Arabidopsis *glabra2* mutant seeds deficient in mucilage biosynthesis produce more oil

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**SUMMARY**

Seed oil, one of the major seed storage compounds in plants, is of great economic importance for human consumption, as an industrial raw material and as a source of biofuels. Thus, improving the seed oil yield in crops is an important objective. The *GLABRA2 (GL2)* gene in *Arabidopsis thaliana* encodes a transcription factor that is required for the proper differentiation of several epidermal cell types. GL2 has also been shown to regulate seed oil levels, as a loss-of-function mutation in the GL2 gene results in plants with a higher seed oil content than wild-type. We have extended this observation by showing that loss-of-function mutations in several positive regulators of GL2 also result in a high seed oil phenotype. The GL2 gene is expressed in both the seed coat and embryo, but the embryo is the main site of seed oil accumulation. Surprisingly, our results indicate that it is loss of GL2 activity in the seed coat, not the embryo, that contributes to the high seed oil phenotype. One target of GL2 in the seed coat is the gene *MUCILAGE MODIFIED 4 (MUM4)*, which encodes a rhamnose synthase that is required for seed mucilage biosynthesis. We found that *mum4* mutant seeds, like those of *gl2* mutants, have an increased seed oil content in comparison with wild-type. Therefore, GL2 regulates seed oil production at least partly through its influence on *MUM4* expression in the seed coat. We propose that *gl2* mutant seeds produce more oil due to increased carbon allocation to the embryo in the absence of seed coat mucilage biosynthesis.

Keywords: seed oil, mucilage, GL2, MUM4, seed coat, Arabidopsis.

**INTRODUCTION**

Higher plants produce seeds to propagate and maintain the species. During the process of seed development, plants fill seeds with reserve compounds that facilitate germination and seedling growth before photosynthesis is established. Seed oil, one of the major storage compounds, accumulates in the middle to late stages of seed development (Beaudoin and Napier, 2004; Gutierrez *et al.*, 2007; Baud *et al.*, 2008). The primary form of seed oil is triacylglycerol, which comprises three fatty acyl chains attached to a glycerol backbone. Seed oils are also important commodities that are used for human consumption and as industrial feed-stocks with a wide range of applications. Furthermore, as a result of the decline in crude oil supplies, plant oils are being increasingly used as renewable resources for biofuel production, replacing conventional fossil fuels. Given the high value and overall demand for seed oils, there is considerable interest in increasing oil production by genetic engineering (Baud *et al.*, 2008; Durrett *et al.*, 2008; Dyer *et al.*, 2008). To accomplish this, a better understanding of oil biosynthesis and its regulation in developing seeds is required.

Recently, Shen *et al.* (2006) showed that a mutation in the GL2 gene resulted in an 8% increase in seed oil content over wild-type levels, indicating that GL2 is a negative regulator of seed oil accumulation. GL2 encodes a homeodomain (HD) transcription factor that, based on its deduced protein sequence, belongs to the class IV homeodomain-leucine zipper (HD-ZIP) gene family (Rerie *et al.*, 1994; Nakamura *et al.*, 2006). GL2 has been shown to control characteristics of several epidermal cell types, including trichomes (Koornneef *et al.*, 1982; Rerie *et al.*, 1994; Szymanski *et al.*, 1998), atrichoblasts (Masucci *et al.*, 1996) and seed coat epidermal cells (Koornneef *et al.*, 1982; Western *et al.*, 2001). In the seed coat epidermis, GL2 is required for seed coat mucilage biosynthesis, at least in part through positive control of the *MUM4/RHM2* gene (Western *et al.*, 2004). *MUM4/RHM2* encodes a rhamnose synthase that is required to produce...
rhamnose (Usadel et al., 2004; Western et al., 2004; Oka et al., 2006), a key substrate for mucilage biosynthesis.

In all cell types, GL2 expression is activated by a transcription complex that includes polypeptides from three classes of transcriptional regulator: a basic helix-loop-helix (bHLH) transcription factor, a MYB transcription factor and the WRKY transcription factor TRANSPARENT TESTA GLABRA 1 (TTG1) (Walker et al., 1999; Zhang et al., 2003; Western et al., 2004; Bernhardt et al., 2005; Zhao et al., 2008; Gonzalez et al., 2009; Li et al., 2009). In the seed coat epidermis, the components of the GL2 regulatory complex are the bHLH protein GLABRA 3 (EGL3) or TRANSPARENT TESTA8 (TT8) and the MYB protein MYB5 or TRANSPARENT TESTA2 (TT2), together with TTG1 (Zhang et al., 2003; Western et al., 2004; Bernhardt et al., 2005; Gonzalez et al., 2009; Li et al., 2009).

Currently, information concerning downstream targets of the GL2 in the seed is limited. In addition to MUM4, in vitro studies have shown that GL2 can bind to the promoter of PHOSPHOLIPASE D ZETA 1 (PLDZ1), and transgenic lines over-expressing PLDZ1 show an ectopic root hair phenotype similar to that of the gl2 mutant, suggesting that GL2 is a negative regulator of PLDZ1 (Ohashi et al., 2003). In this study, we therefore investigated the potential roles of MUM4 and PLDZ in GL2-mediated regulation of seed oil biosynthesis and deposition.

RESULTS

gl2 mutant seeds contain more seed oil than wild-type seeds

Previous studies have shown that gl2-1 mutant seeds have higher oil content than wild-type seeds (Shen et al., 2006).

To verify this result, we obtained a T-DNA insertion line, SALK_130213 (gl2-8; Deal and Henikoff, 2010). GL2 transcript cannot be detected in the gl2-8 mutant (Figure 1b), in which the T-DNA insertion maps to the second intron of the GL2 gene (Figure 1a). Analysis of seed oil content showed a significant increase in oil content in gl2-8 seeds compared with the wild-type (Figure 1c), similar to that described for the gl2-1 allele (Shen et al., 2006). The gl2-8 mutant also showed abnormal trichomes, an increase in root hair density and no extrusion of seed coat mucilage (Figure S1). These results not only indicate that gl2-8 is a loss-of-function allele, but also confirm that mutations in the GL2 gene result in increased seed oil accumulation.

GL2 control over seed oil levels is exerted through maternal tissues

To investigate the expression pattern of GL2 in developing seeds, we generated transgenic plants expressing the uidA reporter gene encoding β-glucuronidase (GUS) under the control of the GL2 promoter (GL2p:GUS). We detected GUS activity in the embryo and seed coat, indicating that the GL2 gene is expressed in both locations during early, middle and late stages of seed development [4, 7 and 10 days post-anthesis (DPA) respectively] (Figure 2). The seed coat and embryo hypocotyl expression patterns were consistent with previous findings using GFP expression driven by the GL2 promoter (Stadler et al., 2005). To determine whether the high seed oil phenotype is due to defects in maternal or embryonic tissue, we crossed a wild-type (WT) plant as the male parent with a gl2-8 plant as the female parent. The F1 (gl2 × WT) progeny were homozygous for gl2 in the seed coat but heterozygous for gl2 in the embryo. The oil content

Figure 1. Plants homozygous for a T-DNA insertion allele of the GL2 gene show an increased seed oil content.

(a) The structure of GL2 gene (At1g79840), with exons shown as black boxes, introns shown as solid lines and untranslated regions shown as gray boxes. The positions of the start codon and the stop codon are indicated by vertical arrows. The location of the T-DNA insertion of gl2-8 (SALK_130213) was mapped and is indicated by the white arrowhead. The locations of primers used for RT-PCR in (b) are indicated by short horizontal arrows.

(b) RT-PCR analysis of steady-state GL2 transcript levels in wild-type (WT; Col-0) and mutant (gl2-8) leaves. RT-PCR was performed using total leaf RNA, and the expression level of GAPC was used as a control.

(c) Seed oil analysis of wild-type (WT, Col-0) and the mutant (gl2-8). Values are mean percentages ± SD (n = 4; replicate analysis was performed on seed lots from each line, with 2–2.5 mg seed analyzed per replicate). Student’s t test was applied to the data; the asterisk indicates that this value was statistically significantly different from wild-type at P < 0.05.
of the F₁ (gl2 × WT) seeds was similar to that of gl2-8 homozygous seeds, and higher than that of wild-type seeds (Figure 3a), suggesting that it is GL2 function in the seed coat that influences seed oil levels.

Seed oils accumulate in both the embryo and the seed coat, although the embryo is the major compartment for seed oil storage (Li et al., 2006). To test whether the loss of GL2 function affects the oil level in the seed coat or in the embryo, we analyzed the oil content in the embryo and the seed coat separately. The oil content in the gl2-8 embryo was higher than that in the wild-type embryo, but no difference was observed between the wild-type and the mutant in terms of seed coat oil levels (Figure 3b). We also compared fatty acid composition on the whole seed, the embryo and the seed coat between wild-type and the gl2-8 mutant. The fatty acid composition of the embryo was different from that of the seed coat in all genotypes tested (Figure S2a), consistent with a previous study (Li et al., 2006). However, no differences in fatty acid composition between wild-type and the gl2-8 mutant in the whole seed, embryo or seed coat were observed (Figure S2b–d). Taken together, these results show that GL2 function in the seed coat influences seed oil accumulation in the embryo.

**PLDZ genes are expressed in the cotyledons and are up-regulated in gl2-8 mutant seeds**

A previous study suggested that PLDZ1 is negatively regulated by GL2 during root cell differentiation (Ohashi et al., 2003). As PLDZ activity could contribute to seed oil synthesis, we tested the hypothesis that PLDZ is a target of GL2 in seeds by examining the expression of PLDZ genes in seeds of the wild-type and the gl2 mutant. In Arabidopsis, there are two PLDZ genes: PLDZ1 (At3g16785) and PLDZ2 (At3g05630). Constructs expressing GUS under the control of the PLDZ1 (PLDZ1p:GUS) and PLDZ2 (PLDZ2p:GUS) promoters were introduced into wild-type plants. Detection of GUS activity in these transgenic plants indicated that both PLDZ genes were expressed in the developing embryos. PLDZ1 was mainly expressed in the cotyledons at the early, middle and late stages of seed development (Figure 4b–d), whereas the PLDZ2 promoter was active in the cotyledons.
only in the middle and late developmental stages (Figure 4f–h). In addition, GUS activity was detected in the funiculus (Figure 4a,e), but not in the seed coats of transgenic plants expressing either construct.

To test whether PLDZ genes are the targets of GL2 in seeds, we determined the expression levels of PLDZ genes in developing seeds of the gl2-8 mutant using quantitative real-time PCR. The results showed that PLDZ1 and PLDZ2 expression levels in the gl2-8 mutant seeds during the early (4 DPA) and middle (7 DPA) stages were the same as those in the wild-type, but both PLDZ genes were up-regulated in the gl2-8 mutant compared with wild-type in late-stage seeds (10 DPA) (Figure 5). These results suggest that the PLDZ genes are GL2 targets in seeds in the late stage of development.

PLDZs are not required for GL2-mediated seed oil accumulation

To determine whether PLDZ is involved in oil biosynthesis, we analyzed the seed oil content of pldz mutants. We obtained T-DNA insertion lines for pldz1 (SALK_083090) and pldz2 (SALK_094369) from the Arabidopsis SALK collection (Alonso et al., 2003) and determined the sites of the T-DNA insertions. The insertion in the PLDZ1 gene mapped to the second exon, while the insertion in the PLDZ2 gene was located in the 11th exon (Figure 6a). Wild-type transcripts of PLDZ1 and PLDZ2 genes were not detected in plants homozygous for pldz1 and pldz2 (Figure 6b). To assess the possibility that PLDZ1 and PLDZ2 may work redundantly, we generated pldz1 pldz2 double mutant plants. The seed oil content of both single mutant plants was not significantly different from wild-type seeds (Figure 6c). The pldz1 pldz2 seeds also had wild-type oil content (Figure 6d), suggesting that PLDZ does not play a role in seed oil biosynthesis. Furthermore, we generated a gl2-8 pldz1 pldz2 triple mutant and compared its seed oil levels to those of gl2-8 and the pldz1 pldz2 double mutant to determine whether the high

Figure 4. Seed-specific expression pattern of PLDZ1 (a–d) and PLDZ2 (e–h) in Arabidopsis seeds.
The uidA reporter gene encoding β-glucuronidase (GUS) under the control of the PLDZ1 or PLDZ2 promoter was expressed in Arabidopsis plants. Developing seeds were incubated in X-gluc assay buffer. GUS activity is indicated by a blue precipitate. Scale bars = 0.1 mm.
(a, e) Seed coat at 7 days post-anthesis (DPA).
(b–d, f–h) Developing embryos at 4 DPA (b, f), 7 DPA (c, g) and 10 DPA (d, h).

Figure 5. Quantitative real-time PCR analysis of PLDZ1 (a) and PLDZ2 (b) expression in developing seeds.
Total RNAs from developing seeds of wild-type (WT, Col-0) and the mutant (gl2-8) at 4, 7 or 10 days post-anthesis were analyzed for expression of PLDZ1 (a) and PLDZ2 (b) by quantitative real-time PCR using gene-specific primer pairs. PLDZ1 and PLDZ2 gene expression values were normalized to GAPC expression. Error bars represent SD (n = 4).
seed oil phenotype of the gl2-8 mutant is dependent on PLDZ function. The oil content of the gl2-8 pldz1 pldz2 seeds was similar to that of the gl2-8 mutant (Figure 6d), demonstrating that PLDZs are not required for GL2-mediated seed oil accumulation.

MUM4 influences seed oil deposition

MUM4 encodes a rhamnose synthase, a mucilage biosynthetic enzyme, and was reported to be a target of GL2 in seed coat mucilage production (Western et al., 2004). To determine whether this is the case, we tested the expression level of MUM4 in developing seeds by quantitative real-time PCR. MUM4 is down-regulated in gl2-8 mutant seeds during the middle stage (7 DPA) of seed development (Figure 7a), consistent with previous data obtained using RT-PCR (Western et al., 2004). To directly assess whether MUM4 is involved in seed oil accumulation, we analyzed the oil content of mum4-1 seeds. As shown in Figure 7(b), mum4-1 seeds contain more oil than wild-type seeds, implying involvement of MUM4 in seed oil accumulation. We also investigated the genetic relationship between GL2 and MUM4 by generating and analyzing gl2-8 mum4-1 double mutant plants. The gl2-8 mum4-1 seed oil levels were similar to those in mum4-1 and gl2-8 mutants (Figure 7b), and the double mutant also had reduced mucilage extrusion as observed in the mum4-1 and gl2-8 mutants (Figure 8). These results are consistent with the hypothesis that the high seed oil phenotype of the gl2 mutant is due to loss of expression of MUM4.

Mucilage-deficient mutants do not always have higher oil content than the wild-type

To determine whether the high oil phenotype is due to loss of mucilage or more specifically loss of MUM4 function, we investigated the seed oil content of mutants disrupted in components of the regulatory pathway for mucilage production, including TTG1, TTG2, MYB5, TT2, EGL3, TT8, GL2 and MUM4 (Figure 9a, Figure S3) (Western et al., 2004; Gonzalez et al., 2009). Our results show that ttg1-1, myb5-1 tt2 and egl3 tt8 mutants have higher seed oil contents than the wild-type. The oil content of myb5-1 tt2 double mutant seeds is higher than that of the single mutants myb5-1 or tt2, indicating that MYB5 and TT2 function redundantly in controlling seed oil levels. Similarly, the egl3 tt8 double mutant had a higher oil content than either the single mutant egl3 or tt8. In contrast, the oil content of ttg2 seeds was indistinguishable from that of the wild-type, suggesting that it is not the loss of mucilage per se, but more specifically the loss of MUM4 function that results in over-production of seed oil.

DISCUSSION

GL2, a homeodomain transcription factor, negatively regulates the level of storage oil in the seed (Shen et al., 2006). We have confirmed and extended this result by showing that the positive regulators of GL2 act in a similar manner, and
that the influence occurs primarily through maternal tissue. To investigate the mechanism by which GL2 controls oil levels, we examined known targets of GL2 that could influence seed oil content, i.e. the PLDZ genes and MUM4. Our data show that the PLDZs are not involved in seed oil biosynthesis or deposition, and that MUM4 may be the primary target through which GL2 acts to control seed oil content. GL2 negatively regulates the PLDZ genes during the late stages of seed development. PLDZs catalyze the hydrolysis of membrane phospholipids to generate a free hydrophilic head group and phosphatidic acid that can serve as a second messenger and/or as a substrate for triacylglycerol synthesis (Wang, 2000). As the PLDZ1 gene is negatively regulated by GL2 in the root (Ohashi et al., 2003), we hypothesized that PLDZ genes may be involved in GL2-mediated control of seed oil levels. Our results showed that PLDZ1 and PLDZ2 are expressed in the embryo and funiculus and are up-regulated in gl2 mutant seeds during the late stages (10 DPA) of seed development (Figures 4 and 5). Thus, in addition to controlling PLDZ1 expression in the root, GL2 is a negative regulator of PLDZ1 and PLDZ2 expression in seeds. However, genetic analysis revealed that PLDZs are not involved in seed oil biosynthesis, as the seed oil contents of pldz single mutants or the pldz1 pldz2 double mutant did not differ from that of wild-type seeds (Figure 6). Consequently, the roles of PLDZs in the seed remain to be elucidated.

MUM4 is a downstream target of GL2

MUM4 encodes a nucleotide diphosphate (NDP)-L-rhamnose synthase, converting NDP-D-glucose to NDP-L-rhamnose, a key precursor for rhamnogalacturonan I (RGI), the major constituent of Arabidopsis seed mucilage (Usadel et al., 2004; Western et al., 2004; Oka et al., 2006). Our quantitative real-time PCR results showed that the MUM4 gene is down-regulated in gl2-8 seeds (Figure 7a), consistent with previous RT-PCR results (Western et al., 2004). Recent analysis of MUM4 promoter sequences suggests that, within the seed, MUM4 is expressed primarily in the seed coat (E. Esfandiari and G. Haughn, unpublished data, Department of Botany, University of British Columbia). These data suggest that GL2 is a positive regulator of MUM4 in the seed coat. We have shown that, like GL2, MUM4 activity in the seed coat is required for normal seed oil accumulation, as loss of MUM4 function results in higher seed oil content. In addition, the fact that the phenotype of the gl2 mum4 double mutant is similar to that of both parents suggests that GL2 and MUM4 function in the same pathway to influence seed oil biosynthesis.
Taken together, our data are consistent with the idea that GL2 negatively regulates seed oil by positively regulating MUM4 (Figures 6 and 7b). Further work is required to determine whether MUM4 is a direct target of GL2, and whether other genes downstream of GL2 are also involved in controlling seed oil levels.

**Carbon partitioning between seed oil biosynthesis and seed coat mucilage production**

In oilseed species such as Arabidopsis, seed storage oil is synthesized and deposited in the embryo. Surprisingly, our data suggest that both GL2 and its downstream target MUM4 negatively regulate seed oil biosynthesis, but act in the seed coat. Thus, this regulation must be indirect. Sucrose, as a major carbon source, is transported from photosynthetic tissues into developing seeds to support accumulation of seed storage compounds. It has been suggested that sucrose is unloaded first into the seed coat through the funiculus, and then imported into the endosperm, before reaching the embryo (Stadler et al., 2005). In the embryonic cells, sucrose is converted by glycolysis into acetyl CoA, the key substrate of fatty acid biosynthesis required for oil production (Baud et al., 2008). Sucrose is also the carbon source for mucilage biosynthesis in the seed coat. Sucrose is first catabolized to glucose and fructose, and the monosaccharides are used for cell-wall polymer biosynthesis. For example, glucose can be converted into rhamnose by MUM4 for use in production of RGI, the key component of mucilage (Usadel et al., 2004; Western et al., 2004; Oka et al., 2006). Therefore, seed oil and seed coat mucilage share the same carbon source. An attractive hypothesis to explain the role of GL2 and MUM4 in negatively regulating oil biosynthesis is that seed mucilage and oil are competing sinks for photosynthate, and that photosynthate in the seed is limiting. In the absence of mucilage formation (gl2 and mum4 mutants), the sucrose normally used for the production of mucilage is instead used for oil biosynthesis.

**ttg2 mutant seeds have a wild-type oil content**

The TTG2 gene encodes a WRKY transcription factor that is reported to be involved in regulation of several development processes in Arabidopsis (Johnson et al., 2002; Western et al., 2004; Ishida et al., 2007; Gonzalez et al., 2009).
2009). It has previously been shown that, like GL2, TTG2 is positively regulated by the TTG1-bHLH-MYB complex and is required for seed coat mucilage biosynthesis (Gonzalez et al., 2009; Western et al., 2004; Figure 9a). However, unlike GL2, TTG2 does not regulate MUM4 and therefore must influence mucilage biosynthesis through control of other, as yet unidentified, genes whose products are required for mucilage biosynthesis. Interestingly, we have shown that ttg2 mutant seeds have a wild-type oil content, indicating that loss of seed mucilage is not sufficient to increase oil biosynthesis. These data raise the question as to why the effect of gl2/mum4 on seed oil is different from that of ttg2. One possible explanation is that, as the mum4 mutation disrupts the conversion of glucose to rhamnose, the unused glucose can easily be transported to and used by the embryo for oil production. If TTG2 is required for a step in mucilage biosynthesis, following the conversion of glucose to rhamnose, then the precursor of mucilage may remain in the seed coat unavailable for oil biosynthesis.

Blocking mucilage biosynthesis may be a way to increase seed oil production

Seed oils are of great value in many food and non-food applications, including biodiesel production, but their supply is limited. Therefore, numerous attempts have been made to increase the seed oil content in plants. Some of these attempts focused on increasing the carbon flow to triacylglycerol biosynthesis (Ekman et al., 2008; Iyer et al., 2008; Weselake et al., 2009) at the expense of the other major seed storage compounds, starch and protein. The biosynthetic pathways involved in the production of seed storage compounds compete with each other for the carbon resources during embryo development (Weselake et al., 2009). For example, swunken seed 1 (sse1) mutant seeds with a disrupted triacylglycerol biosynthetic pathway produced more starch than the wild-type (Lin et al., 2004), whereas embryo-specific over-expression of Biotin Carboxyl Carrier Protein 2 (BCCP2) resulted in a decreased seed oil production and increased carbon flow into synthesis of seed storage proteins (Chen et al., 2009). Our results reveal a regulatory mechanism of carbon partitioning between the maternal seed coat and the filial embryo. Mutations in GL2 and MUM4 do not appear to affect other cell types producing mucilage (root cap and transmitting tract cells), nor do they have obvious deleterious effects on the growth and functioning of Arabidopsis. Although there are reports that germination of some mucilage mutants is delayed relative to wild-type under conditions of limited water supply (Penfield et al., 2001; Arsovski et al., 2009), most species of plants, including some species of Brassicaceae, lack seed mucilage, and some cultivars of Brassica napus extrude little mucilage when imbibed, suggesting that seed mucilage is not critical for agronomic performance.

Thus, it seems possible that manipulation of MUM4 function could be exploited to increase the seed oil content in oil crops with myxospermous seed by blocking conversion of glucose to rhamnose in the seed coat.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis thaliana ecotypes Columbia (Col-0) and Landsberg erecta (Ler) were used in this study. The SALK T-DNA insertional lines SALK_130213 (gl2-8; Dea and Henikoff, 2010), SALK_083909 (pldz1), SALK_094369 (pldz2), SALK_030942 (myb5-1; Li et al., 2009) and SALK_030966 (tt8; Gonzalez et al., 2009) were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org). The mum4-1, ttg1-1 and ttg2-1 mutants have been previously described (Walker et al., 1999; Johnson et al., 2002; Western et al., 2004). The SALK_005260 (tt2), SALK_019114 (egl3), myb5-1 tt2 and egl3 tt8 mutants were kindly provided by Dr Antonio Gonzalez (Molecular, Cell and Developmental Biology, University of Texas at Austin; Gonzalez et al., 2009). Homozygous lines for each gene were identified by genotyping using the primers listed in Table S1. Double and triple mutants were obtained by crossing homozygous lines and identifying the appropriate lines from the F2 generation. The GL2p:GUS line was kindly provided by Dr Jin-Gui Chen (Department of Botany, University of British Columbia, Vancouver, BC, Canada) and has been described previously (Szymanski et al., 1998). Arabidopsis plants were transformed using the floral spray method (Chung et al., 2000). Seeds were germinated on AT medium (Somerville and Ogren, 1982) supplemented with agar (7 g l⁻¹) and appropriate antibiotics. Ten-day-old seedlings were transferred to soil and grown at 20°C under continuous light with a light intensity of 100 μmol m⁻² sec⁻¹. The plants used for oil content analysis in each biological replicate were grown in the same chamber, and the positions of the plants were rotated every other day.

Plasmid construction

To generate the PLDZ1p:GUS construct, a 1662 bp fragment that includes 1107 bp immediately upstream and 555 bp downstream of the putative PLDZ1 start codon was amplified from Col-0 genomic DNA using the forward primer 5′-attB1-GGTAACCAAGAAGACTCGA-3′ and the reverse primer 5′-attB2-GTTACATAAAGCGCTA AAAGT-3′. To generate the PLDZ2p:GUS construct, a 2068 bp fragment that includes 1570 bp immediately upstream and 498 bp downstream (i.e. the first exon and the first intron) of the putative PLDZ2 start codon was amplified from Col-0 genomic DNA using the forward primer 5′-attB1-GTTGAACCCCTATAGCTTA-3′ and the reverse primer 5′-attB2-CTATACATATACAAAAACAG-3′. The PCR fragments were recombined into pDONR207 (Invitrogen, http://www.invitrogen.com) and then into the binary GUS vector pMDC162 (Curtis and Grossniklaus, 2003) using the Gateway cloning strategy (Invitrogen).

RNA isolation, RT-PCR and quantitative real-time PCR

Rosette leaves and developing seeds of the wild-type (Col-0) and the gl2-8 mutant or the pldz1 pldz2 double mutant were collected and immediately frozen in liquid nitrogen. RNA isolation and reverse transcription were performed using protocols described previously (Zhao et al., 2010). Gene-specific primers used in RT-PCR and quantitative real-time PCR are listed in Table S1. IQ SYBR Green Supermix (Bio-Rad, http://www.bio-rad.com) was used to perform quantitative real-time PCR in an iQ5 Multicolor real-time PCR
Muclilage-deficient gl2 seeds produce more oil

Figure S2. Fatty acid composition in whole seeds, embryos and seed coats of wild-type and the mutant gl2-8.

Figure S3. Muclilage phenotypes of wild-type and the mutants myb5-1 tt2, egl3 tt8, ttg1-1 and ttg2-1.

Table S1. Genotyping primers for mutants.

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