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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 F_1 (*gl2* × WT) progeny were homozygous for *gl2* in the seed coat but heterozygous for *gl2* in the embryo. The oil content

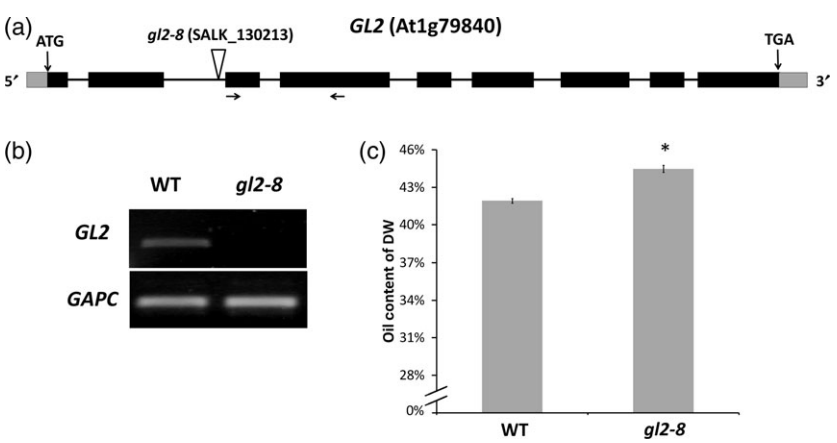


Figure 1. Plants homozygous for a T-DNA insertion allele of the *GL2* gene show an increased seed oil content.
(a) The structure of *GL2* gene (At1g79840), with exons shown as black boxes, introns shown as solid lines and untranslated regions shown as gray boxes. The positions of the start codon and 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 $P < 0.05$.

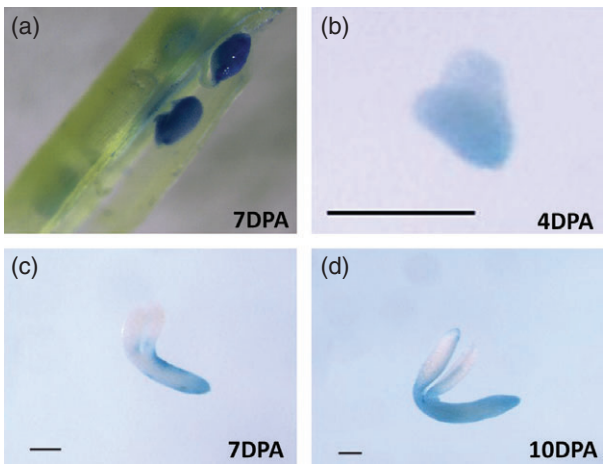
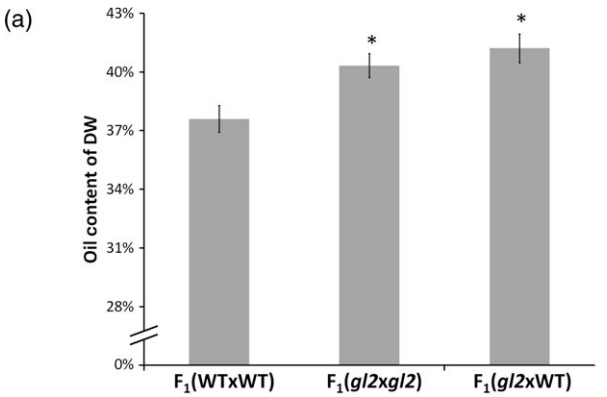


Figure 2. Seed-specific expression pattern of *GL2* in Arabidopsis seeds.
The *uidA* reporter gene encoding β-glucuronidase (GUS) under the control of the GL2 promoter was expressed in Arabidopsis plants. Developing seeds were incubated in X-gluc assay buffer. GUS activity is indicated by a blue precipitate. Scale bars = 0.1 mm.
(a) Seed coat at 7 days post-anthesis (DPA).
(b–d) Developing embryos at 4 DPA (b), 7 DPA (c) and 10 DPA (d).



♀	WT	*gl2-8*	*gl2-8*
♂	WT	*gl2-8*	WT
Seed coat	***GL2GL2***	***gl2gl2***	***gl2gl2***
Embryo	*GL2GL2*	*gl2gl2*	*GL2gl2*

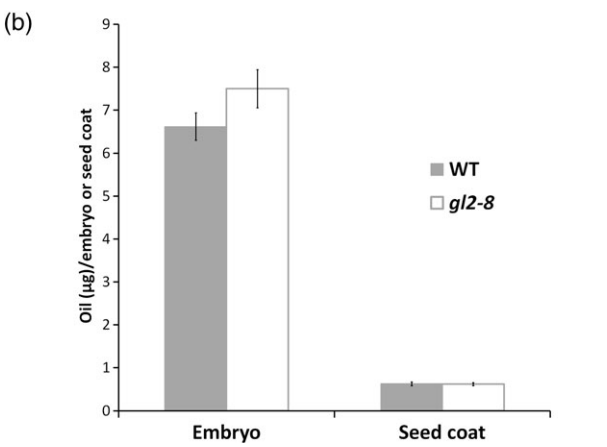


Figure 3. Oil content of seeds, embryos and seed coats for wild type and *gl2* mutant plants. (a) Oil content analysis of seeds homozygous for *gl2* in the seed coat and heterozygous in the embryo. We crossed *gl2-8* plants as the female parent (♀) with wild-type (WT, Col-0) plants as the male parent (♂)to generate F_1 seeds (F_1; *gl2* × WT). The genotypes of the seed coat and embryo of the F_1 progeny are shown below. The seed oil content was analyzed and compared with that of wild-type (F_1; WT × WT) and *gl2-8* plants (F_1; *gl2* × *gl2*). Values are mean percentages ± SD (n = 4; replicate analysis was performed on seed lots from each line, with 2–2.5 mg of seed analyzed per replicate). Student's t test was applied to the data; asterisks indicate that these values were statistically significantly different from wild-type at $P < 0.05$.
(b) Fatty acid analysis of mature embryos and seed coats of wild-type (WT, Col-0) and the mutant (*gl2-8*). Values are mean percentages ± SD (n = 3; replicate analysis was performed on seed lots from each line).

of the F_1 (*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

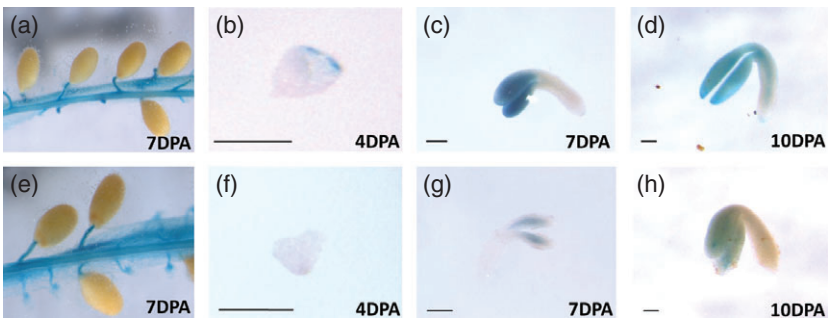


Figure 4. Seed-specific expression pattern of *PLDZ1* (a–d) and *PLDZ2* (e–h) in Arabidopsis seeds.
The *uidA* reporter gene encoding β-glucuronidase (GUS) under the control of the *PLDZ1* or *PLDZ2* promoter was expressed in Arabidopsis plants. Developing seeds were incubated in X-gluc assay buffer. 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).

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

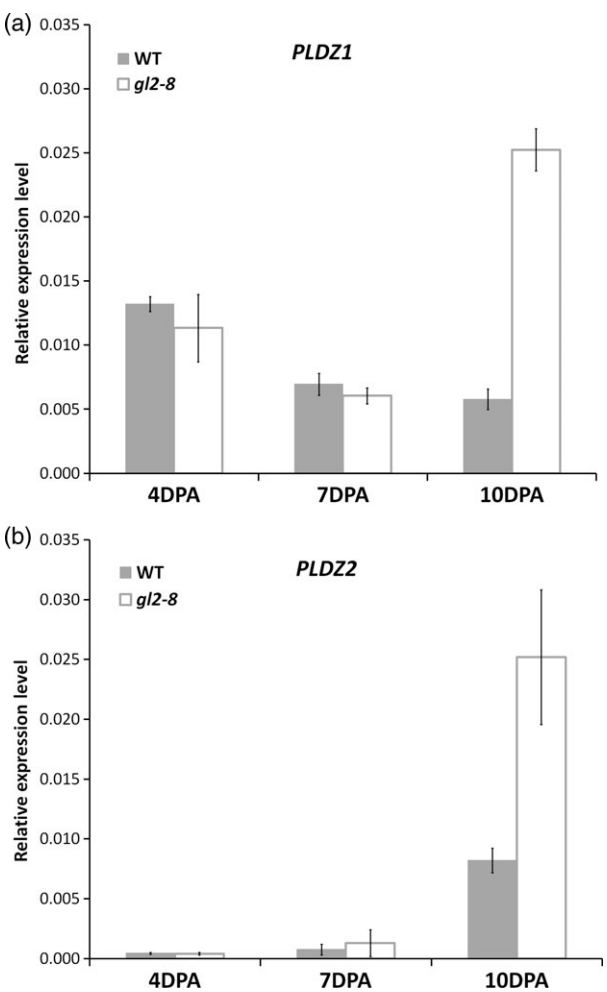


Figure 5. Quantitative real-time PCR analysis of *PLDZ1* (a) and *PLDZ2* (b) expression in developing seeds.
Total RNAs from developing seeds of wild-type (WT, Col-0) and the mutant (*gl2-8*) at 4, 7 or 10 days post-anthesis were analyzed for expression of *PLDZ1* (a) and *PLDZ2* (b) by quantitative 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).

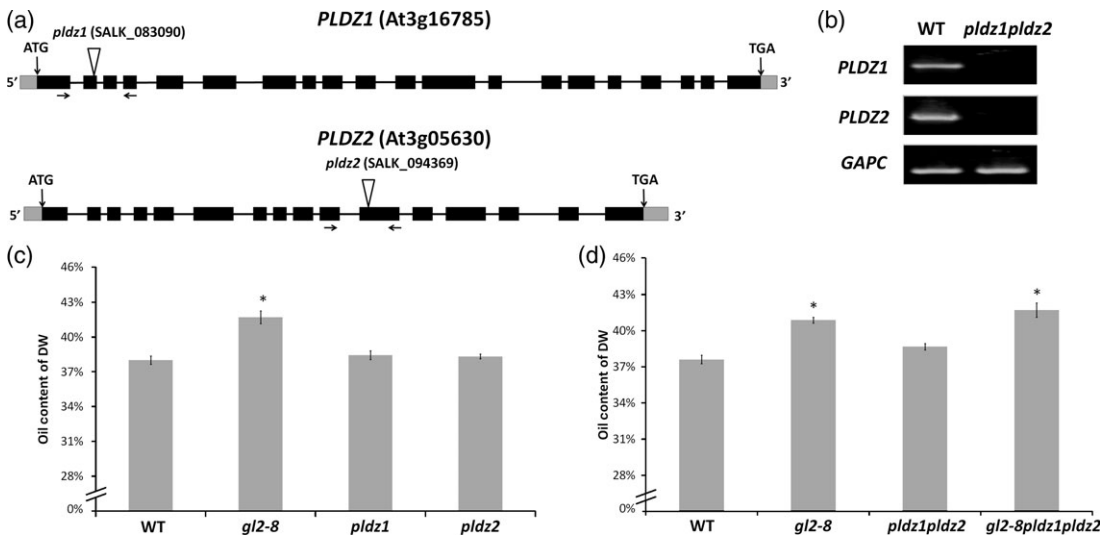


Figure 6. T-DNA insertion alleles of *pldz1* and *pldz2* mutants have a wild-type seed oil phenotype.
(a) The structures of the *PLDZ1* gene (At3g16785) and the *PLDZ2* gene (At3g05630), with exons shown as black boxes, introns shown as solid lines and untranslated regions shown as gray boxes. The start codon and the stop codon are indicated by vertical arrows. The locations of the T-DNA insertions of *pldz1* (SALK_083090) and *pldz2* (SALK_094369) were mapped and are indicated by white arrowheads. The locations of primers used for RT-PCR in (b) are indicated by short horizontal arrows.
(b) RT-PCR analysis of steady-state *PLDZ1 and PLDZ2* transcript levels in mRNA from wild-type (WT, Col-0) and the double mutant (*pldz1 pldz2*) leaves. RT-PCR was performed using total leaf RNA, and the expression level of *GAPC* was used as a control.
(c) Oil content analysis of wild-type (WT, Col-0) and the mutants *gl2-8*, *pldz1* and *pldz2*.
(d) Oil content analysis of the double mutant *pldz1 pldz2* and the triple mutant *gl2-8 pldz1 pldz2*.
Oil analysis data are expressed as mean percentages ± SD (n = 4; replicate analysis was performed on seed lots from each line, with 2–2.5 mg seeds analyzed per replicate). Student's t test was applied to the data; asterisks indicate that these values were statistically significantly different from wild-type at $P < 0.05$.

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

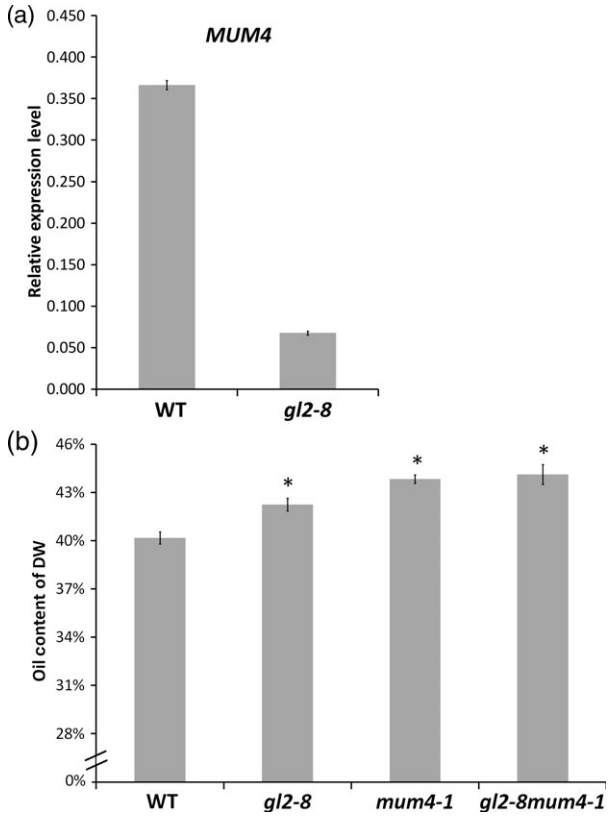


Figure 7. Expression level of the *MUM4* gene in developing seeds, and levels of seed oil in the *mum4* mutant.
(a) Total RNAs from developing seeds of wild-type (WT, Col-0) and the *gl2-8* mutant at 7 days post-anthesis (DPA) were analyzed for expression of the *MUM4* gene, using the *GAPC* gene as a standard, by quantitative real-time PCR. *MUM4* gene expression was normalized to *GAPC* expression values. Error bars represent SD (n = 4).
(b) Oil content analysis of wild-type (WT, Col-0), the single mutants *gl2-8* and *mum4-1*, and the double mutant *gl2-8 mum4-1*. Values are mean percentages ± SD (n = 4; replicate analysis was performed on seed lots from each line, with 2–2.5 mg seeds analyzed per replicate). Student's t test was applied to the data; asterisks indicate that these values were statistically significantly different from wild-type at $P < 0.05$.

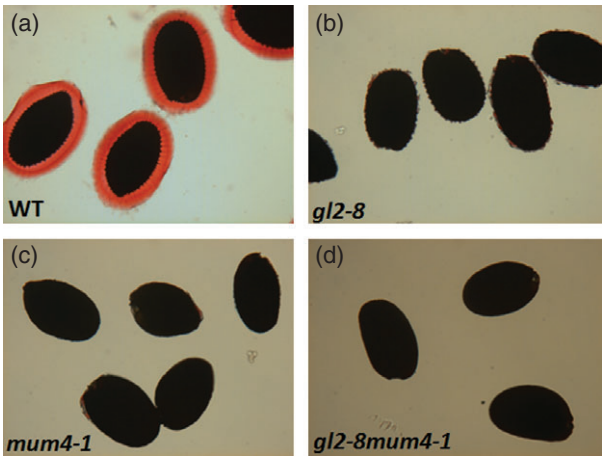


Figure 8. Seed coat mucilage phenotypes of wild-type (WT, Col-0) (a), *gl2-8* (b), *mum4-1* (c) and *gl2-8 mum4-1* (d).
The mature wild-type and mutant seeds were stained with Ruthenium red. Note the red staining of the mucilage capsule surrounding the seeds.

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.

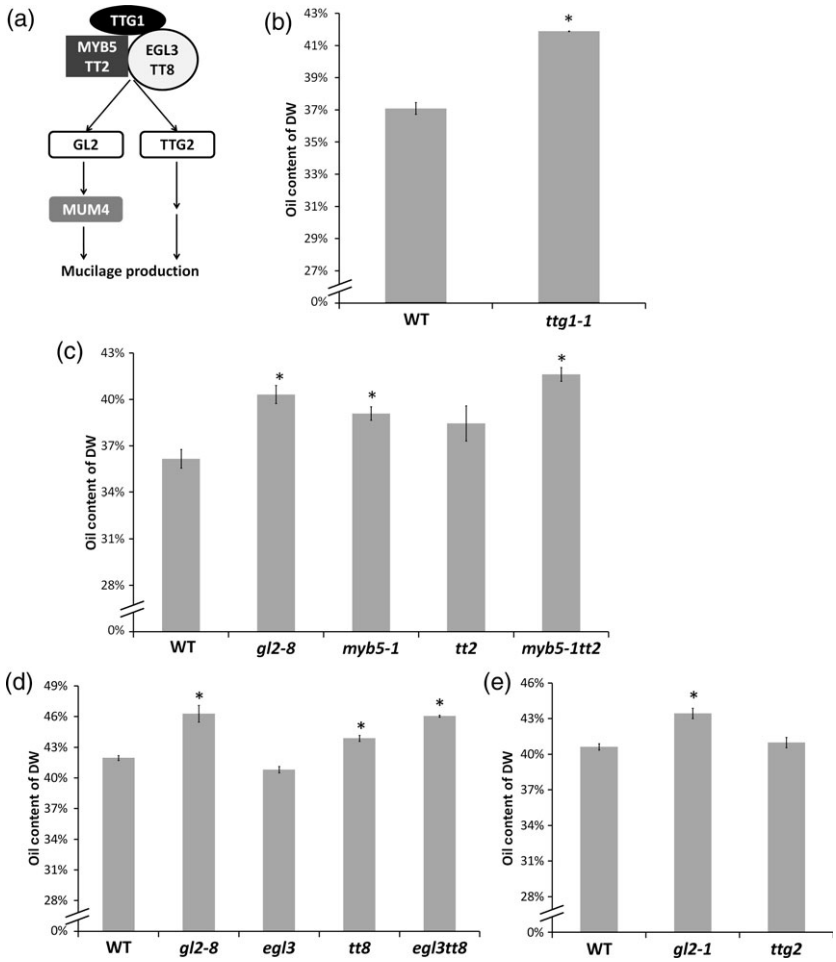


Figure 9. Oil content phenotypes of the mutants with decreased production of seed mucilage.
(a) Proposed pathway for the regulation of seed coat mucilage (Western *et al.*, 2004; Gonzalez *et al.*, 2009).
(b) Oil content phenotypes of wild-type (WT, L*er*) and the mutant *ttg1-1*.
(c) Oil content phenotypes of wild-type (WT, Col-0), the single mutants *gl2-8*, *myb5-1* and *tt2*, and the double mutant *myb5-1 tt2*.
(d) Oil content phenotypes of wild-type (WT, (Col-0), the single mutants *gl2-8*, *egl3* and *tt8*, and the double mutant *egl3 tt8*.
(e) Oil content phenotypes of wild-type (WT, L*er*) and the mutants *gl2-1* and *ttg2-1*.
Oil analysis data are expressed as mean percentages ± SD (n = 4; replicate analysis was performed on seed lots from each line, with 2–2.5 mg seeds analyzed per replicate). Student's t test was applied to the data; asterisks indicate that these values were statistically significantly different from wild-type at $P < 0.05$.

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). 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, *shrunken 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* (L*er*) were used in this study. The SALK T-DNA insertional lines SALK_130213 (*gl2-8*; Deal and Henikoff, 2010), SALK_083090 (*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 F_2 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 geminated 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-GGTAACCAAGAAGAAGTCGA-3′ and the reverse primer 5′-attB2-GTTACATAAAAGCGCTAAAGT-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-GTTTGAACCCCTATAGCTTA-3′ and the reverse primer 5′-attB2-CTATACATATACCAAAAACAG-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

detection system (Bio-Rad) as specified by the manufacturer. The gene used for normalization in PCR experiments was GLYCERALDEHYDE-3- PHOSPHATE DEHYDROGENASE C SUBUNIT (GAPC).

GUS histochemical assays

Seeds at various developmental stages were collected from transgenic plants containing the *GL2p:GUS*, *PLDZ1:GUS* or *PLDZ2:GUS* constructs. The seed coats and embryos were carefully separated using needles under a dissecting microscope. Tissues were immersed in GUS staining buffer [100 mM Na-phosphate, pH 7.0, 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$, 0.2% Triton X-100 and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc)] and incubated for 1–4 h or overnight. Chlorophyll was removed by incubating with 70% ethanol. Stained samples were examined under a dissecting microscope.

Seed oil content analysis

The seed oil content analyses were performed as described by Li *et al.* (2006) with minor modifications. Briefly, for each of four replicates, 2–2.5 mg of dry seeds from each line were weighed and transferred into 1 × 10 cm glass tubes (pre-rinsed thoroughly with chloroform and dried) with screw caps, to which 1 ml of freshly prepared 5% v/v sulfuric acid in methanol and 300 μl of 0.1 μg μl^{-1} triheptadecanoin in toluene as internal standard were added. The samples were mixed and incubated at 90°C for 1.5 h. After cooling on ice, 1.5 ml of 0.9% NaCl was added to each tube, and fatty acid methyl esters were extracted with 2 ml of hexane, followed by evaporation under nitrogen gas. Samples were dissolved in 50 μl of hexane and transferred into glass vials. Fatty acid methyl esters were analyzed by gas chromatography with flame ionization detection (GC-FID) as described previously (Kunst *et al.*, 1992). Each experiment was repeated at least twice.

For oil analysis in the embryo and the seed coat, mature seeds were imbibed in water at 4°C overnight. The imbibed seeds were broken by gently pressing between two slides. The embryos and the seed coats were carefully separated under the dissection microscope. Ten embryos or 100 seed coats per replicate were collected for fatty acid methyl ester analysis.

Ruthenium red staining for seed coat mucilage

Dry seeds from each line were shaken in water for 1 h before staining in a 0.01% w/v aqueous solution of Ruthenium red. The stained seeds were inspected under a compound light microscope.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phenotypes of wild-type and *gl2-8* mutant plants with regard to trichome development, root hair differentiation and mucilage production.

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

Figure S3. Mucilage 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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