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Duplicate Maize *Wrinkled1* Transcription Factors Activate Target Genes Involved in Seed Oil Biosynthesis^{1[C][W]}

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WRINKLED1 (WRI1), a key regulator of seed oil biosynthesis in *Arabidopsis* (*Arabidopsis thaliana*), was duplicated during the genome amplification of the cereal ancestor genome 90 million years ago. Both maize (*Zea mays*) coorthologs *ZmWri1a* and *ZmWri1b* show a strong transcriptional induction during the early filling stage of the embryo and complement the reduced fatty acid content of *Arabidopsis wri1-4* seeds, suggesting conservation of molecular function. Overexpression of *ZmWri1a* not only increases the fatty acid content of the mature maize grain but also the content of certain amino acids, of several compounds involved in amino acid biosynthesis, and of two intermediates of the tricarboxylic acid cycle. Transcriptomic experiments identified 18 putative target genes of this transcription factor, 12 of which contain in their upstream regions an AW box, the cis-element bound by AtWRI1. In addition to functions related to late glycolysis and fatty acid biosynthesis in plastids, the target genes also have functions related to coenzyme A biosynthesis in mitochondria and the production of glycerol backbones for triacylglycerol biosynthesis in the cytoplasm. Interestingly, the higher seed oil content in *ZmWri1a* overexpression lines is not accompanied by a reduction in starch, thus opening possibilities for the use of the transgenic maize lines in breeding programs.

While intensive agricultural and industrial use of the maize (*Zea mays*) kernel is largely due to its high starch content, the oil stored in the maize kernel also has considerable importance. In compound feeds for poultry, pigs, and ruminant animals, the oil is appreciated for its calorific value and its fatty acid composition, mainly oleic and linoleic acids. Maize oil is also a value-added by-product of the starch industry, reflected by a worldwide production of 2,294,470 tons, used mainly for human food purposes, in 2009 (<http://faostat.fao.org>). Maize oil is highly regarded for its low level of saturated fatty acids, on average 11% palmitic acid and 2% stearic acid, and its rela-

tively high levels of polyunsaturated fatty acids such as linoleic acid (24%). Furthermore, maize oil is relatively stable, since it contains only small amounts of linolenic acid (0.7%) and high levels of natural antioxidants (Bressani et al., 1990).

Oil and starch are accumulated in different compartments of the maize kernel: 85% of the oil is stored in the embryo, whereas 98% of the starch is located in the endosperm (Watson, 1987). Consequently, the relative amounts of oil and starch are correlated with the relative sizes of the embryo and endosperm, and successful breeding for high oil content in the Illinois High Oil strains has mainly been achieved through an increase in embryo size (Moose et al., 2004). Whereas the embryo represents less than 10% of the kernel weight in normal or high-protein lines, it can contribute more than 20% in high-oil lines (Bressani and Mertz, 1958). However, genetic components may also modulate oil content in the embryo, independently of its size, as shown by the cloning of a high-oil quantitative trait locus in maize that is caused by an amino acid insertion in an acyl-CoA:diacylglycerol acyltransferase catalyzing the last step of oil biosynthesis (Zheng et al., 2008).

The oil stored in most seeds is composed of triacylglycerols (TAGs). An extensive genetic knowledge of storage oil biosynthetic pathways, and their regulation,

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^[C] Some figures in this article are displayed in color online but in black and white in the print edition.

^[W] The online version of this article contains Web-only data.

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has been generated in the model plant *Arabidopsis* (*Arabidopsis thaliana*; Beisson et al., 2003; Baud and Lepiniec, 2010). Fatty acids are synthesized from acetyl-CoA in plastids and then exported to the cytoplasm as acyl-CoA (Ohlrogge and Browse, 1995). At the endoplasmic reticulum, they are used for the acylation of the glycerol-3-phosphate backbone either by the relatively straightforward Kennedy pathway or by acyl exchange between lipids (Napier, 2007; Bates et al., 2009). The resulting TAGs are stored in specialized structures called oil bodies.

Late steps of plastidial glycolysis necessary for the production of acetyl-CoA and several steps of fatty acid synthesis are regulated by WRINKLED1 (WRI1; Ruuska et al., 2002; Baud et al., 2007, 2009; Maeo et al., 2009). WRI1 encodes a transcription factor of the large APETALA2/ethylene-responsive element-binding protein (AP2/EREBP) family (Cernac and Benning, 2004). Loss-of-function mutants have no obvious phenotype during vegetative development but produce wrinkled, incompletely filled seeds with an 80% reduction in seed oil content (Focks and Benning, 1998). In addition, a delay in embryo elongation and a modification of fatty acid composition toward longer and more desaturated fatty acids were observed in *wri1* mutants (Baud et al., 2007). Expression of WRI1 under the control of the 35S promoter can lead to a slight increase of seed oil content and cause the ectopic accumulation of TAGs in developing seedlings, which show aberrant development consistent with a prolonged embryonic state (Cernac and Benning, 2004).

Expression of the *WRI1* gene has been shown to be under the direct control of the transcription factor LEAFY COTYLEDON2 (LEC2; Baud et al., 2007), which is considered, together with LEC1, FUSCA3 (FUS3), and ABA INSENSITIVE3 (ABI3), as a master regulator of seed development (Braybrook and Harada, 2008; Suzuki and McCarty, 2008; North et al., 2010). The pleiotropic phenotypes of the respective mutants suggest that the four gene products act on separate developmental or metabolic pathways and, in so doing, coordinate the timing of developmental events or the fluxes through particular pathways (Santos-Mendoza et al., 2008). In maize, the knowledge of the regulatory network formed by LEC1, LEC2, ABI3, and FUS3 remains incomplete. The best characterized gene is *Viviparous1* (*VP1*; McCarty et al., 1991), the maize ortholog of *ABI3*. However, the extensive characterization of the *vp1* mutant focused largely on a single aspect, the role of *Vp1* in abscisic acid-mediated regulation of seed dormancy (Suzuki et al., 2003). After the cloning and molecular characterization of *ZmLec1* in maize (Zhang et al., 2002), recent work demonstrated that its overexpression increases seed oil production but reduces seed germination and plant growth. In contrast, overexpression of the putative downstream factor *ZmWri1* stimulates oil accumulation without undesirable side effects (Shen et al., 2010).

Published data, therefore, suggest that the influence of WRI1 on seed oil content may be conserved between *Arabidopsis* and maize. However, it remains to be demonstrated whether the underlying mechanisms are conserved and whether *ZmWri1* activates the same target genes as *AtWRI1* in *Arabidopsis* (Ruuska et al., 2002; Baud et al., 2007; Maeo et al., 2009). More generally, clarification of the extent to which knowledge gained in the model species *Arabidopsis* can be generalized to other plants, and more precisely whether the regulatory networks active in the exalbuminate seed of *Arabidopsis* are conserved in cereal grains with persistent endosperms, is required. This question is complicated by independent whole genome duplications that happened in the monocot and dicot lineages (Abrouk et al., 2010). In monocots, a first duplication occurred in the common ancestor of all cereals and was followed by multiple chromosome fusions, translocations, and other rearrangements (Salse et al., 2008; Devos, 2010). Later on, maize was involved in an alloduplication, which probably resulted from hybridization of two maize progenitors and which occurred after its split from sorghum (*Sorghum bicolor*; Wei et al., 2007). Here, we present the molecular characterization of duplicate *ZmWri1* genes in maize, their functionality in the *Arabidopsis wri1-4* mutant, and the effect of the overexpression of *ZmWri1a* in maize on the kernel transcriptome and metabolome.

RESULTS

Wri1 Is Duplicated in Maize

To identify the maize ortholog of *AtWRI1*, we performed a TBLASTN search of the maize genome with the amino acid sequence of *AtWRI1*. The highest scores were obtained for the two closely related gene models GRMZM2G124524 and GRMZM2G174834 (release 4a.53), which we termed *ZmWri1a* and *ZmWri1b*, respectively. To clarify the phylogenetic relationship between *AtWRI1* and the two maize genes, we enlarged the search to the related *Arabidopsis* proteins *AtWRI2* (At2g41710), *AtWRI3* (At1g16060), and *AtWRI4* (At1g79700) and included sequences from the dicot grape (*Vitis vinifera*) and from the monocots rice (*Oryza sativa*) and sorghum. The construction of a phylogenetic tree using the whole amino acid sequence of the transcription factors clearly demonstrated that *ZmWri1a* and *ZmWri1b* fell into the same clade as *AtWRI1* and that they were the closest maize relatives. Other maize proteins were present in the clades defined by *AtWRI2* or *AtWRI3/AtWRI4* (Fig. 1). Consequently, *ZmWri1a* and *ZmWri1b* appeared to be coorthologs of *AtWRI1*.

To determine the evolutionary timing of the *AtWRI1* duplication, we evaluated synteny between the respective chromosome regions in maize, sorghum, and rice with the SynMap and GEvo tools (<http://synteny.cnr.berkeley.edu/CoGe>). There was not only substantial synteny between the regions around *ZmWri1a* and

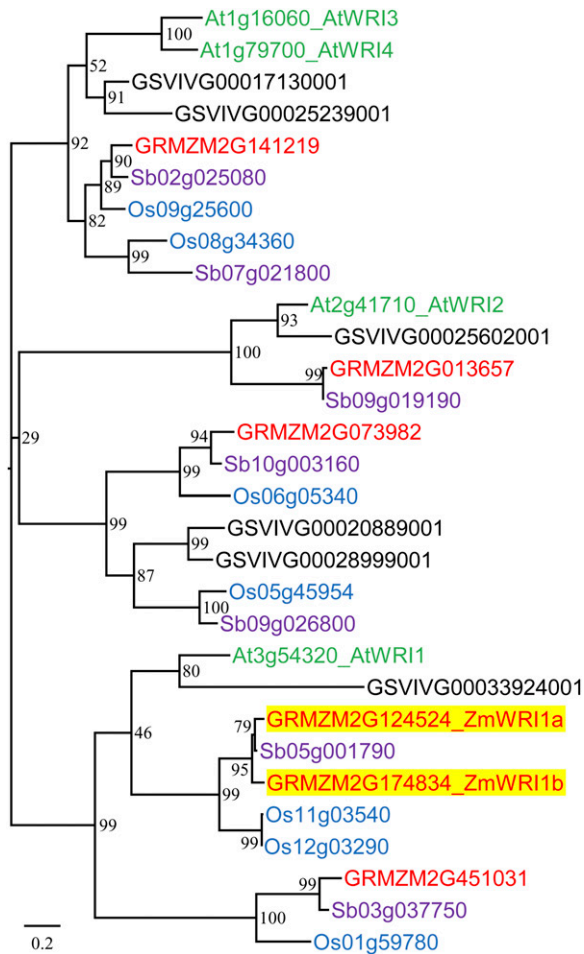


Figure 1. Phylogenetic tree of WRI proteins. A maximum likelihood phylogenetic tree was generated using all full-length grapevine (GSVIV; black), maize (GRMZM; red), rice (Os; blue), and sorghum (Sb; purple) amino acid sequences related to AtWRI1, AtWRI2, AtWRI3, and AtWRI4 from Arabidopsis (At; green) using Treefinder software. Percentage values on each branch represent the corresponding bootstrap probability. [See online article for color version of this figure.]

ZmWri1b in maize but also with the regions around the sorghum gene (Sb05g001790) and the two rice genes (Os11g03540 and Os12g03290) present in the AtWRI1 clade. In addition, all five regions showed synteny with a region at the top of chromosome 8 of sorghum, which did not contain an *AtWRI1*-related gene. The most parsimonious interpretation of these data is that the duplication did not occur during the last whole genome duplication of maize 5 million years ago but that it was already present in the common ancestor of maize and rice 60 million years ago and that one copy was subsequently lost in sorghum after the split from maize 12 million years ago.

***ZmWri1a* and *ZmWri1b* Exhibit Highest Expression in the Embryo**

To assess functional conservation between Arabidopsis and maize *WRI1* genes, we first established

expression profiles of both maize genes in major organs of the maize plant by quantitative reverse transcription (qRT)-PCR experiments using gene-specific primers. Both *ZmWri1a* and *ZmWri1b* showed strongest expression in young maize kernels, although they were expressed to various extents in all organs tested (Fig. 2A). *ZmWri1a* relative mRNA levels were higher than *ZmWri1b* mRNA levels in reproductive tissues, whereas the opposite was true in leaves, where both genes, in particular *ZmWri1b*, showed considerably higher expression in sheaths than in blades. The preferential expression of *ZmWri1a* and *ZmWri1b* in maize kernels mirrored the preferential expression of *AtWRI1* in Arabidopsis siliques (Cernac and Benning, 2004).

During the development of the maize caryopsis, *ZmWri1a* and *ZmWri1b* were expressed at low levels in immature or mature ovules (data not shown) and in kernels younger than 7 d after pollination (DAP; Fig. 2B). A first expression maximum between 7 and 12 DAP was shared by both genes, although with different kinetics. Finally, *ZmWri1a* showed a second expression peak between 30 and 50 DAP (Fig. 2B).

To gain further insight into the spatial expression pattern of *ZmWri1* genes at the onset of the filling stage, which lasts approximately from 12 to 30 DAP in our material, qRT-PCR was performed on dissected embryos and endosperms. Both genes showed an almost constant expression level in the endosperm and a sharp increase in expression between 9 and 12 DAP in the embryo (Fig. 2C). At 12 DAP, expression was predominant in the embryo, *ZmWri1a* and *ZmWri1b* mRNA levels being more than 50-fold and more than 5-fold higher than in the endosperm, respectively. The relative expression levels of the two genes somewhat differed between embryo and endosperm, *ZmWri1a* being stronger in the embryo and *ZmWri1b* stronger in the endosperm.

Both *ZmWri1a* and *ZmWri1b* Complement the *wri1* Mutation in Arabidopsis

To further investigate the functionality of *ZmWri1a* and *ZmWri1b* in planta, complementation experiments were carried out in Arabidopsis. Homozygous Arabidopsis *wri1-4* mutants (Baud et al., 2007) were transformed with maize *ZmWri1a* or *ZmWri1b* cDNA, the expression of which was driven by the seed-specific *AT2S2* (*At4g27150*) promoter. For each construct, seven independent primary transformants were selected and propagated; the progeny of T3 lines were subjected to detailed analyses. A microscopic observation of mature dry seeds showed a complete reversion of the *wri* seed phenotype usually observed in the *wri1-4* mutant background (Fig. 3A). Fatty acid analyses confirmed the ability of both *ZmWri1a* and *ZmWri1b* to restore the defect in fatty acid accumulation previously described in *wri1-4* seeds (Fig. 3, B and C).

It is noteworthy that the fatty acid profile of *wri1-4* seeds complemented either with *ZmWri1a* or *ZmWri1b*

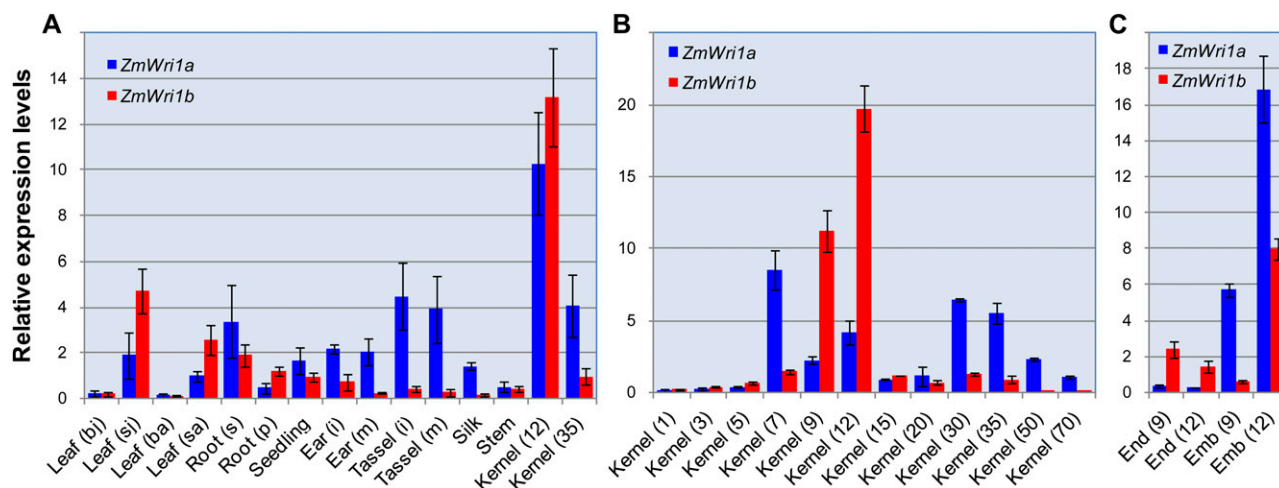


Figure 2. Expression profiles of *ZmWri1a* and *ZmWri1b* in the maize plant. Expression profiles of *ZmWri1a* and *ZmWri1b* were established by qRT-PCR in major maize organs (A), during kernel development (B), and in dissected endosperm and embryo (C). Leaf blade (b) or sheath (s) of juvenile (j) or adult (a) leaves, roots of seedlings (s) or plantlets (p), immature (i) or mature (m) ears and tassels, as well as kernels at 12 or 35 DAP were used in A, kernels of the indicated DAP in B, and dissected endosperms (End) or embryos (Emb) of the indicated DAP in C. Error bars correspond to the SD calculated from technical quadruplicates on organ or tissue pools of various size harvested from two plants. [See online article for color version of this figure.]

differed from the profile in wild-type seeds. In particular, complemented seeds exhibited an increased content of 18:3 fatty acid species and a concomitant reduction of 18:1 and 18:2 fatty acid species (Supplemental Fig. S1). This might be the consequence of a delay in *AT2S2* promoter activity compared with *AtWRI1* promoter activity. Experiments using a *Pro_{AT2S2}::uidA* transgene have shown that *Pro_{AT2S2}* activity was specifically detected in the embryonic tissues of maturing seeds from the early bent stage onward (data not shown), whereas the *Pro_{AtWRI1}* activity was already detected in torpedo-shaped embryos (Baud et al., 2007). The delayed induction of maize *ZmWri1* cDNAs in complemented seeds compared with that of *AtWRI1* in wild-type seeds may postpone the production of de novo fatty acids until midmaturation, when FATTY ACID DESATURASE3 is already highly active (Zimmermann et al., 2004).

Overexpression of *ZmWri1a* Increases Fatty Acid Content of the Maize Kernel

Since overexpression of *AtWRI1* under the control of a strong and constitutive promoter had limited effect (Cernac and Benning, 2004) or no effect (Baud et al., 2009) on the oil content of oleaginous *Arabidopsis* seeds, we wanted to test whether a similar approach could impact more significantly on the composition of nonoleaginous maize kernels. To this end, we placed the *ZmWri1a* cDNA under the control of the constitutive cassava vein mosaic virus (CsVMV) promoter and generated 11 independent transformation events in maize. In the T1 generation, three events were selected for further analysis on the basis of complete T-DNA

transfer, single transgene copy number, and high expression level of the transgene.

RT-PCR experiments on T1 leaves established comparable expression levels of the transgene in the three selected events, and a near infrared spectrum analysis indicated that the transgenic, hemizygous T2 kernels of all three events had a higher content of certain fatty acids (linoleic acid, oleic acid, total fatty acids) and certain amino acids (Pro, Ser, Tyr) than wild-type kernels from the same ears (data not shown). The transgenic kernels did not show any macroscopically visible defects such as wrinkled or plump phenotypes, and there was no significant difference in kernel weight between transgenic and wild-type kernels (data not shown).

To further characterize the impact of the *ZmWri1a* transgene on the carbon metabolism of the maize kernel, we performed a gas chromatography-time of flight-mass spectrometry metabolomics analysis on T4 kernels from self-pollinated ears of three homozygous *ZmWri1a*-overexpressing (OE) plants and three wild-type siblings. Out of 152 metabolites analyzed, 79 were actually detected, and the relative abundance of 22 of these was significantly different between mature *ZmWri1a*-OE and wild-type kernels (Table I). In parallel, principal component analysis confirmed the strong difference between the *ZmWri1a*-OE and wild-type samples, the first three components explaining 69% of the variability. Among the 10 metabolites with the most strongly significant differences ($P < 0.01$ by Student's *t* test) were four fatty acids (palmitic, linolenic, oleic, and stearic acid), three short organic acids (succinic, citric, and glyceric acid), and two free amino acids (Lys and Glu), all of which showed an increased

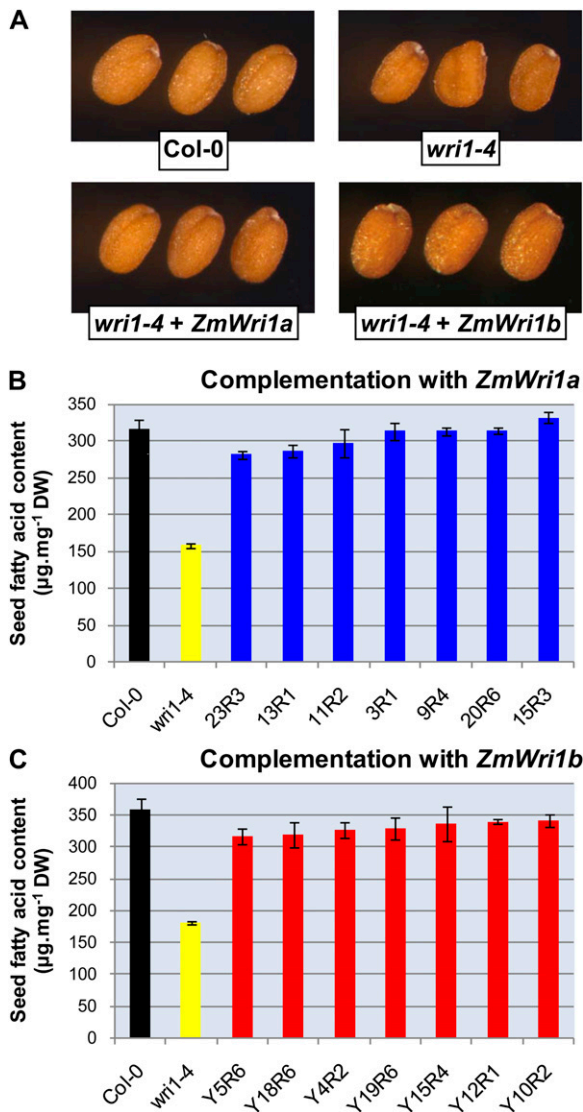


Figure 3. Complementation of the Arabidopsis *wri1-4* mutant with maize *ZmWri1a* and *ZmWri1b*. A, Images of mature seeds of the wild type (ecotype Columbia-0 [Col-0]), mutant (*wri1-4*), and complemented mutant (*wri1-4 + ZmWri1a*, *wri1-4 + ZmWri1b*). B and C, Seed fatty acid contents of Columbia-0, *wri1-4*, and six independent *wri1-4* lines complemented with *ZmWri1a* (B) or *ZmWri1b* (C) from maize. For each transformation event, five distinct plants were cultured. Twenty seeds were harvested from each plant and pooled. Error bars correspond to the sd calculated from five technical replicates per pool of 100 seeds. DW, Dry weight.

content in transgenic kernels. An independent gas chromatography analysis of total fatty acid content (from C16 to C22 in their saturated and unsaturated forms; up to three double bonds) allowed the quantification of the increase between transgenic kernels (71.09 µg mg⁻¹ dry weight) and wild-type kernels (64.68 µg mg⁻¹ dry weight).

Since starch, the major storage product of the maize kernel, is a polymer and consequently not captured by

gas chromatography-mass spectrometry-based techniques, we used spectroscopic methods to measure starch content as well as the relative amounts of amylose and amylopectin. No statistically significant difference was detected between mature *ZmWri1a*-OE and wild-type kernels despite a consistent trend for slightly lower starch, amylose, and amylopectin contents in transgenic kernels (data not shown).

Finally, the microscopic observation of cytological sections of 24-DAP kernels did not reveal any morphological defect in *ZmWri1a*-OE kernels, and size measurements of the embryo at 24 DAP and at maturity did not show any significant size differences between *ZmWri1a*-OE and wild-type embryos. In summary, the increased content in oil and certain free amino acids in *ZmWri1a*-OE kernels impacted neither on the deposition of other storage compounds nor on kernel morphology.

Transcriptome Analysis Identifies 18 Putative Target Genes of *ZmWri1a*

To identify genes regulated by the transcription factor *ZmWri1a*, a transcriptome comparison between transgenic *ZmWri1a*-OE maize plants overexpressing *ZmWri1a* and wild-type sister plants was carried out. RNA was extracted from the aerial parts of plantlets at 18 d after sowing (DAS) and used to hybridize a genome-wide 46 K microarray. A first gene list of only five differentially expressed genes was established based on a value of $P < 0.05$ for the biological triplicate and strong expression differences ($\log_R > 0.3$ or < -0.3). Using lower stringency for the second parameters ($\log_R > 0.2$ or < -0.2) but including high spot intensity ($\log_I > 2.3$) as an additional criterion, the list was extended to 25 candidates. The differential expression was confirmed for 18 of the 25 candidates by qRT-PCR experiments based on the same samples that had been used for the initial microarray analysis (Table II). In four cases, the up-regulation observed after array hybridization was not confirmed by qRT-PCR. Two candidates were eliminated due to redundancy because two distinct oligonucleotides on the array, which had been designed based on EST assemblies, corresponded in reality to a single gene in the more recently established maize genome sequence (MZ00042163 and MZ00016943 as well as MZ00044044 and MZ00026553). Among the confirmed candidates, two genes corresponding to MZ00024552 (chromosome 1) and MZ00043500 (chromosome 9) were near isogenic paralogs, so that it was not possible to design gene-specific primers and test whether the up-regulation in *ZmWri1a*-OE plantlets concerned one or both genes.

Since the function of *ZmWri1a* has been attributed to the kernel, the up-regulation of the 18 confirmed target genes was also assessed in developing *ZmWri1a*-OE and wild-type kernels at 16, 24, 32, and 40 DAP (Table II; Fig. 4). At 24 DAP, all 18 genes were significantly up-regulated, and for 15 of them this was also true

Table 1. Metabolites with significantly increased or decreased content in ZmWri1a-OE kernels

Metabolite ^a	Trend in Wri1a-OE	Mean ^b Wri1a-OE	Mean ^b Wild Type	SD Wri1a-OE	SD Wild Type	Ratio Wri1a-OE/Wild Type ^c
Palmitic acid	Up	0.02362	0.01254	0.00217	0.00039	1.88**
Succinic acid	Up	0.00152	0.00105	0.00010	0.00006	1.45**
Linolenic acid	Up	0.00133	0.00069	0.00015	0.00006	1.94**
Lys	Up	0.00188	0.00128	0.00011	0.00011	1.47**
Oleic acid	Up	0.00085	0.00039	0.00010	0.00007	2.18**
Glyceric acid	Up	0.00050	0.00032	0.00002	0.00004	1.53**
Stearic acid	Up	0.00230	0.00111	0.00017	0.00036	2.07**
Citric acid	Up	0.00411	0.00236	0.00052	0.00031	1.74**
Glu	Up	0.00049	0.00000	0.00017	0.00000	98.76**
Phosphoric acid	Up	0.00806	0.00446	0.00107	0.00073	1.81**
Phe	Up	0.00076	0.00049	0.00010	0.00003	1.54*
Ara	Up	0.00333	0.00244	0.00030	0.00020	1.36*
Linoleic acid	Up	0.00147	0.00081	0.00022	0.00016	1.82*
Decanoic acid	Down	0.00754	0.01061	0.00025	0.00132	0.71*
Pyro-Glu	Up	0.00723	0.00470	0.00117	0.00037	1.54*
Nor-Leu	Up	0.00024	0.00016	0.00003	0.00003	1.51*
Nicotinic acid	Up	0.00021	0.00009	0.00004	0.00005	2.39*
Ala (peak A)	Up	0.09383	0.04840	0.00922	0.02302	1.94*
Val	Up	0.02484	0.01708	0.00403	0.00160	1.45*
Aminoadipic acid	Up	0.00023	0.00011	0.00006	0.00004	2.14*
Nonanoic acid	Down	0.00001	0.00003	0.00000	0.00001	0.25*
Orn	Up	0.00124	0.00068	0.00034	0.00009	1.82*

^aMetabolites are in order of statistical significance of the difference between transgenic and wild-type kernels. ^bArbitrary units representing peak areas of biological triplicates. ^cStudent's *t* test value: ** $P < 0.01$, * $P < 0.05$.

at all other developmental stages tested. However, in two cases (MZ00031529 and MZ00039375), the up-regulation in ZmWri1a-OE kernels was limited to the stages 16 and 24 DAP, whereas expression was much stronger in wild-type kernels at 32 DAP and particularly at 40 DAP. The corresponding genes, therefore, may be controlled by two distinct regulatory pathways, with ZmWri1a either promoting the first or inhibiting the second. Similar reasoning would also explain the up-regulation of the gene corresponding to MZ00042142 in wild-type kernels at 16 DAP but not in transgenic kernels at later stages.

Putative Targets of ZmWri1a Act in the Glycolysis, Fatty Acid, and TAG-Related Biosynthetic Pathways

To determine whether the 18 putative target genes acted in common metabolic or developmental pathways, complete protein sequences were assembled starting from the 70-nucleotide oligonucleotide deposited on the microarray, exploiting the very rich maize EST data available (Alexandrov et al., 2009; Soderlund et al., 2009) as well as the recently established maize genome sequence (Schnable et al., 2009). Based on sequence similarities, most of the 18 genes encoded enzymes of the glycolysis and the fatty acid or TAG-related biosynthetic pathways (Table II).

The deduced amino acid sequence of the first gene showed some homology to the phosphoenolpyruvate (PEP)-utilizing domain (pfam00391) of a bacterial PEP-protein phosphotransferase, which has been shown

to produce pyruvate from PEP and use the phosphate, via carrier proteins, for various processes such as sugar transport (Rabus et al., 1999). Two near isogenic genes (MZ00024552 and MZ00043500) coded for closely related plastidial pyruvate kinases catalyzing the irreversible synthesis of pyruvate and ATP from PEP and ADP. The first step of fatty acid synthesis is executed by the pyruvate dehydrogenase multi-enzyme complex (PDHC), which catalyzes the decarboxylation of pyruvate into acetyl-CoA. Among the targets isolated, three genes encoded either α (MZ00024718) or β (MZ00016632 and MZ00014741) components of the pyruvate dehydrogenase (E1 subunit of PDHC) and two genes (MZ00017663 and MZ00043050) encoded dihydrolipoyl acyltransferase (E2 subunit of PDHC). In addition, the lipoyltransferase encoded by MZ00017651 likely catalyzes the covalent attachment of lipoic acid to the PDHC-E2 apoenzyme, thereby creating the active holoenzyme.

The first gene clearly involved in acyl chain production (MZ00056535) encoded 3-ketoacyl-acyl carrier protein (ACP) synthase III, the isoform responsible for the initial condensation of acetyl-CoA with malonyl-ACP. This reaction, like the subsequent cycles responsible for fatty acid elongation, requires ACPs, one of which was also encoded by a confirmed candidate gene (MZ00016866). Two other candidates encoded enzymes involved downstream in the plastidial fatty acid biosynthetic process: oleoyl-ACP thioesterase (MZ00041636) releases free fatty acid from ACP at the inner plastid membrane, whereas the long-chain acyl-CoA synthetase

Table II. Expression level, annotation, and predicted subcellular localization of *ZmWri1a* target genes

M, Mitochondrion; P, plastid.

Identifier of No. Oligonucleotide on the Array ^a	Gene Model (Release 4a.53)	Annotation	Ratio OE/Wild-Type Leaf ^b (Array)	Ratio OE/Wild-Type Leaf ^b (qRT-PCR)	Ratio OE/Wild-Type Kernel ^{b,c} (qRT-PCR)	Predicted Subcellular Localization (SignalP)	Predicted Subcellular Localization (Predotar)	Arabidopsis Ortholog(s)	
1	MZ00042142	GRMZM2G019923_P01	Phosphoenolpyruvate-protein phosphotransferase	2.29*	2.61*	4.00*	P	M	No hit
2	MZ00024552	GRMZM2G033526_P02	Pyruvate kinase	1.73**	3.11**	4.20**	P	None	At1g32440 ^d
3	MZ00043500	GRMZM2G144730_P01	Pyruvate kinase	1.79**			P	None	At1g32440 ^d
4	MZ00024718	GRMZM2G033894_P01	Pyruvate dehydrogenase E1 α -subunit	1.93**	4.89**	3.63**	P	P	At1g01090 ^d
5	MZ00016632	GRMZM2G127546_P01	Pyruvate dehydrogenase E1 β -subunit	2.99**	27.33**	1.97*	P	P	At2g34590, At1g30120 ^d
6	MZ00014741	GRMZM2G088565_P01	Pyruvate dehydrogenase E1 β -subunit	1.64**	3.40**	3.84**	P	P	At2g34590, At1g30120 ^d
7	MZ00017663	GRMZM2G165176_P01	Pyruvate dehydrogenase E2 subunit	1.63**	1.92*	2.57**	None	None	At1g34430
8	MZ00043050	GRMZM2G058702_P01	Pyruvate dehydrogenase E2 subunit	1.71**	2.09**	2.91**	P	P	At3g25860
9	MZ00017651	GRMZM2G038922_P01	Lipoyltransferase	1.60**	3.27**	2.81**	P	P	At4g31050, At1g47580
10	MZ00056535	GRMZM2G127623_P01	3-Ketoacyl-acyl carrier protein synthase III	1.62**	2.69**	5.06**	None	None	At1g62640 ^d
11	MZ00016866	GRMZM2G032878_P01	Acyl carrier protein 3	1.82**	2.50**	3.14**	P	P	At1g54580, At1g54630
12	MZ00041636	GRMZM2G102878_P01	Oleoyl-acyl carrier protein thioesterase	1.89**	6.48**	1.45**	None	None	At4g13050, At3g25110 ^d
13	MZ00017355	GRMZM2G117064_P01	Long-chain acyl-CoA synthetase 9	2.00**	6.03**	4.77**	P	Endoplasmic reticulum	At1g77590
14	MZ00040095	GRMZM2G085474_P01	Cytosolic acetyl-CoA acetyltransferase	1.60**	2.92**	2.96**	None	None	At5g47720, At5g48230
15	MZ00042163, MZ00016943	GRMZM2G010596_P01	Ketopantoate hydroxymethyltransferase	1.96**	2.37**	3.42**	M	M	At2g46110, At3g61530
16	MZ00044044, MZ00026553	GRMZM2G145101_P02	β -Type carbonic anhydrase	1.94**	2.16**	5.17**	M	M	At1g58180, At4g33580
17	MZ00015977	Not in release 4a.53	Glycerol-3-phosphate dehydrogenase (NAD+)	2.06**	3.11**	9.22**	None	None	At5g40610
18	MZ00031529	GRMZM2G113875_P02	Outer membrane lipoprotein	1.98**	5.00*	1.61**	P	Too short	No hit
19	MZ00039375	GRMZM2G016004_P01	No BLAST hit	2.02**	1.79*	2.09*	None	No ATG	No hit

^aIdentification number of the corresponding oligonucleotide deposited on the microarray. ^bStudent's *t* test value: ** $P < 0.01$, * $P < 0.05$. ^cValue at 24 DAP. ^dPublished as a target gene of AtWRI1 (Baud et al., 2009; Maeo et al., 2009).

MZ00017355 activates free fatty acids to CoA esters in the outer plastid envelope (Shockey et al., 2002).

Despite its suggestive annotation, the function of the acetyl-CoA acetyltransferase encoded by MZ00040095 in fatty acid synthesis remains unclear. This cytoplasmic enzyme is thought to be involved in isoprenoid biosynthesis (mevalonate pathway), Ile degradation, or Lys degradation and may influence the CoA pool (Carrie et al., 2007). The ketopantoate hydroxymethyltransferase encoded by MZ00042163/MZ00016943 has a more direct link to CoA biosynthesis, since it catalyzes the reaction from Val to 2-ketoisovalerate, a precursor of pantoate, the universal precursor for the synthesis of the 4'-phosphopantetheine moiety of CoA and ACP. The CoA biosynthetic pathway also contains a decarboxylation reaction, which may be facilitated by the action of a β -type carbonic anhydrase regulating the balance between carbon dioxide and carbonic acid and possibly encoded by MZ00044044/MZ00026553.

Finally, MZ00015977 encodes a cytosolic NAD-dependent glycerol-3-phosphate dehydrogenase providing glycerol backbones necessary for TAG biosynthesis. No precise function could be attributed to the outer membrane lipoprotein MZ00031529, while the last confirmed candidate has no informative annotation (MZ00039375).

DISCUSSION

WRI1, a key regulator of oil biosynthesis discovered in Arabidopsis, is duplicated in maize. Our expression and complementation data indicate that both *ZmWri1a* and *ZmWri1b* fulfill a role similar to that of AtWRI1 in the developing embryo. We have shown that at least *ZmWri1a* acts via the transcriptional regulation of enzymes involved in glycolysis or in the fatty acid and TAG biosynthetic pathways and that overexpression of *ZmWri1a*

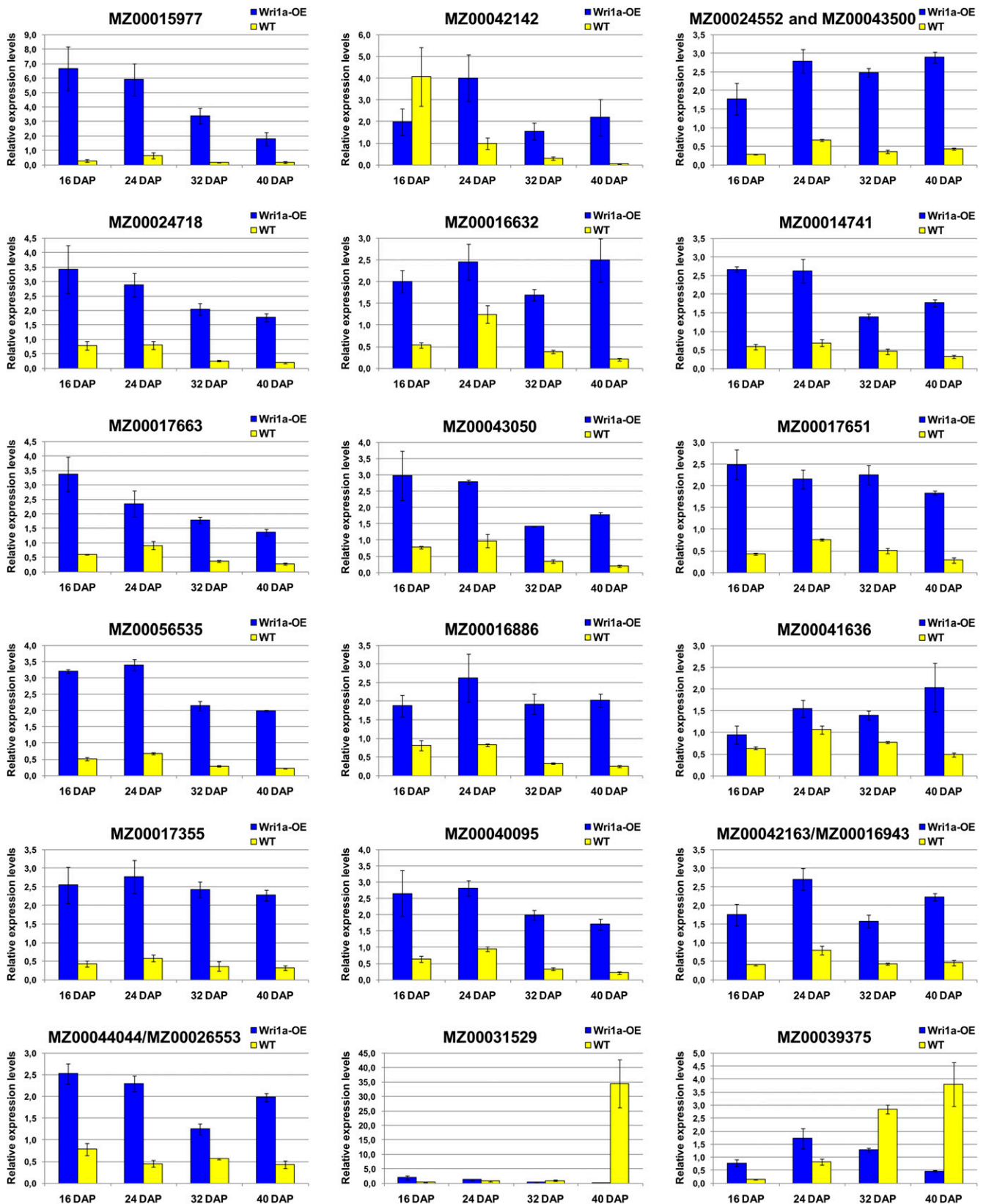


Figure 4. Expression profiles of *ZmWri1a* target genes in *ZmWri1a*-OE and wild-type (WT) kernels. Expression profiles of direct or indirect target genes of *Wri1a* were established by qRT-PCR in *ZmWri1a*-OE and wild-type kernels at four developmental stages indicated in DAP. Genes are identified by their MZ000xxxx number used in Table II. Error bars correspond to the SD calculated from technical duplicates of the gene of interest and the reference gene on pools of five kernels. For each data point, the difference between *ZmWri1a*-OE and wild-type kernels is statistically significant at $P < 0.05$ in Student's *t* test. [See online article for color version of this figure.]

is sufficient to increase the oil content in maize kernels.

Conserved Function and Expression between *ZmWri1* Genes and *AtWRI1*

The duplication of *WRI1* is not specific to maize but is also found in rice and probably occurred 90 million years ago during the genome duplication of the common ancestor of cereals. Both maize genes encode functional proteins, since they both complement the wrinkled phenotype and the low fatty acid content of *Arabidopsis wri1-4* mutant seeds. These data suggest that the overall molecular function (i.e. the transcriptional activation of multiple target genes involved in fatty acid biosynthesis) has been conserved over more than 150 million years between *AtWRI1*, *ZmWri1a*, and *ZmWri1b*. The qualitative differences in fatty acid composition between *wri1-4* mutants complemented with maize genes and wild-type controls might be explained by a heterochronic effect of the *AT2S2* promoter used for complementation and/or by ongoing coevolution between each individual transcription factor and its respective cis-elements/targets within species.

Despite the overall conservation of their molecular function, the two maize genes *ZmWri1a* and *ZmWri1b* may not play the same role in the maize plant. Although both *ZmWri1* genes mirror the expression pattern of *AtWRI1*, with a preferential expression in the kernel and a strong induction in the embryo at the onset of the maturation phase, the expression patterns of *ZmWri1a* and *ZmWri1b* are clearly distinct. Contrary to *ZmWri1a*, *ZmWri1b* is barely expressed in reproductive tissues prior to pollination, whereas the opposite is true in leaf sheaths. In the kernel, *ZmWri1a* has a biphasic expression pattern with a second peak at the end of the filling stage, which is missing for *ZmWri1b*. In the embryo, *ZmWri1a* is the predominant gene, while *ZmWri1b* but not *ZmWri1a* shows substantial expression in the endosperm. All these expression differences likely reflect an ongoing specialization on an evolutionary time scale, which may imply subtle changes in the sets of target genes and/or altered responses to regulators of carbon partitioning. One may speculate that *ZmWri1b* is evolving toward a housekeeping gene maintaining TAG biosynthesis for various cellular processes while *ZmWri1a* remains mainly devoted to storage fatty acid biosynthesis during grain filling.

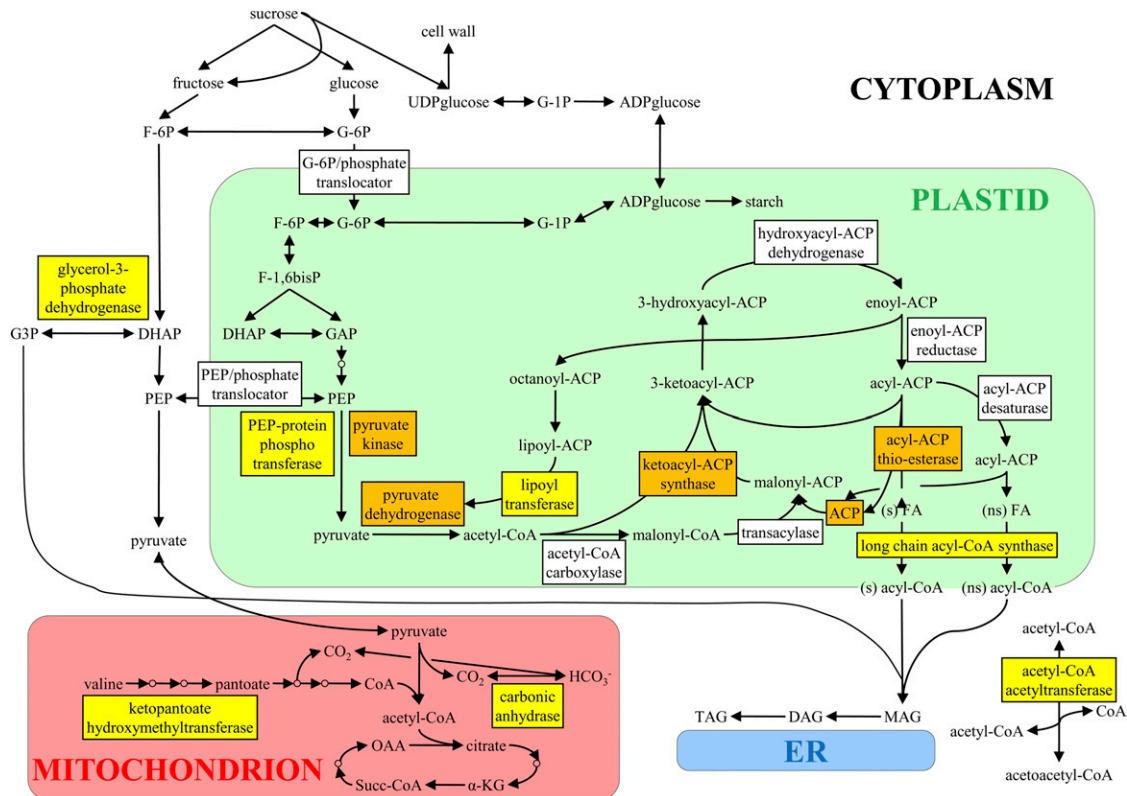


Figure 5. Putative metabolic functions of up-regulated *ZmWri1a* target genes. Enzymatic functions encoded by up-regulated target genes of *ZmWri1a* and *AtWRI1* are in orange, those encoded only by *ZmWri1a* target genes are in yellow, and those encoded only by *AtWRI1* target genes are in white. Abbreviations not defined in the text: DAG, diacylglycerol; (ns) FA, nonsaturated fatty acid; (s) FA, saturated fatty acid; DHAP, dihydroxyacetone phosphate; F-1,6bisP, Fru-1,6-bisP; F-6P, Fru-6-P; GAP, glycerol aldehyde phosphate; G-1P, Glc-1-P; G-6P, Glc-6-P; G3P, glycerol-3-phosphate; MAG, monoacylglycerol.

Conserved Regulation of Oil Biosynthesis between Maize and Arabidopsis

The overexpression of *ZmWri1a* under the control of the constitutive *CsVMV* promoter was sufficient to increase the fatty acid content of the maize kernel and confirmed recent results obtained by overexpression of the same gene under the control of the embryo-preferred *OLE* promoter (Shen et al., 2010). The underlying mechanism involves the transcriptional up-regulation of at least 18 target genes identified by a comparative transcriptome analysis between *ZmWri1a*-OE and wild-type leaves and confirmed by qRT-PCR experiments in leaves and kernels. The manually improved annotations of the deduced amino acid sequences clearly indicate that the majority of these genes have functions related to late glycolysis and fatty acid or TAG biosynthesis. This reflects the situation in Arabidopsis, where a list of putative target genes of AtWRI1 has been previously established by a combination of microarray analysis, targeted qRT-PCR, yeast one-hybrid analysis, and electrophoretic mobility shift assay experiments (Ruuska et al., 2002; Baud et al., 2007, 2009; Maeo et al., 2009). Common targets between maize and Arabidopsis include subunits of the pyruvate kinase and pyruvate dehydrogenase complexes, ketoacyl-ACP synthase, acyl-ACP thioesterase, and ACPs (Fig. 5). While our confirmed candidates did not include all putative targets isolated in Arabidopsis (Fig. 5), such as Glc-6-P/phosphate translocator, PEP/phosphate translocator, acetyl-CoA carboxylase, transacylase, hydroxyacyl-ACP dehydrogenase, enoyl-ACP reductase, or acyl-ACP desaturase, genes encoding these enzymes may be up-regulated in *ZmWri1a*-OE plants but have escaped classification as differentially expressed for a variety of technical reasons, such as expression levels close to background or low spot quality of one of the replicas. Similarly, novel target genes with differential expression detected only in maize may also be targets of AtWRI1 in Arabidopsis. These include genes encoding glycerol-3-phosphate dehydrogenase, PEP-protein phosphotransferase, lipoyltransferase, and long-chain acyl-CoA synthase (Fig. 5). It is noteworthy that two of these enzymes are predicted to be located at the plastid membrane (long-chain acyl-CoA synthase) or in the cytoplasm (glycerol-3-phosphate dehydrogenase) and have functions related to TAG biosynthesis.

Among the remaining four *ZmWri1a* targets, one has no similarity to proteins or domains with known function, whereas the other three have roles related to CoA biosynthesis and/or homeostasis (Fig. 5). At first sight, their predicted localization in mitochondria (ketopantoate hydroxymethyltransferase, carbonic anhydrase) or the cytoplasm (acetyl-CoA acetyltransferase) makes it difficult to establish a direct link to fatty acid biosynthesis localized in plastids. However, it is known that CoA is present in all compartments and that precursors are shuttled between compartments

(Rubio et al., 2008). In addition, the overexpression of a CoA biosynthetic enzyme has been shown to lead to increased seed oil content in Arabidopsis (Rubio et al., 2008). The maize acetyl-CoA acetyltransferase shows similarity to ACAT1 and ACAT2 from Arabidopsis, suggesting a function in the mevalonate pathway leading to isoprenoid biosynthesis rather than in the degradation of Lys or Ile (Carrie et al., 2007). The increase rather than decrease in Lys content in *ZmWri1a*-OE kernels further substantiates this hypothesis. While the mevalonate pathway consumes rather than produces acetyl-CoA, the reaction releases CoA, which may be transported to the plastid and have a beneficial effect on fatty acid synthesis.

AW Boxes Are Present in the Upstream Regions of *ZmWri1a* Target Genes

In Arabidopsis, the AW box 5'-CnTnG(n)₇CG-3' has been identified as the AtWRI1 binding site in the promoter sequences of three target genes, and 28 AW boxes have been found in the upstream regions of 19 genes involved in fatty acid biosynthesis out of 46 examined (Maeo et al., 2009). A survey of the putative promoter sequences, 3 kb upstream of the predicted

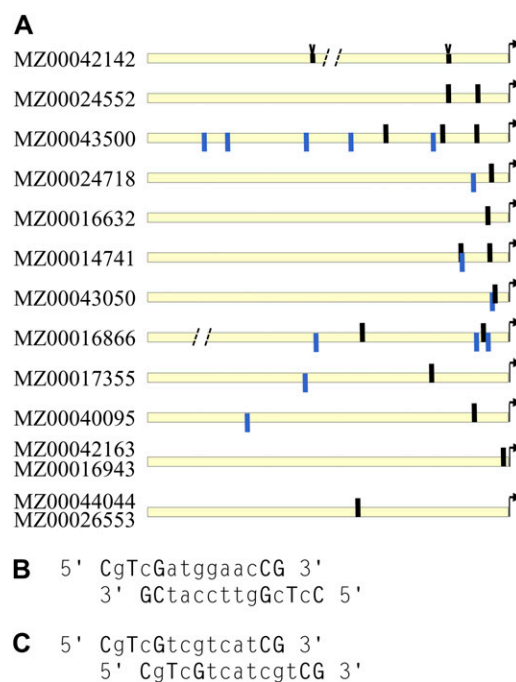


Figure 6. AW boxes in upstream sequences of *ZmWri1a* target genes. A, Schematic drawing of the sequence 3 kb upstream of the ATG start codon (arrows) of *ZmWri1a* target genes. AW boxes in the sense strand are indicated by black vertical bars shifted to the top, AW boxes in the antisense strand by blue vertical bars shifted to the bottom. B, Palindromic AW boxes in the upstream region of MZ00014741. C, Overlapping AW boxes in the upstream region of MZ00042142. The fixed bases of the consensus sequence CnTnG(n)₇CG are in uppercase letters. [See online article for color version of this figure.]

ATG start codons, of the 18 *ZmWri1a* target genes isolated in this study demonstrated the presence of 33 AW boxes in 12 of the 18 sequences analyzed (Fig. 6A). As in the case of Arabidopsis, at least one AW box per sequence was located close to the start codon, with one possible exception (MZ00044044/MZ00026553). Our analysis also showed that an AW box can overlap with a second AW box, both on the sense strand (Fig. 6B) and the antisense strand (Fig. 6C). The existence of a palindromic AW box may hint at the binding of *ZmWri1a* as a homodimer or as a heterodimer with another transcription factor of the AP2/EREBP family.

Metabolic Adjustments in *ZmWri1a*-OE Kernels

Transgenic *ZmWri1a*-OE kernels did not only show a significant increase in saturated and unsaturated fatty acids with 16 to 18 carbon atoms but also a significant increase for several free amino acids (Lys, Glu, Phe, Ala, Val), intermediates or cofactors of amino acid biosynthesis (pyro-Glu, aminoadipic acid, Orn, nor-Leu), and intermediates of the tricarboxylic acid cycle (citric acid, succinic acid). Since the transcriptome analysis suggests that *ZmWri1a* essentially activates genes coding for enzymes in late glycolysis, fatty acid, CoA, and TAG biosynthesis, and considering that no misregulated candidates participate in any additional pathways, the increase in amino acids and TCA intermediates probably reflects secondary adjustments of the carbon and nitrogen metabolism to the increased oil biosynthesis triggered by *ZmWri1a*. The three amino acids Phe, Ala, and Val are derived from PEP or pyruvate, and their increase may simply be a by-product of a strongly increased carbon flux through glycolysis. Likewise, the increased amounts of citric and succinic acid may reflect an increased activity of the TCA cycle. Glu is the storage form of ammonia in the plant, and the very steep increase in Glu content of transgenic kernels may be indicative of an excess of nitrogen or rather an imbalance between nitrogen and available carbon skeletons. The increase in aminoadipic acid, Orn, and pyro-Glu is possibly linked to subsequent adjustments of the ammonia assimilation cycle between Gln and Glu and downstream pathways leading to Pro and Arg synthesis.

Use of *ZmWri1a*-OE Plants in Plant Breeding

Our results with greenhouse-grown transgenic plants overexpressing *ZmWri1a* under the control of the constitutive *CsVMMV* promoter confirm recent results in field-grown transgenic plants using the embryo-preferred *OLE* promoter in showing that the increase in seed oil content is not linked to an increase in embryo size but in seed oil (Shen et al., 2010). This increase in oil does not compromise the other uses of the maize kernel, and in particular the use of starch for nutritional or industrial purposes, since neither study detected significant changes in the overall starch content of the kernel, despite a decrease in the embryo,

where very minor amounts of starch are stored (Shen et al., 2010). While it does not appear to be possible to produce oil rather than starch in maize endosperm by the simple overexpression of *ZmWri1a* in the starchy endosperm (Shen et al., 2010), increased yield of nutritionally improved oil from transgenic embryos is still of sufficient interest to the milling industry to warrant the use of *ZmWri1a*-OE lines in plant breeding. In addition, the differences in oil quality seen in Arabidopsis between wild-type seed and mutant seed complemented with maize *ZmWri1* genes may provide valuable information for the engineering of oil-seed crops. Once it has been determined whether a heterochronic effect of the *AT2S2* promoter or the altered coding sequence of the maize genes is responsible for the effect, the identified component could be transferred to rapeseed (*Brassica napus*) or other crops to produce oil with higher levels of polyunsaturated fatty acids.

Beyond nutritional aspects, TAGs also present considerable interest for energy production. In vegetative tissues of Arabidopsis, the ectopic expression of *AtWRI1* leads to a 3-fold increase in TAG levels, which can be further increased by the concomitant repression of ADP-Glc pyrophosphorylase (Sanjaya et al., 2011). This approach can likely be transferred to maize, since mRNA levels of all 18 target genes of *ZmWri1a* are significantly increased in leaves of *ZmWri1a*-OE plants (Table II). Crosses to ADP-Glc pyrophosphorylase mutants may provide a means to shift carbon partitioning at least partially from starch to TAG biosynthesis not only in vegetative tissues but also in the endosperm.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The maize (*Zea mays*) inbred line A188 and transgenic A188 plants overexpressing *ZmWri1a* were grown in a S2 greenhouse with a 16-h illumination period (100 W m⁻²) at 24°C/19°C (day/night) and without control of the relative humidity. Kernels were germinated in 0.2 L of Favorit MP Godets substrate (Eriterre) and were transferred at 21 DAS to 10 L of Favorit Argile substrate (Eriterre) supplemented with 4 g L⁻¹ Osmocote Exact hi-end 15+9+12 fertilizer (Scotts). All plants were propagated by hand pollination.

Seeds of the Arabidopsis (*Arabidopsis thaliana*) *wri1-4* (ecotype Columbia-0 background) mutant line (Baud et al., 2007) were surface sterilized and germinated on Murashige and Skoog medium (Murashige and Skoog, 1962). After a cold treatment of 48 h at 4°C in the dark, plates were kept in a growth chamber (16/8-h light photoperiod at 150 μmol m⁻² s⁻¹, 15°C night/20°C day temperature). After 10 d, the plantlets were transferred to compost, grown in a greenhouse under similar conditions, and irrigated twice a week with mineral nutrient solution.

T-DNA Constructs and Plant Transformation

The coding sequences of *ZmWri1a* and *ZmWri1b* were amplified on cDNA from 7-DAP kernels (genotype A188) with primers FT126-F7 and FT126-R7 and on cDNA from 16-DAP kernels (genotype B73) with primers attB1-Wri2 and attB2-Wri2, respectively. After BP recombination into pDONZeo (Invitrogen), the resulting entry vectors L698 and L1129 were sequenced prior to LR recombination. Only L698 was recombined into the maize destination vector pBIOS886, which was based on the backbone of vector pSB11 (Ishida et al., 1996) and contained between T-DNA borders a Basta resistance cassette

for selection, a GFP cassette for the tracking of transgenics in segregating material, as well as attR1 and attR2 sites downstream of the constitutive CsV_{MV} promoter and upstream of the *Sac66* terminator. Primer sequences are given in Supplemental Table S1.

Agrobacterium tumefaciens-mediated transformation of maize inbred line A188 with the resulting plasmid was based on a published protocol (Ishida et al., 2007). Among 11 independent transformation events, three events with single-copy insertions and high expression levels were chosen for this study.

For the construction of the *Pro*_{AT2S2}-*RIR2*-*HYGRO* destination vector, the AT2S2 (At4g27150) promoter was amplified from the proofreading Pfu Ultra DNA polymerase (Stratagene) on Arabidopsis genomic DNA using primers *HindIII*pAT2S2up and *AscI*pAT2S2low (Supplemental Table S1). The PCR product representing positions -984 to -1 relative to the AT2S2 translational start codon was digested with *HindIII* and *AscI* and cloned into *pMDC32* (Curtis and Grossniklaus, 2003) digested with *HindIII* and *AscI* to replace the 2× 35S cassette. *ZmWri1a* and *ZmWri1b* were recombined from their respective entry clones into this vector by LR recombination.

The binary vectors were electroporated into *Agrobacterium* C58C1 strain and used for agroinfiltration of Arabidopsis *wri1-4* flower buds (Bechtold et al., 1993). Primary transformants were selected on Murashige and Skoog medium containing hygromycin (50 mg L⁻¹) and then transferred to soil. Homozygous T3 lines producing 100% resistant plantlets were selected and used for complementation analyses. For each construct, seven independent transformants were considered.

Sequence Analysis

The cDNA sequences corresponding to the 70-mers present on the microarray were established by BLASTN individual EST sequences or full-length cDNA sequences at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST/). Consensus sequences were obtained using VectorNTI ContigExpress software (Invitrogen) and regularly updated. Genomic sequences were obtained by BLASTN of the cDNA sequence against the maize genome (www.maizesequence.org). Deduced amino acid sequences were annotated by BLASTP against the Arabidopsis genome at the National Center for Biotechnology Information and screened for known, conserved domains using the conserved domain database.

After amino acid sequence alignment with ClustalW and conserved block selection with SeaView (<http://pbil.univ-lyon1.fr/software/seaview.html>), maximum likelihood phylogenetic trees were generated with Treefinder software (www.treefinder.de) using the substitution model WAG_optimumG4 and 1,000 bootstrap replicates.

qRT-PCR

Total RNA was extracted with the TRIzol reagent, treated with DNase, and reverse transcribed as described previously (Javelle et al., 2010). Real-time PCR was carried out with the Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) on a StepOne Real-Time PCR System (Applied Biosystems). Dilutions series (2ⁿ with *n* = 0–7) of a mixture of all cDNA within a comparison were used to fix the threshold cycle (C_T). Gene expression levels relative to the 18S rRNA reference gene were calculated by the $\Delta\Delta C_T$ method (Schmittgen and Livak, 2008). The primers used are listed in Supplemental Table S1.

Microarray Analysis

Hybridization of the maize 46 K (version 1) 70-mer oligoarray printed on a single glass slide (<http://www.maizearray.org>) was performed in biological triplicate and with Cy5/Cy3 dye swap as technical replicates. Probes were synthesized from total RNA isolated from the aerial parts of 18-DAS maize seedlings from wild-type and *ZmWri1a*-OE plants using an Eberwine-based amplification method and indirect labeling with dyes. Briefly, 2 μg of total RNA was reverse transcribed with an oligo(dT) primer bearing a T7 promoter. After second-strand synthesis, double-stranded cDNA served as a template for amplification by in vitro transcription with the Amino Allyl MessageAmp aRNA Kit (Ambion). The incorporated amino-allyl dUTP was then labeled by covalent binding with activated dye esters using Cy5 Mon-Reactive Dye Pack and Cy3 Mon-Reactive Dye Pack (GE Healthcare). Each microarray was hybridized with a mix of 3 μg of Cy3-labeled target and 3 μg of Cy5-labeled target resuspended in 60 μL of 1× hybridization buffer (50% formamide, 5× SSC, 0.1% SDS, 0.4 μg μL⁻¹ tDNA, and 0.2 μg μL⁻¹ salmon sperm DNA).

Hybridizations were carried out overnight at 42°C in a SlideBooster hybridization station (Advantix). After slide washing (most stringent wash for 5 min in 0.05× SSC at room temperature), slides were immediately scanned on a ScanArray 4000XL scanner (Perkin-Elmer).

Raw data were normalized using a quantile normalization approach consisting of adjusting each individual signal distribution to a common one. Normalization was performed with the open source R software with packages dedicated to microarray analysis. Gene lists were constituted of differentially expressed genes presenting a minimum 2-fold change between the two conditions with a statistical threshold at *P* < 0.01.

Metabolomic Measurements

The extraction of metabolites from plant tissues and their analysis by gas chromatography coupled to time of flight mass spectrometry on a LECO Pegasus III with an Agilent 6890 N gas chromatography system and an Agilent 7683 automatic liquid sampler were performed as described (Tcherkez et al., 2009). The integration of peaks was performed using the LECO Pegasus software. Because automated peak integration was occasionally erroneous, integration was verified manually for each compound in all analyses.

Fatty acid analyses were performed on pools of 20 seeds (Arabidopsis) and on meals of lyophilized grain tissues (maize) as described previously (Li et al., 2006). Starch content was determined with the starch kit according to the instructions of the manufacturer (Roche). Amylose and amylopectin were detected simultaneously by multiwavelength analysis (Sené et al., 1997).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Seed fatty acid composition of the Arabidopsis *wri1-4* mutant complemented with maize *ZmWri1a* or *ZmWri1b*.

Supplemental Table S1. Primers used in this study.

ACKNOWLEDGMENTS

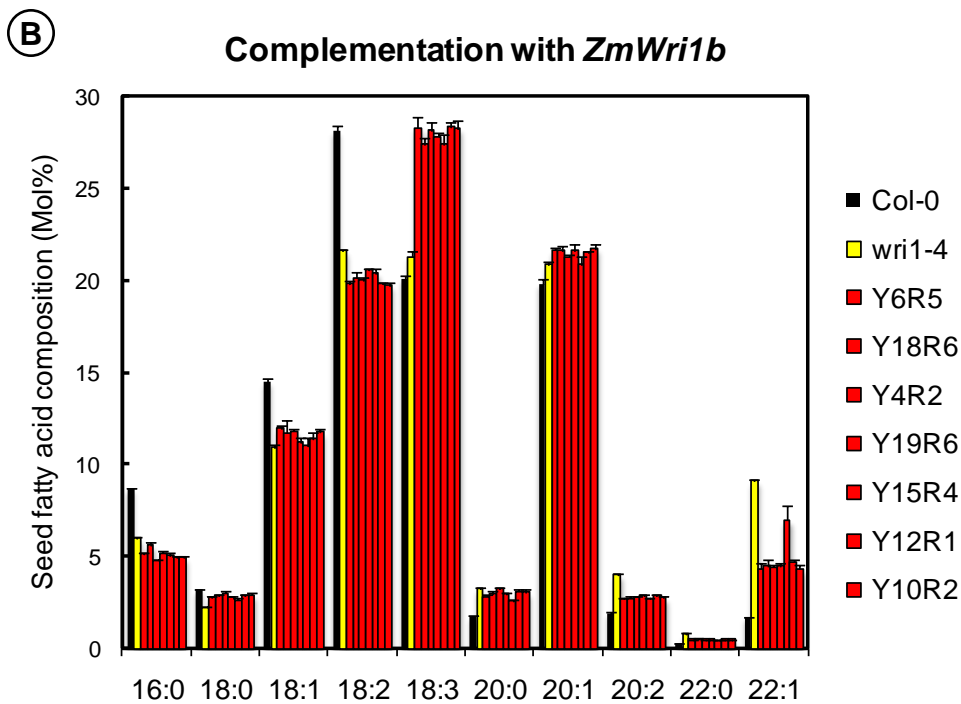
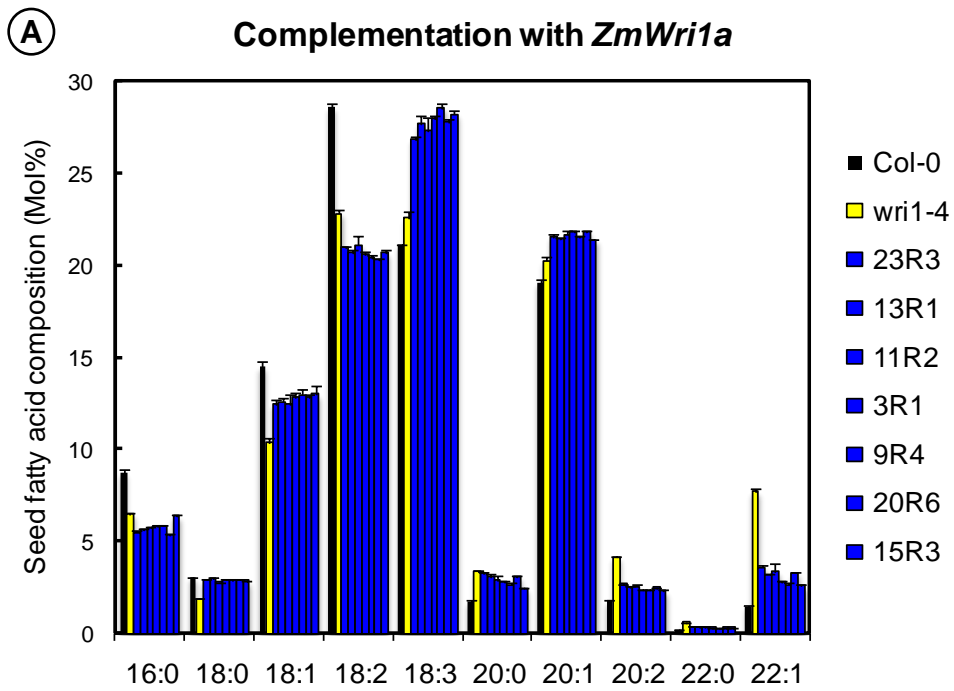
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Supp. Fig. S1: Seed fatty acid composition of the Arabidopsis *wri1-4* mutant complemented with maize *ZmWri1a* or *ZmWri1b*

Seed fatty acid composition of Col-0, *wri1-4* and 6 independent *wri1-4* lines complemented with *ZmWri1a* (A) or *ZmWri1b* (B) from maize.

Supplementary Table S1: Primers used in this study

Oligo ID ¹ or gene name	Primer name	Primer sequence (5' to 3')	Use
MZ00015977	15977-F2	TCAATCCCAAGGACGAAATGG	Expression <i>Wri1a</i> -OE/WT
	15977-R2	CCGTGGTTATGGTTCGCTTAGATA	
MZ00016632	16632-F	CCATCATGTGCCTGTCATCTCA	Expression <i>Wri1a</i> -OE/WT
	16632-R	TGGCAACAACAGTCTCCCAAAC	
MZ00017355	17355-F	AGGCTGGTAAGCAAGCACGGTT	Expression <i>Wri1a</i> -OE/WT
	17355-R	AGAAACTGTAGCGGCAGCGTCA	
MZ00042142	42142-F	TCTGGTGAACCTTGACCTGTGTTCTG	Expression <i>Wri1a</i> -OE/WT
	42142-R	ATTAGCTTGAAGCAGCGGCAC	
MZ00014741	14741-F	AGATCTCGAACAACCTGCGGCA	Expression <i>Wri1a</i> -OE/WT
	14741-R	AAGGCCAGGAATCGACTGGAA	
MZ00042163	42163-F	TGCCTGTTGTTTTCAGCAGAT	Expression <i>Wri1a</i> -OE/WT
	42163-R	ACCTTTCGGAGGCTATCCAA	
MZ00044044	44044-F2	GTTGCAAGGTGAAAGTCTGTGG	Expression <i>Wri1a</i> -OE/WT
	44044-R2	TGTGGCGACAATTACACGC	
MZ00040095	40095-F	AAGATCGGTGTTGCTGGTGTCTG	Expression <i>Wri1a</i> -OE/WT
	40095-R	CCGTTGAATTAAGAAGACAGTGGCAC	
MZ00026553	26553-F	AATAACTGTGGCAAGGCTGTTGG	Expression <i>Wri1a</i> -OE/WT
	26553-R	TTGCACCTCACTCCATTGTGGT	
MZ00024718	24718-F	ACCTGCTCGCTGGGAACAA	Expression <i>Wri1a</i> -OE/WT
	24718-R	GACAGCGCATGGACATGAT	
MZ00017663	17663-F	CAGCATCCTGTGGTGAACCTCA	Expression <i>Wri1a</i> -OE/WT
	17663-R	TTTGCTCGCGCCTTATCAACTAAC	
MZ00041636	41636-F	TCTGCTCCATTTTGCTTCTCGC	Expression <i>Wri1a</i> -OE/WT
	41636-R	AAACGCAGGTTATTGCATGTGC	
MZ00031529	31529-F	AAGCACTCTACATGGATGCTCGATC	Expression <i>Wri1a</i> -OE/WT
	31529-R	TACGTTTGTTCCTTGCCGACG	
MZ00024552	24552-F	GCACTGTCAGCGTGAACCTATGATGA	Expression <i>Wri1a</i> -OE/WT
	24552-R	CATTTAGGTGGCGCCTTGATTT	
MZ00016866	16866-F	TGAGGAGGCTTTCAAGATCACTG	Expression <i>Wri1a</i> -OE/WT
	16866-R	AAGTGATGCCCTGGTACAGCAC	
MZ00043050	43050-F	GGCAATTGATGGTGGCCTTATT	Expression <i>Wri1a</i> -OE/WT
	43050-R	CCAAACATACCCAGGTTGGACA	
MZ00017651	17651-F	AGGAGGAGGTGCGAATGCTTT	Expression <i>Wri1a</i> -OE/WT
	17651-R	TGTCGGTGCCCAATGTGTAGAC	
MZ00016943	16943-F	CGATGTAATCAACAAGGCGCTATC	Expression <i>Wri1a</i> -OE/WT
	16943-R	ATCTGCGGCTTCACTCAAACC	
MZ00039375	39375-F	CTGAAGATGCACTTTGAGGAGG	Expression <i>Wri1a</i> -OE/WT
	39375-R	GTTGTAGCCACTCCTTCCTGCT	
MZ00056535	Oxa-F1	CATGGTGTTCCCTGGATTTCCGC	Expression <i>Wri1a</i> -OE/WT
	Oxa-R1	GCAGCCGCATCAATAATCCGCTGA	
<i>Wri1a</i>	FT126-F7	GGGACAAGTTTGTACAAAAAAGCAGGCTTAGGGA CGGAGACCACGGACAT	Cloning <i>Wri1a</i> cDNA
	FT126-R7	GGGACCACCTTTGTACAAGAAAGCTGGGTCGACAC CTGGAAAGCGAAAACCTC	

<i>Wri1b</i>	attB1-Wri2	GGGGACAAGTTTGTACAAAAAAGCAGGCTCACGTT CATGCATGACCATGGA	Cloning <i>Wri1b</i> cDNA
	attB2-Wri2	GGGGACCACTTTGTACAAGAAAGCTGGGGGTCTCA GAATCAATTGCACAC	
<i>Napin S2</i>	HindIIIpAT2S2up	CCCAAGCTTGGGACGAATCATGTACAATTGTGG	Cloning <i>Napin</i> S2 promoter
	AscIpAT2S2low	TTGGCGCGCCAATTTTTTGCTATTTGTGTTTGTATTC	
<i>Wri1a</i>	FT126-F3	CCGAGGAGGTTTTCGCTTT	Expression profile A188
	FT126-R3	GCTAAGTGAGACAATGACAAACCC	
<i>Wri1b</i>	Wri2-new-F	AACCGCAGCACCAGCAGTCT	Expression profile A188
	Wri2-new-R	GCCCTCTTATTGGCCTTG	