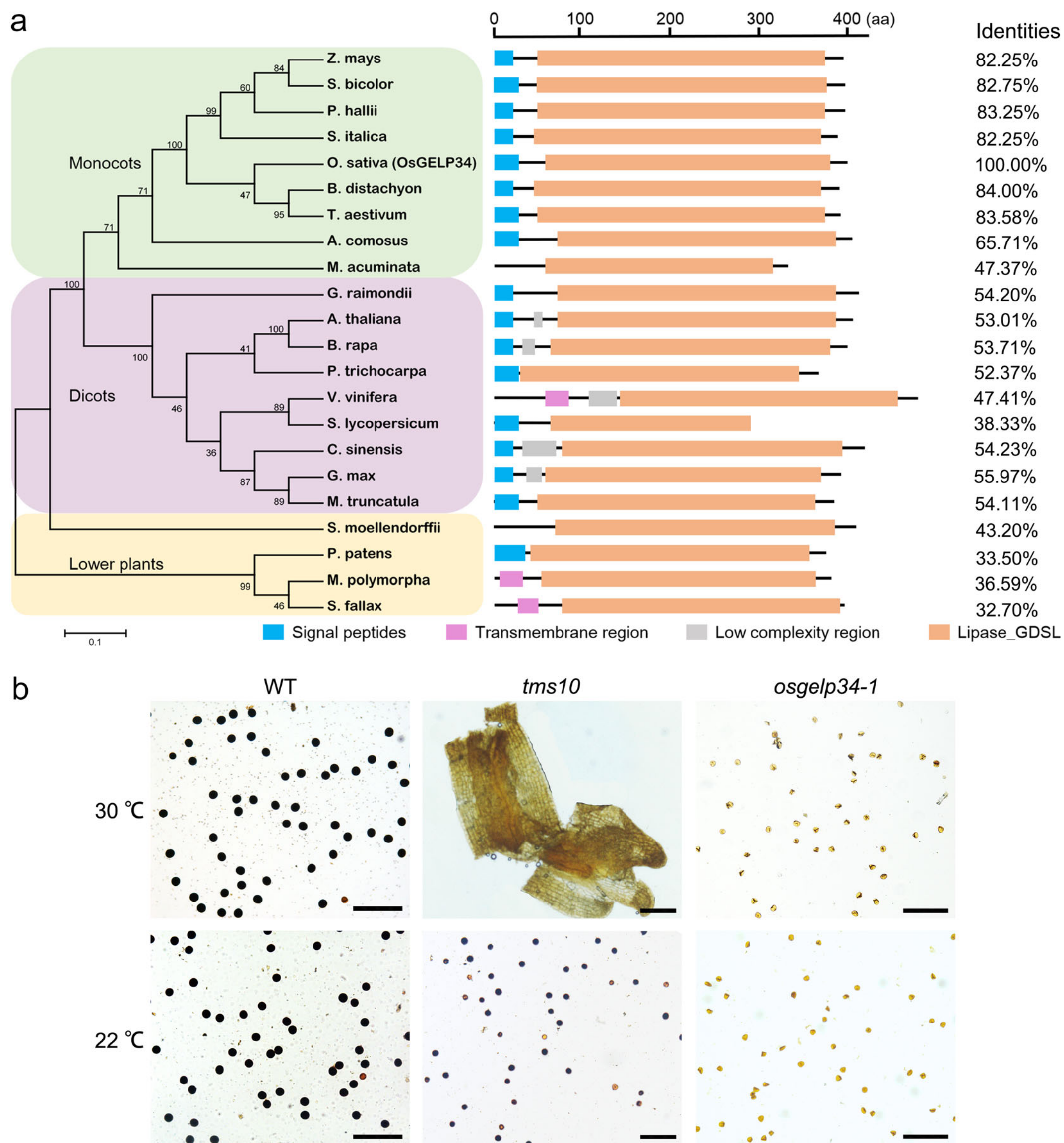

Fig. 7 Phylogenic analysis of OsGELP34-related proteins and different temperature treatment of the *osgelp34-1* mutant. **a** The phylogenic tree of OsGELP34-related proteins in land plants. The tree was constructed based on the alignment result in Supplementary Fig. S4. Bootstrap values were indicated by the numbers at the nodes. The percentage numbers indicate the identities between corresponding orthologs and OsGELP34. **b** Pollen viability of the WT and *osgelp34-1* at different growth temperature. I_2-IK staining of pollen grains from the WT and *osgelp34-1* grown under 30 °C(August, 2018) and 22 °C (October, 2019) average environmental temperature in paddy field at Chengdu, China. These observations were consistent for two consecutive years (2018–2019). The *thermo-sensitive genic male-sterile 10* (*tms10*) mutant was used as a control. Bars = 200 μm in each panel

Potentiality of *OsGELP34* in hybrid breeding

Non-EGMS line has been used in rice hybrid breeding via the Seed Production Technology (SPT) (Chang et al. 2016). To explore the potential application of *OsGELP34* in breeding, we designed another two independent targets in the first exon of *OsGELP34* for further KO analysis (Fig. 8a and S5). An elite maintainer line (B48, an *indica* rice cultivar) with excellent agronomic traits, such as long grain and fragrance, was used as the receptor for genetic transformation. By direct sequencing and cloned sequencing, we identified three positive lines from T_0 plants (Fig. 8a). Compared with B48, all these three mutants had no apparent differences in vegetative growth (Fig. 8b, c)

except for the inactive pollen grains (Fig. 8d–f). These results suggested that stable non-GMS lines can be generated from different background of rice by functional KO of *OsGELP34*. *OsGELP34* is therefore a possible candidate genetic resource for rice hybrid breeding system.

Discussion

Male sterile lines are indispensable for rice hybrid breeding, and have contributed greatly to rice yield improvement (Chen and Liu 2014; Xing and Zhang 2010). Therefore, identification of male sterile mutant in different genetic background can provide useful germplasm resources for hybrid seed production. Here, we characterized *osgelp34-1*, a mutant of *OsGELP34*, which belonged to the rice GELP family (Chepyshko et al. 2012). Cytological observations indicated that the *osgelp34-1* mutation caused delayed degradation of tapetal cells and middle layers (Fig. 2m–p), compact cuticle nanoridges on the anther epidermal surface, abnormal pollen vacuolation (Fig. 3e, l), and hence a completely male sterile phenotype (Fig. 1d–f). These cellular defects were similar to that of *rice male sterile 2* (*rms2*) (Zhao et al. 2020) and *osgelp34* (Zhang et al. 2020), another two allelic mutants of *OsGELP34* reported very recently. Additionally, we also created eight KO lines of *OsGELP34* in different genetic backgrounds, including a *japonica* ZH11 and an *indica* B48, by using the CRISPR/Cas9 genomic editing tool. All these mutants were male sterile (Figs. 5g–l and 8d–f). Taken together, these findings collectively demonstrated that *OsGELP34* is required for both anther and pollen development during male reproduction in rice.

The *osgelp34* mutation caused an amino acid substitution from Gly to Ser (Zhang et al. 2020), which positioned in the conserved GDSL block I of OsGELP34 protein (Fig. S3). In this work, we found that a Tyr deletion in block II of OsGELP34 protein also resulted in a male sterile phenotype (Fig. 5g–f, Fig. S2), suggesting the important roles of blocks I and II of this protein. In *rms2* mutant, the mutation replaced a conserved Leu residue between blocks III and IV by His and led to a low enzymatic activity of OsGELP34 protein (Fig. S3) (Zhao et al. 2020). Our protein sequence analysis indicated that the *osgelp34-1* mutation changed a Ser residue between the GDSL blocks I and II into Phe (Fig. S1). Besides, in the *ko-1-1* and *ko-3-1* mutants, the OsGELP34 protein was predicted to harbor a single amino acid deletion between the GDSL blocks I and II (Fig. S2 and S5). These amino acid residues showed a high conversation among the orthologs of OsGELP34 from various plant species (Fig. 7a and Fig. S3). Based on the male sterile phenotype of these mutants, we proposed that these amino acids located between the GDSL blocks may also be critical for the normal function of OsGELP34 protein.

It was reported that GELP was associated with lipid metabolism. For example, OsGELP78 was identified to modulate lipid metabolism to regulate rice disease resistance (Gao et al. 2017). Wilted Dwarf and Lethal1/OsGELP112 is required for cuticle formation of rice leaf surface by its esterase/lipase activity (Park et al. 2010). Mutation of ZmMs30, an anther-specific and active GELP in maize, resulted in defective lipid metabolism and pollen development in anther (An et al. 2019). In rice, OsGELP110 and OsGELP115 are two homologs of ZmMS30 and display a similar and temporal expression pattern in developing anthers (Zhang et al. 2020). *osgelp110/osgelp115* double mutant also showed a male sterile phenotype with production of non-viable pollen grains (Zhang et al. 2020), similar to that of *zmms30* and *osgelp34-1* (Fig. 1d-f). *Arabidopsis* RVMS, an ortholog of OsGELP34 with lipase activity, is specifically expressed in floral organs (Fig. S4) and is required for maintain male fertility (Zhu et al. 2020). OsGELP34 also has in vitro lipase activity (Zhao et al. 2020), and our results showed that *OsGELP34* transcripts are highly accumulated in reproductive organs (Fig. 5a). These evidences suggested that these GELP proteins, with high expression in male reproductive organs, have roles in pollen development possibly through the function of lipase activity during lipid metabolism.

Remarkably, in *Arabidopsis*, the *rvms* mutant showed a reversible male sterile phenotype, which was male sterile at normal temperature (24 °C) but restored the fertility at low temperature (17 °C) (Zhu et al. 2020). However, our data indicated that the sterility of *osgelp34-1* could not be recovered under low temperature (Fig. 7b). One possible explanation is, though the OsGELP34 proteins are conserved among various species (Fig. 7a and S3), the actual biochemical functions of OsGELP34 and RVMS may be divergence. Alternatively, though it has been proposed that the developmental processes of pollen between rice and *Arabidopsis* are generally conserved (Gómez et al. 2015; Wilson and Zhang 2009), the fine structures of their mature pollens

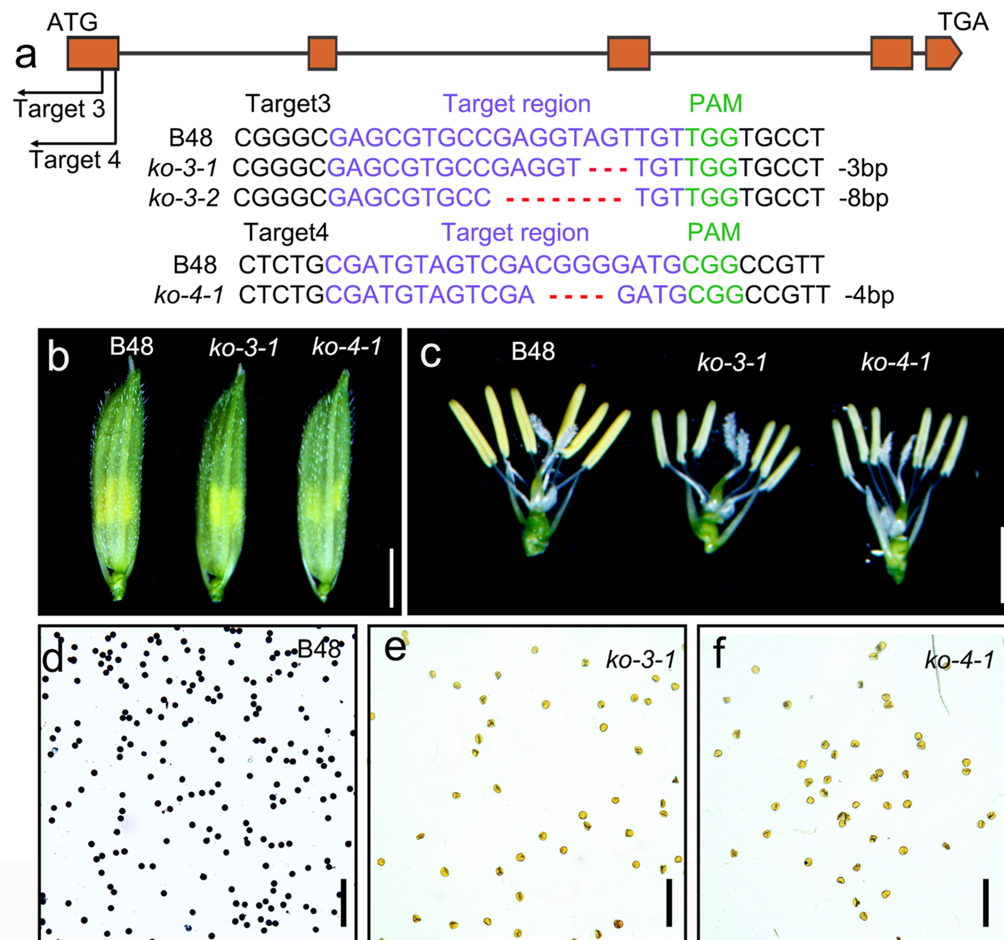

Fig. 8 Generation of MS lines in B48 background by knocking-out of *OsGELP34*. **a** Schematic representation of target site positions for the CRISPR/Cas9 genomic editing (upper) and sequencing analysis of the target regions in transgenic lines. The rectangles indicate the directions of two independent targets. **b** The floret of B48 and transgenic lines at heading stage. **c** The floret of B48 and transgenic lines at heading stage after removal of lemma and palea. **d**–**f** Mature pollen grains of B48 and the mutant transgenic lines stained by I_2-KI solution. Bars = 0.2 mm (**b**, **c**); 50 μm (**d**–**f**)

are different and may lead to different sensibility to environmental temperature. Rice pollen grains have a continuous and smooth exine with much less tryphine filled in the cavities between tectum and nexine, whereas *Arabidopsis* pollen shows a sculptured exine surface with abundant tryphine filled in reticulate cavities (Shi et al. 2015). Additionally, rice reproductive development undergoes at higher temperatures than which of *Arabidopsis*. It is therefore possible that the tested temperature is not low enough for the fertility restoring of *osgelp34-1*.

In rice, hybrid seed production requires male sterile lines. CMS lines and EGMS lines have been widely used for commercial three-line and two-line hybrid breeding, respectively (Chen and Liu 2014; Yu-Jin and Zhang 2018). However, their intrinsic problems, such as narrow germplasm resources and strict environmental conditions, limit their applications. In contrast, the non-EGMS lines, which are caused by mutations of recessive nuclear male sterile genes, have more stable male sterile phenotypes with fewer limitations. However, due to the lacking of the corresponding maintainer lines, these non-EGMS lines have not been commercially used in rice hybrid breeding system. Recently, with the advance of the genomic editing system and the SPT technology, it has become possible to apply non-EGMS to hybrid breeding. The SPT maintainer line is developed by the co-transformation of the tightly linked

restoration gene, pollen-killing gene, and selection marker gene into a homologous non-EGMS line, which enables the simultaneous production of transgenic-free male sterile seeds and transgenic maintainer seeds (Wu et al. 2016). This technology has begun to apply in maize and rice hybrid breeding (An et al. 2019; Chang et al. 2016; Wu et al. 2016; Zhang et al. 2017). Our results have showed that the CRISPR/Cas9 genomic editing system could create stable homologous non-EGMS lines in different rice varieties by KO of *OsGELP34* (Figs. 5g and 8d–f). Further efforts on creating the SPT maintainer lines based on the KO mutants of *OsGELP34* will be helpful for expanding the genetic resource of rice hybrid breeding.

Authors' contributions S.L. and P. L. designed and directed the experiments. Z. H. and Y. T. performed the expression analysis and tissue localization and subcellular localization. J.Z., Q.D., and S.W. performed the genetic transformations. D.Z., H.Z., S.L., R.L., and M.Z. performed the phenotypic characterization of the mutant and the transgenic plants. Y.L., A. Z., A.W., and L.W. constructed all the vectors. G. Y., T. Z., X. Z., M.L., and T. L. performed the cloning and functional analysis and collected almost all the data. T. Z. and G. Y. analyzed the data and wrote the manuscript.

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Compliance with ethical standards

Competing interests The authors declare that they have no conflict of interest.

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