Expression of ZmLEC1 and ZmWRI1 Increases Seed Oil Production in Maize[^W][^OA]

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Increasing seed oil production is a major goal for global agriculture to meet the strong demand for oil consumption by humans and for biodiesel production. Previous studies to increase oil synthesis in plants have focused mainly on manipulation of oil pathway genes. As an alternative to single-enzyme approaches, transcription factors provide an attractive solution for altering complex traits, with the caveat that transcription factors may face the challenge of undesirable pleiotropic effects. Here, we report that overexpression of maize (Zea mays) LEAFY COTYLEDON1 (ZmLEC1) increases seed oil by as much as 48% but reduces seed germination and leaf growth in maize. To uncouple oil increase from the undesirable agronomic traits, we identified a LEC1 downstream transcription factor, maize WRINKLED1 (ZmWRI1). Overexpression of ZmWRI1 results in an oil increase similar to overexpression of ZmLEC1 without affecting germination, seedling growth, or grain yield. These results emphasize the importance of field testing for developing a commercial high-oil product and highlight ZmWRI1 as a promising target for increasing oil production in crops.

Maize (Zea mays) grain is the most important feedstock for meat, egg, milk, and fuel production in the world. Approximately 65% of maize grain is used for feeding animals. High-oil maize shows a greater feed efficiency than normal-oil maize in animal feed trials because the caloric content of oil is 2.25 times greater than that of starch on a weight basis (Han et al., 1987; Perry, 1988). Maize oil is the most valuable coproduct from industrial processing of maize grain through wet milling or dry milling and is high-quality oil for human consumption. Compared with soybean (Glycine max) oil, which contains 6.8% linolenic acid (18:3) and is susceptible to oxidation, maize oil is stable because it contains very little (less than 1.0%) linolenic acid (Weber, 2003). With the rapid growth of human consumption and industrial use for biodiesel production, the demand for vegetable oil has increased significantly. Therefore, high oil content is a desirable trait for the maize end-users and becomes an important goal for genetic engineering.

Plant oil is synthesized from glycerol-3-phosphate and fatty acyl-CoA in the endoplasmic reticulum as triacylglycerols (TAGs). Fatty acids are synthesized from acetyl-CoA exclusively in the plastid and then transported to the cytoplasm in the form of fatty acyl-CoA (Ohlrogge and Browse, 1995). In the endoplasmic reticulum, TAGs are synthesized by the stepwise acylation of glycerol-3-phosphate, known as the Kennedy pathway. First, fatty acyl moieties are added to the sn-1 and sn-2 positions of glycerol-3-phosphate by glycerol-3-phosphate acyltransferase and lysophosphatic acid acyltransferase, respectively, to form phosphaticidic acid. Phosphaticidic acid is then hydrolyzed by phosphatidate phosphatase to yield diacylglycerol (DAG). DAG can be used to form TAGs or it can be used as a substrate for membrane lipid biosynthesis. Diacylglycerol acyltransferase (DGAT), the last enzyme for TAG synthesis, adds a third acyl chain to DAG and yields TAGs (Voelker and Kinney, 2001). An alternative pathway for TAG formation may also exist in plants. For example, phospholipid:diacylglycerol acyltransferase can transfer the sn-2 acyl chain from phosphatidylcholine to DAG and form lysophosphatidylcholine and TAG and has overlapping functions with DGAT for TAG synthesis in both seed and pollen in Arabidopsis (Arabidopsis thaliana; Zhang et al., 2009). Finally, TAGs are stored in seeds in specialized structures termed oil bodies.

Plant seed oil content is controlled by multiple steps in the oil biosynthetic pathway. Manipulation of single steps in the pathway often shows a moderate effect on seed oil content (Thelen and Ohlrogge, 2002; Durrett et al., 2008). For example, expression of a fungal DGAT...
in soybean results in an approximately 7.5% relative increase in seed oil content (Lardizabal et al., 2008). Transcription factors regulate multiple steps simultaneously, and they provide an attractive alternative to single-enzyme approaches for altering complex traits in crops (Broun, 2004, 2005; Grothewold, 2008). In Arabidopsis, LEAFY COTYLEDON1 (LEC1) and WRINKLED1 (WRI1) have been identified as two key transcription factors involved in the regulation of oil accumulation. Mutations in both genes lead to reduced oil content in seeds. LEC1 encodes a HAP3 subunit of the CCAAT-binding factor and plays an important role in Arabidopsis embryo development. Ectopic expression of Arabidopsis LEC1 leads to the formation of embryo-like structures containing oil and storage protein in leaves (Lotan et al., 1998). WRI1 encodes a transcription factor containing two AP2 domains and may play an important role in the regulation of carbon metabolism. Overexpression of WRI1 in Arabidopsis results in an increase in oil accumulation in seeds and leaves (Cernac and Benning, 2004). Expression profiling and genetic analyses also indicate that WRI1 functions downstream of LEC1 and is a key transcription factor controlling fatty acid biosynthesis (Baud et al., 2007; Mu et al., 2008; Santos-Mendoza et al., 2008; Maeo et al., 2009).

While these results are exciting, much of this has been done in Arabidopsis. The effect of transcription factors on seed oil production in major crops has not been determined. Furthermore, altered expression of transcription factors may show undesirable pleiotropic effects on plant growth and development in addition to oil increase. Rigorous field trials of transgenic events in elite, high-yielding commercial varieties at various locations and multiple environments are necessary to determine whether oil increase is associated with yield penalty or poor agronomic performance. Here, we report that overexpression of maize LEC1 (ZmLEC1) increases seed oil production but reduces seed germination and plant growth. To uncouple oil increase from undesirable changes in germination and growth, we identify maize WRI1 (ZmWRI1) as a transcription factor downstream of LEC1 and demonstrate that overexpression of ZmWRI1 increases seed oil content without the undesirable effects caused by ZmLEC1. The results presented highlight the potential application of transcription factors for increasing oil production in major crops.

RESULTS

Expression of ZmLEC1 in Transgenic Maize Plants

Previous studies indicated that overexpression of Arabidopsis LEC1 induced the formation of embryo-like structures and increased the expression of fatty acid pathway genes in Arabidopsis leaves (Lotan et al., 1998). We have identified a maize homolog that shares 41% identity with Arabidopsis LEC1 in amino acid sequence. ZmLEC1 is expressed specifically in early embryo development and is not expressed in endosperm, leaf, and root (Supplemental Fig. S1A). ZmLEC1 protein accumulated in embryos at 15 and 20 d after pollination (DAP) and diminished after 25 DAP (Supplemental Fig. S1B). Expression of ZmLEC1 under the control of a constitutive synthetic SCP1 promoter in Arabidopsis lec1 mutant plants can complement the lec1 mutant seed phenotype, indicating that ZmLEC1 is functionally equivalent to Arabidopsis LEC1 (Supplemental Fig. S2). To test whether alteration of seed development in maize can increase seed oil production, ZmLEC1 was expressed under two embryo-preferred promoters, a strong OLEOSIN (OLE) promoter and a weaker EARLY EMBRYO PROTEIN (EAP1) promoter. Each construct also contained a DS-RED2 marker gene driven by an aleurone-specific LIPID-TRANSFER PROTEIN2 promoter to facilitate the identification of transgenic and null seeds for phenotypic analysis. Transgenic seeds with red fluorescence can be separated from null seeds easily. Analysis of 15 transgenic maize lines expressing ZmLEC1 under the control of the EAP1 promoter revealed average increases in T1 seed oil content by 35% and in embryo oil concentration by 24% (Fig. 1A). Because maize seed oil content is determined by the amount of oil in the seed divided by seed weight and the amount of oil in the seed is determined by the oil concentration in the embryo, embryo size, and oil in the endosperm, we determined the effect of EAP1: ZmLEC1 on oil accumulation in endosperm and on embryo size. Endosperm oil was extracted by hexane and determined by the amount of oil divided by endosperm dry weight. ZmLEC1 endosperm contained 0.55% oil, which was not significantly different from null endosperm oil content (0.49%). Transgenic lines showed an average increase of 14.4% in embryo size compared with null, but only line 103.1.12 showed a significant increase as determined by Student’s t test (Supplemental Table S1). An increase in seed oil content by ZmLEC1 may be driven primarily by a higher embryo oil concentration and a small increase in embryo size. The T1 seeds were propagated to obtain T3 homozygous seeds. The high-oil trait was stable across three generations in different locations. T3 homozygous transgenic seeds showed a level of oil increase similar to that seen in the T1 generation. The best transgenic ZmLEC1 line (line 103.1.12) showed as much as 48.7% increase in seed oil content relative to its null (Fig. 1B). Detailed analysis, however, found that overexpression of ZmLEC1 reduced seed germination and leaf growth in addition to elevating oil content. The first and second leaves of transgenic ZmLEC1 plants were 40% to 50% shorter than those of the null plants and were narrow and dark green (Fig. 1D). In germination tests, root and shoot growth of transgenic ZmLEC1 seedlings were slower than their corresponding nulls (Fig. 1C), resulting in a poor early stand count and reduced plant height in the field. Expression of ZmLEC1 by the OLE promoter...
increased seed oil content similar to that by the EAP1 promoter, but these lines showed a more severe reduction in seed germination and leaf growth than lines expressing ZmLEC1 under the control of the EAP1 promoter (data not shown).

Identification and Expression of ZmWRI1 in Transgenic Maize Plants

It is not surprising that alteration of a master switch transcription factor such as LEC1 may lead to pleiotropic effects on seed metabolism, development, and seedling growth. We hypothesized that a transcription factor downstream of LEC1 might uncouple the high-oil phenotype from the negative effects on germination and growth. In Arabidopsis, WRI1 is another key transcription factor affecting seed oil accumulation (Cernac and Benning, 2004). We identified a maize WRI1 that showed 43% identity with AtWRI1 in the amino acid sequence and was up-regulated by approximately 2-fold in ZmLEC1-expressing embryos. ZmWRI1 showed an expression pattern similar to ZmLEC1, with peak expression in embryos at approximately 20 DAP and decreased expression after 25 DAP (Supplemental Fig. S3). In contrast to ZmLEC1, which expressed specifically in embryos, ZmWRI1 showed very weak expression in leaf, root, and stalk. The up-regulation of ZmWRI1 by ZmLEC1 was confirmed by coexpression of the ZmLEC1 protein and a ZmWRI1 promoter:GUS reporter in maize Black Mexican Sweet (BMS) culture cell. Coexpression of ZmLEC1 protein increased GUS activity significantly (Fig. 2), indicating that ZmLEC1 regulated the expression of ZmWRI1 directly or indirectly. Similar to ZmLEC1, expression of ZmWRI1 by the embryo-preferred OLE promoter increased T1 seed oil content by an average of 30.6% across 15 transgenic lines analyzed (Fig. 3A). In contrast to ZmLEC1-expressing lines, the embryo size of transgenic ZmWRI1 seeds was not significantly different from the nulls (Supplemental Table S2). Transgenic ZmWRI1 endosperm contained 0.81% oil, which was significantly higher than the 0.47% in null endosperm. The increase of seed oil by ZmWRI1 may be primarily due to higher embryo and endosperm oil concentrations. In endosperm, oil bodies were found in aleurone cells but not in starchy endosperm cells. To determine whether ZmWRI1 increases oil in starchy endosperm or in aleurone cells, ZmWRI1 was expressed in starchy endosperm under a maize 19 KD ZEIN promoter (Lappegard and Martino-Catt, 2001). Expression of ZmWRI1 under
the control of the 19 KD ZEIN promoter did not lead to an increase in seed oil content (Supplemental Fig. S4), suggesting that higher oil content in endosperm expressing ZmWRII under the control of the OLE promoter could be due to the expression of ZmWRII by the OLE promoter in the aleurone layer. Furthermore, we determined the protein and starch levels in the ZmWRII embryos to understand the source of the additional carbon needed for the biosynthesis of the increased embryo oil. Expression of ZmWRII did not affect protein content in the embryo but did reduce starch content by approximately 60% compared with nulls (Fig. 4), suggesting that ZmWRII may enhance oil biosynthesis by reducing carbon flux to starch biosynthesis in the embryo. The high-oil trait was stable in three genetic backgrounds at three locations. T3 homozygous transgenic seeds showed an increase in oil similar to the T1 generation, with a 46% increase in the best line (line 25.2.1; Fig. 3B). To determine whether oil quality was affected by ZmWRII expression, we analyzed major fatty acid composition in seed oil. There were no significant changes in fatty acid composition between mature transgenic ZmWRII seeds and their corresponding null seeds (Supplemental Table S3). In contrast to ZmLEC1-expressing lines, transgenic ZmWRII seeds germinated normally compared with null seeds (Fig. 3C). Transgenic ZmWRII plants did not show any significant growth differences from the null plants in the length of the first and second leaves (Fig. 3D). Expression of ZmWRII by the weaker EAP1 promoter also increased T1 seed oil content, but to a lesser extent, averaging 16.9% increase in the top 15 transgenic lines (data not shown).

Figure 3. Transgenic overexpression of ZmWRII in maize. A, Effects of overexpression of ZmWRII under the control of the OLE promoter on embryo oil concentration and seed oil content. Each point represents an average of 10 null seeds (white triangles) and 10 transgenic seeds (black triangles) from each transgenic line. A total of 15 transgenic lines were analyzed. B, Seed oil content of T3 homozygous transgenic lines (black bars) and their corresponding null segregants (white bars). For each transgenic line, 10 seeds per ear, eight homozygous transgenic or null ears, were analyzed. Data shown are means ± sd. All five transgenic lines showed a significant increase in seed oil content compared with null segregants as determined by Student’s t test (P < 0.01). C, Warm germination test of transgenic seeds. Transgenic and null seeds were placed between two sheets of filter paper and germinated at 25°C for 5 d. D, Transgenic and null plants at the three- to four-leaf stage. Seeds were planted in soil mixture and grown in the greenhouse. The photograph shows a typical line 7 d after planting.

Figure 4. Protein and starch contents of ZmWRII transgenic embryos. The data represent means ± sd of three replicate samples. Each sample was run in triplicate in starch and protein assays. All three transgenic lines showed no significant difference in embryo protein content (P > 0.1 by Student’s t test) but did show a significant reduction in embryo starch content as determined by Student’s t test (P < 0.05).
Field Test of Transgenic ZmWRI1 Plants

To determine whether the expression of OLE: ZmWRI1 in transgenic lines affects agronomic traits such as early stand count and plant height, five transgenic lines were field tested with six rows of transgenic plants and three rows of null plants for each line planted side by side. None of the five transgenic lines was significantly different from nulls in early stand count and plant height at maturity in the field (Fig. 5). To determine further if the high oil content in transgenic ZmWRI1 grain affects hybrid yield, the EAP1: ZmWRI1 construct was retransformed into an inbred line, PHWWE, and was outcrossed to a male tester line (PH1B5) to produce F1 hybrid for field yield tests. Hybrids from five transgenic ZmWRI1 lines with grain oil increase from 10% to 22% and their corresponding null lines were tested in eight locations in the United States maize belt with three repeats for each line at each location. All five transgenic ZmWRI1 lines showed no significant difference from their corresponding nulls in grain yield (Fig. 6). The average yield of the five transgenic ZmWRI1 lines was 9.45 tons ha$^{-1}$, which was not significantly different from the average null yield of 9.55 tons ha$^{-1}$ (Fig. 6). In addition, we did not observe any significant differences between transgenic lines and null lines in early stand count, seedling vigor, flowering time, grain moisture, grain test weight, or plant height.

DISCUSSION

Relative to a single-enzyme approach, transcription factors provide an attractive solution for increasing plant oil production (Broun, 2004; Grotewold, 2008). However, possible pleiotropic effects of transcription factors are a key challenge for using them in a commercial product (Century et al., 2008). For example, knockout of a homeobox gene, GLABRA2, increased seed oil content in Arabidopsis, but GLABRA2 also affected seed coat, trichome, and root hair development (Shen et al., 2006). LEC1 encodes a CCAAT-binding transcription factor that is critical for seed development. Mutation of Arabidopsis LEC1 resulted in desiccation-intolerant seeds with reduced oil content. Ectopic expression of AtLEC1 in Arabidopsis led to the formation of embryo-like structures that accumulate oil and seed storage proteins (Lotan et al., 1998) and up-regulation of the fatty acid biosynthetic pathway (Mu et al., 2008). We have identified a maize LEC1 gene with 41% identity to Arabidopsis LEC1 and have demonstrated that overexpression of maize LEC1 increased seed oil content by as much as 48.7%. However, transgenic seeds germinated poorly, and plants showed stunted growth with dark green, narrow leaves. Construct optimization with different promoters giving different expression levels and tissue specificities reduced the undesirable phenotypes but was unable to eliminate them. To uncouple the high-oil phenotype from undesirable agronomic traits, such as poor germination and plant growth, we have identified a downstream transcription factor, ZmWRI1, that appears to be more specific for oil biosynthesis. Expression of ZmWRI1 increased seed oil content by as much as 46% but did not affect seed germination and plant growth. Our work demonstrates that it is possible to uncouple a desired trait from unwanted side effects by identifying a downstream transcription factor that is more specific for the trait. Assuming that the grain yield of the transgenic line is equal to current commercial hybrids, an average 25% increase in maize seed oil content will add an additional 87.5 kg of oil or $70 per ha based on a current yield of 10 tons ha$^{-1}$, 3.5% kernel oil content, and an oil price of $0.80 kg$^{-1}$. If U.S. farmers plant all their approximately 35 million ha with high-oil maize, then an additional approximately 3.0 million tons of oil will be produced.

Maize WRII is a transcription factor containing two AP2 domains and showing 43% identity to Arabidopsis WRII in amino acid sequence. In Arabidopsis, WRII is involved in the regulation of seed storage accumulation. Mutation in the WRII gene causes reduction of oil accumulation and wrinkled seeds. Ectopic expression of AhWRII results in abnormal seedlings with the accumulation of oil in the presence of Glc in the growth medium (Cernac and Benning, 2004). Molecular and genetic analyses identified WRII as a target of LEC2 (Baud et al., 2007). Function of WRII is necessary for LEC2 or LEC1 in the regulation
of fatty acid biosynthesis (Baud et al., 2007; Mu et al., 2008). Because LEC2 expression is not affected by overexpression of LEC1, it is less likely that LEC1 regulates WRII1 through LEC2. AtWRII1 functions downstream of LEC1 and LEC2, possibly in two parallel pathways (Mu et al., 2008; Santos-Mendoza et al., 2008). Gene expression profiling and quantitative reverse transcription-PCR experiments have identified a few putative targets of AtWRII1, including genes in late glycolysis and the fatty acid synthetic pathway, such as pyruvate kinase, pyruvate dehydrogenase, acetyl-CoA carboxylase BCCP2 subunit, and enoyl-ACP reductase (Baud et al., 2007). It was confirmed recently that AtWRII1 protein binds to a conserved AW-box sequence identified from the promoter region of genes involved in fatty acid synthesis (Maéo et al., 2009). Those results suggest that WRII1 is a key transcription factor that regulates glycolysis and fatty acid pathways directly in Arabidopsis. In maize, we found a similar regulatory network on oil biosynthesis. ZmWRII1 is a key transcription factor controlling the expression of glycolysis and fatty acid pathway genes (B. Shen, W.B. Allen, P. Zheng, and C. Li, unpublished data) and is regulated by ZmLEC1. Unlike Arabidopsis, an ortholog of AtLEC2 was not identified in maize. It is not clear whether the LEC2 pathway is present in maize. Overexpression of ZmWRII1 does not affect embryo protein content but reduces embryo starch content by approximately 60%, suggesting that ZmWRII1 may regulate carbon flux between starch and oil biosynthesis in the embryo. It needs to be determined whether ZmWRII1 regulates starch and oil pathway genes directly or indirectly. Furthermore, overexpression of WRII1 in Arabidopsis and maize embryos up-regulated key glycolytic pathway genes, such as pyruvate kinase and pyruvate dehydrogenase, suggesting that ZmWRII1 may enhance sugar metabolism to support carbon needed for additional oil biosynthesis.

Most of maize seed oil is located in the embryo. Seed oil content is thus primarily determined by the oil concentration of the embryo and embryo size (Weber, 2003). Maize endosperm consists of a central mass of starchy endosperm cells, a single layer of aleurone cells surrounding the starchy endosperm, and a basal layer of transfer cells (Olsen, 2001). Only aleurone cells accumulate oil, while starchy endosperm cells do not. Because starch endosperm accounts for 80% to 90% of seed mass, conversion of starch to oil in starchy endosperm cells will increase seed oil content dramatically and potentially make maize a C4 oil crop. We expressed ZmWRII1 under the control of the 19 KD ZEIN promoter to promote oil biosynthesis in endosperm and did not detect a significant increase in seed oil content (Supplemental Fig. S4). Overexpression of ZmWRII1 in embryo up-regulated multiple genes in fatty acid biosynthesis, but not the genes involved in oil biosynthesis, such as glycerol-3-phosphate acyltransferase, DGAT, and oleosin (B. Shen, W.B. Allen, P. Zheng, and C. Li, unpublished data). Failure of ZmWRII1 to increase oil in starchy endosperm could be due to a lack of expression of genes involved in oil biosynthesis and oil body formation. Interestingly, long-term recurrent selection for high oil in maize resulted in high-oil lines with as much as 22% kernel oil content but did not increase oil content in starchy endosperm (Lambert et al., 2004). High oil content resulted from higher oil concentration in embryo, larger embryo, smaller seed, and more oil in aleurone cell. In contrast, recurrent selection for high oil in oat (Avena sativa) led to oil accumulation in starchy endosperm (Peterson and Wood, 1997). It needs to be determined why oat and maize respond differently in endosperm to recurrent selection for high oil. Expression of WRII1 may be able to increase oil in oat endosperm, while maize is selected for starch accumulation and is not competent for oil biosynthesis in endosperm.

Development of high-oil maize has been a breeding goal for many years. Alexander et al. started a high-oil breeding program using recurrent selection in synthetic in 1956 and has developed the ASK high-oil population with grain oil content as high as 22% (Lambert et al., 2004). However, commercialization of high-oil maize has not been successful, mainly because of significant grain yield reduction and poor agronomic traits associated with high-oil germplasm. It is not known if grain yield reduction is caused by high oil content directly due to the high energy input for oil biosynthesis or rather by genetic linkage drag of old nonelite germplasm. Maize seed oil content is a complex trait affected by multiple quantitative trait loci (Berke and Rocheford, 1995; Clark et al., 2006). One major oil quantitative trait locus on chromosome 6 (qHO6) affecting seed oil content and oleic acid content
was cloned recently (Zheng et al., 2008). qHO6 encodes DGAT1-2, which catalyzes the final step of oil biosynthesis. Overexpression of the unique high-oil DGAT1-2 allele increased seed oil content by up to 41%. With the qHO6 cloned, a rigorous field yield test of DGAT1-2 transgenic plants should be able to answer whether high oil content in grain affects grain yield or not. Interestingly, transgenic expression of a fungal DGAT2 resulted in a relative increase in oil of 7.5% in soybean with no significant difference from the control in seed yield and other agronomic traits at multiple location field trials (Lardizabal et al., 2008). Overexpression of BnDGAT1 in canola (Brassica napus) increased seed oil content by approximately 13% in the greenhouse, but the increase in oil dropped to approximately 3% under field conditions. The effect on seed yield and other agronomic traits was not determined (Weselake et al., 2008). These results highlight DGAT as a promising target for increasing oil content in important crops. The work reported here has identified another promising target, ZmWRII, for increasing oil content in crops. Transgenic expression of ZmWRII in maize increases seed oil content with no significant difference from controls in agronomic traits and grain yield, suggesting that modest high oil content in seeds does not result in yield loss. A higher resolution yield trial with more replicates and locations, however, is needed to determine whether ZmWRII transgenic plants have impacted yield at the one to three bushels per acre level. Because ZmWRII primarily affects glycolysis and fatty acid pathways and not DGAT expression, stacking ZmWRII with DGAT should up-regulate the whole pathway from fatty acid biosynthesis to oil biosynthesis and should increase seed oil content more than either single gene can do. The combination of the two most promising high-oil genes may provide the best opportunity for future commercial high-oil transgenic crops.

MATERIALS AND METHODS

Vector Construction and Maize Transformation

Maize (Zea mays) LEC1 (GenBank accession no. AF410176) and WRII (GenBank accession no. AY103852) were cloned behind the EAP1 promoter (Abbitt et al., 2006), the 19 KD ZEIN promoter (Lappegard and Martinez-Catt, 2001), or the OLE promoter and before an EAP1 terminator or NOS terminator. The OLE promoter is from the 16 KD OLE gene of maize (GenBank accession no. BD 23503, including the 81-bp 5’ untranslated region of OLE [U13701]). The DS-RED2 gene encodes a variant of original red fluorescent protein from Clontech. Vector construction and maize transformation were conducted as described previously (Zheng et al., 2008).

Oil Analysis

Maize seed oil content was determined as described previously (Zheng et al., 2008). For embryo oil and weight, seeds were soaked in water overnight at room temperature. Embryos were then dissected from their endosperm and lyophilized. Embryo oil content was determined by NMR. Because oil content in endosperm is too low to be detected by NMR, the hexane extraction method was used to determine endosperm oil content. Endosperm oil was extracted by hexane from 5 to 10 g of endosperm meal. Hexane supernatant was transferred to a preweighed aluminum boat after being centrifuged at 20,000 rpm for 5 min. The hexane was evaporated in the hood, and the boat was baked at 100°C for 5 min. After cooling down to room temperature, the boat was weighed. Endosperm oil content was calculated by the amount of oil in the boat divided by the endosperm dry weight.

Warm Germination Test

Seeds were placed in a row on a piece of wet filter paper, covered by another piece of wet filter paper, rolled up, then wrapped with a piece of waxed paper and placed in a large beaker with 1 inch of water at the bottom. The beaker was placed in a growth chamber at 25°C. Seed germination was evaluated 5 d after seeding.

Coexpression of LEC1 with ZmWRII Promoter:GUS

Maize WRII promoter (796 bp from the start codon) was cloned from the B73 inbred line by PCR and linked to the reporter gene GUS. To confirm that ZmLEC1 regulates ZmWRII expression in vivo, maize BMS cells were transformed by a construct containing LEC1 driven by the maize ubiquitin promoter and ZmWRII promoter:GUS reporter or a control construct with ZmWRII:GUS alone. Expression of GUS from the ZmWRII promoter was monitored 24, 48, 72, 96, and 120 h after transformation. BMS cell culture, transformation by Agrobacterium tumefaciens, and GUS staining procedures were described in detail previously (Gao et al., 2004).

Protein and Starch Measurement in Embryo

Maize embryos were manually separated from endosperms and ground into a fine meal for starch and protein analysis. Maize embryo protein content was determined by combustion using a total combustion nitrogen/protein analyzer (Flash EA 1112 Series) manufactured by Thermo Electron Corpora- tion, and protein was determined by multiplying the nitrogen concentration by 6.25. Embryo starch content was determined as described previously (Seebauer et al., 2010). Approximately 100 mg of embryo meal was digested in 0.9 mL of MOPS buffer (50 mM MOPS, pH 7.0, 5 mM CaCl2, and 0.02% Na- azide) containing 100 units of heat-stable a-amylase (A-4551; Sigma-Aldrich). Following incubation at 90°C for 75 min, 0.6 mL of acetate buffer (285 mM Na-acetate, pH 4.5, and 0.02% Na-azide) containing 5 units of amyloglucosidase (catalogue no. 1120236701; Roche Applied Science) was added. Reactions were held at 55°C overnight, stopped by boiling, and centrifuged (14,000g) for 5 min. Glc concentration was determined by hexokinase/Glc-6-P dehydrogenase reactions. A minimum of duplicate digests were processed for all samples, and the results were corrected for moisture content.

Field Yield Trial

ZmWRII was transformed into an inbred line, PHW3E. Five homozygous ZmWRII transgenic inbred lines and their corresponding null segregants were crossed onto a proprietary tester (PH1B5) to evaluate the agronomic performance associated with the gene. Agronomic field trials were conducted as two row plots planted and thinned to a density of 30,000 plants per acre. The experiment was designed as a random complete block nested by hybrid tester genotype. Entries were randomized within nests. Yield, harvest moisture, test weight, and plant and ear height were tested in eight Midwest maize belt locations (Johnston, IA; Marion, IA; Maconh, IL; Princeton, IL; Champaign, IL; Windsor, IN; York, NE; and Janeville, WI) with three replicates in each location. Statistical analyses were run using Student’s t test.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AF410176 and AY103852.

Supplemental Data

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

Supplemental Figure S1. Expression of ZmLEC1.

Supplemental Figure S2. Complementation of Arabidopsis lec1 mutant seed with maize LEC1.

Supplemental Figure S3. Massively parallel signature sequencing expression profiles of ZmWRII, plotted in parts per million, of transcript levels in different tissues.
Supplemental Figure S4. Overexpression of ZmWRII in maize endosperm does not increase seed oil content.

Supplemental Table S1. Effects of ZmLEC1 on embryo weight, seed weight, embryo oil content, and seed oil content in maize.

Supplemental Table S2. Effects of ZmWRII on embryo weight, seed weight, embryo oil content, and seed oil content in maize.

Supplemental Table S3. Fatty acid composition of seed oil from OLE: ZmWRII and null seeds.

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