Running title: ZmLEC1 and ZmWRI1 Increase Oil in Maize

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Expression of ZmLEC1 and ZmWRII Increases Seed Oil Production in Maize

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ABSTRACT

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 over-expression of maize LEAFY COTYLEDON 1 (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 down-stream transcription factor, maize WRINKLED1 (ZmWRI1). Over-expression of ZmWRI1 results in an oil increase similar to over-expression 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.
INTRODUCTION

Maize 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 co-product from industrial processing of maize grain through wet milling or dry milling and is high quality oil for human consumption. Compared to soybean oil which contains 6.8% linolenic acid (18:3) and is susceptible to oxidation, maize oil is stable because it contains very little (<1.0%) linolenic acid (Weber, 2003). With the rapid growth of human consumption and industrial use for biodiesel production, the demanding 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 lyso-phosphatidic acid acyltransferase, respectively, to form phosphatidic acid. Phosphatidic 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. An alternative pathway for TAG formation may also exist in plants. For example, phospholipid:diacylglycerol acyltransferase (PDAT) can transfer the sn-2 acyl chain from phosphatidylcholine (PC) to DAG and form lyso-PC and TAG, and has overlapping functions with DGAT for TAG synthesis in both seed and pollen in
Arabidopsis (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 diacylglycerol acyltransferase in soybean results in an approximate 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; Broun, 2005; Grotewold, 2008). In Arabidopsis, LEAFY COTYLEDON 1 (LEC1) and WRINKLED 1 (WRI1) have been identified as two key transcription factors involved in 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). WRII encodes a transcription factor containing two AP2 domains and may play an important role in regulation of carbon metabolism. Over-expression of WRII 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 WRII 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 genes 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 over-expression of
maize \textit{LEAFY COTYLEDON 1} (\textit{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 \textit{WRINKLED1} (\textit{ZmWRI1}) as a transcription factor down-stream of \textit{LEC1} and demonstrate that over-expression of \textit{ZmWRI1} increases seed oil content without the undesirable effects caused by \textit{ZmLEC1}. The results presented highlight the potential application of transcription factors for increasing oil production in major crops.

\section*{RESULTS}

\subsection*{Expression of \textit{ZmLEC1} in Transgenic Maize Plants}

Previous studies indicated that over-expression of \textit{Arabidopsis LEC1} induced the formation of embryo-like structures and increased expression of fatty acid pathway genes in \textit{Arabidopsis} leaves (Lotan et al., 1998). We have identified a maize homolog that shares 41\% identity to \textit{Arabidopsis LEC1} in amino acid sequence. \textit{ZmLEC1} is expressed specifically in early embryo development and is not expressed in endosperm, leaf, and root (Supplemental Fig. S1A). \textit{ZmLEC1} protein accumulated in embryos at 15 and 20 days after pollination (DAP) and diminished after 25 DAP (Supplemental Fig. S1B).

Expression of \textit{ZmLEC1} under a constitutive synthetic SCP1 promoter in \textit{Arabidopsis lec1} mutant plants can complement the \textit{lec1} mutant seed phenotype, indicating \textit{ZmLEC1} is functionally equivalent to \textit{Arabidopsis LEC1} (Supplemental Fig. S2). To test whether alteration of seed development in maize can increase seed oil production, \textit{ZmLEC1} was expressed under two embryo-preferred promoters, a strong \textit{OLEOSIN} promoter (\textit{OLE pro}) and a weaker \textit{EARLY EMBRYO PROTEIN} promoter (\textit{EAP1 pro}). Each construct also contained a \textit{DS-RED2} marker gene driven by an aleurone-specific \textit{LIPID-TRANSFER PROTEIN 2} (\textit{LTP2}) promoter to facilitate 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 \textit{ZmLEC1} under the \textit{EAP1} promoter revealed average increases in \textit{T1} seed oil content by 35\% and embryo oil concentration by 24\% (Figure 1A). Because maize seed oil content is determined by the amount of oil in seed divided by seed weight and the amount of oil
in seed is determined by the oil concentration in embryo, embryo size and oil in endosperm, we determined the effect of \textit{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. \textit{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 to null but only line 103.1.12 showed a significant increase as determined by Student’s \textit{t}-test (Supplemental Table S1). An increase in seed oil content by \textit{ZmLEC1} may be driven primary by a higher embryo oil concentration and a small increase in embryo size. The T\textsubscript{1} seeds were propagated to obtain T\textsubscript{3} homozygous seeds. The high oil trait was stable across three generations in different locations. T\textsubscript{3} homozygous transgenic seeds showed a level of oil increase similar to that seen in the T\textsubscript{1} generation. The best transgenic \textit{ZmLEC1} line (line 103.1.12) showed as much as 48.7\% increase in seed oil content relative to its null (Figure 1B). Detailed analysis, however, found that over-expression of \textit{ZmLEC1} reduced seed germination and leaf growth in addition to elevating oil content. The first and second leaves of transgenic \textit{ZmLEC1} plants were 40-50\% shorter than those of the null plants and were narrow and dark green (Figure 1D). In germination tests, root and shoot growth of transgenic \textit{ZmLEC1} seedlings were slower than their corresponding nulls (Figure 1C), resulting in a poor early stand count and reduced plant height in the field. Expression of \textit{ZmLEC1} by the \textit{OLE} promoter increased seed oil content similar to that by the \textit{EAP1} promoter, but these lines showed a more severe reduction in seed germination and leaf growth than lines expressing \textit{ZmLEC1} under the \textit{EAP1} promoter (data not shown).

\textbf{Identification and Expression of Zm\textit{WRI1} in Transgenic Maize Plants}

It is not surprising that alteration of a master switch transcription factor such as \textit{LEC1} may lead to pleiotropic effects on seed metabolism, development, and seedling growth. We hypothesized that a transcription factor down-stream of \textit{LEC1} might uncouple the high oil phenotype from the negative effects on germination and growth. In \textit{Arabidopsis}, \textit{WRI1} is another key transcription factor affecting seed oil accumulation (Cernac and
Benning 2004). We identified a maize WRII which showed 43% identity with AtWRII in the amino acid sequence and was up-regulated by ~2 fold in ZmLEC1-expressing embryos. ZmWRII showed an expression pattern similar to ZmLEC1 with a peak expression in embryos at ~20 DAP and decreased expression after 25 DAP (Supplemental Fig. S3). In contrast to ZmLEC1, which expressed specifically in embryos, ZmWRII showed very weak expression in leaf, root and stalk. The up-regulation of ZmWRII by ZmLEC1 was confirmed by co-expression of the ZmLEC1 protein and a ZmWRII promoter:GUS reporter in maize culture BMS cell. Co-expression of ZmLEC1 protein increased GUS activity significantly (Figure 2), indicating that ZmLEC1 regulated expression of ZmWRII directly or indirectly. Similar to ZmLEC1, expression of ZmWRII by the embryo-preferred OLE promoter increased T1 seed oil content by an average of 30.6% across 15 transgenic lines analyzed (Figure 3A). In contrast to ZmLEC1-expressing lines, the embryo size of transgenic ZmWRII seeds was not significantly different from the nulls (Supplemental Table S2). Transgenic ZmWRII endosperm contained 0.81% oil, which was significantly higher than 0.47% in null endosperm. The increase of seed oil by ZmWRII may be primarily due to higher embryo and endosperm oil concentration. In endosperm, oil bodies were found in aleurone cells but not in starchy endosperm cells. To determine whether ZmWRII increases oil in starchy endosperm or in aleurone cells, ZmWRII was expressed in starch endosperm under a maize 19 KD ZEIN promoter (Lappegard and Martino-Catt, 2001). Expression of ZmWRII under 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 OLE promoter could be due to expressions 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 reduced starch content by ~60% compared to nulls (Figure 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) (Figure 3B). To determine whether oil
quality was affected by \textit{ZmWRII} expression, we analyzed major fatty acid composition in seed oil. There were no significant changes in fatty acid composition between mature transgenic \textit{ZmWRII} seeds and their corresponding null seeds (Supplemental Table S3). In contrast to \textit{ZmLEC1}-expressing lines, transgenic \textit{ZmWRII} seeds germinated normally compared to null seeds (Figure 3C). Transgenic \textit{ZmWRII} plants did not show any significant growth differences from the null plants in the length of the first and second leaves (Figure 3D). Expression of \textit{ZmWRII} by the weaker \textit{EAPI} promoter also increased T\textsubscript{1} seed oil content, but to a lesser extent, averaging 16.9\% increase in the top 15 transgenic lines (data not shown).

\textbf{Field Test of Transgenic \textit{ZmWRII} Plants}

To determine whether expression of \textit{OLE:ZmWRII} 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 (Figure 5). To determine further if high oil content in transgenic \textit{ZmWRII} grain affects hybrid yield, the \textit{EAP1:ZmWRII} construct was re-transformed into an inbred line, PHWWE, and was out-crossed to a male tester line (PH1B5) to produce F\textsubscript{1} hybrid for field yield tests. Hybrids from 5 transgenic \textit{ZmWRII} lines with grain oil increase from 10\% to 22\% and their corresponding null lines were tested in 8 locations in the United States maize belt with 3 repeats for each line at each location. All 5 transgenic \textit{ZmWRII} lines showed no significant difference from their corresponding nulls in grain yield (Figure 6). The average yield of the 5 transgenic \textit{ZmWRII} lines was 9.45 tonne /ha, which was not significantly different from the average null yield of 9.55 tonne/ha (Figure 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.

\textbf{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 formation of embryo-like structures which 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 the Arabidopsis LEC1 and have demonstrated that over-expression 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 down-stream transcription factor, ZmWRI1, which 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 down-stream transcription factor that is more specific for the trait. Assuming the grain yield of the transgenic line is equal to current commercial hybrids, an average 25% increase in maize seed oil content will add additional 87.5 kg oil or $70 per hectare based on a current yield of 10 tonne/ha, 3.5% kernel oil content, and an oil price of $0.80/Kg. If US farmers plant all their ~35 million hectares with high oil maize, then an additional ~3.0 million tonnes of oil will be produced.

Maize WRI1 is a transcription factor containing two AP2 domains and showing 43% identity to Arabidopsis WRI 1 in amino acid sequence. In Arabidopsis, WRI 1 is involved
in regulation of seed storage accumulation. Mutation in the \textit{WRII} gene causes reduction of oil accumulation and wrinkled seeds. Ectopic expression of \textit{AtWRII} results in abnormal seedlings with accumulation of oil in the presence of glucose in the growth medium (Cernac and Benning, 2004). Molecular and genetic analyses identified \textit{WRII} as a target of \textit{LEC2} (Baud et al., 2007). Function of \textit{WRII} is necessary for \textit{LEC2} or \textit{LEC1} in regulation of fatty acid biosynthesis (Baud et al., 2007; Mu et al., 2008). Because \textit{LEC2} expression is not affected by over-expression of \textit{LEC1}, it is less likely that \textit{LEC1} regulates \textit{WRII} through \textit{LEC2}. \textit{AtWRII} functions downstream of \textit{LEC1} and \textit{LEC2}, possibly in two parallel pathways (Mu et al., 2008; Santos-Mendoza et al., 2008). Gene expression profiling and quantitative RT-PCR experiments have identified a few putative targets of \textit{AtWRII} including genes in late glycolysis and 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 \textit{AtWRII} protein binds to a conserved AW-box sequence identified from the promoter region of genes involved in fatty acid synthesis (Maeo et al. 2009). Those results suggest that \textit{WRII} is a key transcription factor that regulates glycolysis and fatty acid pathways directly in \textit{Arabidopsis}. In maize, we found a similar regulatory network on oil biosynthesis. \textit{ZmWRII} is a key transcription factor controlling expression of glycolysis and fatty acid pathway genes (Shen et al., unpublished data) and is regulated by \textit{ZmLEC1}. Unlike \textit{Arabidopsis}, an ortholog of \textit{AtLEC2} was not identified in maize. It is not clear whether the \textit{LEC2} pathway is present in maize. Over-expression of \textit{ZmWRII} does not affect embryo protein content but reduces embryo starch content by \textit{~60\%}, suggesting that \textit{ZmWRII} may regulate carbon flux between starch and oil biosynthesis in the embryo. It needs to be determined whether \textit{ZmWRII} regulates starch and oil pathway genes directly or indirectly. Furthermore, over-expression of \textit{WRII} in \textit{Arabidopsis} and maize embryo up-regulated key glycolytic pathway genes, such as pyruvate kinase and pyruvate dehydrogenase, suggesting that \textit{ZmWRII} 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 starch endosperm cells do not. Because starch endosperm accounts for 80-90% of seed mass, conversion of starch to oil in starchy endosperm cells will increase seed oil content dramatically and make maize a C4 oil crop potentially. We expressed ZmWRII under 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 ZmWRII 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 (Shen et al., unpublished data). Failure of ZmWRII to increase oil in starchy endosperm could be due to 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 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 WRII 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 synthetics 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 non-elite germplasm. Maize seed oil content is a complex trait affected by multiple QTLs (Berke and Rocheford, 1995; Clark et al., 2006). One major oil QTL on
chromosome 6 (qHO6) affecting seed oil content and oleic acid content was cloned recently (Zheng et al., 2008). qHO6 encodes a diacylglycerol acyltransferase (DGAT1-2) which catalyzes the final step of oil biosynthesis. Over-expression 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 control in seed yield and other agronomic traits at multiple location field trials (Lardizabal et al., 2008). Over-expression of BnDGAT1 in canola increased seed oil content by ~13% in the greenhouse but the increase in oil dropped to ~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, ZmWRI1, for increasing oil content in crops. Transgenic expression of ZmWRI1 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 ZmWRI1 transgenic plants have impacted yield at the 1-3 bushels/acre level. Because ZmWRI1 primarily affects glycolysis and fatty acid pathways and not DGAT expression, stacking ZmWRI1 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.

MATERIAL AND METHODS

Vector Construction and Maize Transformation. Maize LEC1 (Genbank #AF410176) and WRI1 (GenBank #AY103852) were cloned behind the EAP1 promoter (Abbitt et al., 2006), 19 KD Zein promoter (Lappegard and Martino-Catt, 2001) or OLE promoter and before an EAP1 terminator or NOS terminator. OLE promoter is from the 16-Kda
OLEOSIN gene of maize (GenBank no. BD 235503, including the 81-bp 5’untranslated region of OLEOSIN, U13701). The DS-RED2 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 5-10 g endosperm meal. Hexane supernatant was transferred to a pre-weighed 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 boat divided by 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 days after seeding.

Co-expression of LEC1 with ZmWRII promoter::GUS. Maize WRII promoter (796-bp from start codon) was cloned from B73 inbred 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 maize ubiquitin promoter and ZmWRII PRO::GUS reporter or a control construct with ZmWRII::GUS alone. Expression of GUS from ZmWRII promoter was monitored 24, 48, 72, 96 and 120 hours after transformation. BMS cell culture, transformation by Agrobacterium and GUS staining procedures were described in details 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 N/protein analyzer (Model Flash EA 1112 Series) manufactured by Thermoelectron Corporation and protein was determined by multiplying the N concentration by 6.25. Embryo starch content was determined as described previously (Seebauer et al., 2009). Approximately 100 mg embryo meal was digested in 0.9 ml of MOPS buffer (50 mM MOPS, pH 7.0, 5 mM CaCl₂, 0.02% Na-azide) containing 100 units of heat stable α-amylase (A-4551; Sigma-Aldrich, St Louis, MO). Following incubation at 90 °C for 75 min, 0.6 ml of acetate buffer (285 mM Na-acetate, pH 4.5, 0.02% Na-azide) containing 5 units of amyloglucosidase (catalogue number 11202367001; Roche Applied Science, Indianapolis, IN) was added. Reactions were held at 55 °C overnight, stopped by boiling, and centrifuged (14,000 g) for 5 min. Glucose concentration was determined by hexokinase/glucose-6-phosphate dehydrogenase reactions. A minimum of duplicate digests were processed for all samples and the results were corrected for moisture content.

Field Yield Trial. ZmWRI1 was transformed into an inbred line, PHWWE. Five homozygous ZmWRI1 transgenic inbred lines and their corresponding null segregates 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 RCB nested by hybrid tester genotype. Entries were randomized within nests. Yield, harvest moisture, test weight, along with plant and ear height were tested in 8 mid-west maize belt locations (Johnston, IA; Marion, IA; Macomb, IL; Princeton, IL; Champaign, IL; Windfall, IN; York, NE; and Janeville, WI) with 3 replicates in each location. Statistical analyses were run using Student’s t-test.

Supplemental Data

The following materials are available in the online version of this article.
Supplemental Table S1. Effect of ZmLEC1 on embryo weight, seed weight, embryo oil content, and seed oil content in maize.

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

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

Supplemental Fig. S1. Expression of ZmLEC1.

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

Supplemental Fig. S3. Massively Parallel Signature Sequencing (MPSS) expression profiles of ZmWRII plotted in parts per million of transcript levels in different tissues.

Supplemental Fig. S4. Over-expression of ZmWRII in maize endosperm does not increase seed oil content.
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Figure legends

Figure 1. Transgenic over-expression of ZmLEC1 in maize. A, Effects of over-expression of ZmLEC1 under EAP1 promoter on embryo oil concentration and seed oil content. Each point represents an average of null seeds (open triangle) and transgenic seeds (closed triangle) from each transgenic line. Total 15 transgenic lines were analyzed. B, Seed oil content of T3 homozygous transgenic lines (closed) and its null segregates (open). For each line, 10 seeds per ear, 5 homozygous transgenic or null ears were analyzed. Data were shown as mean ± SD. All 3 transgenic lines showed a significant increase in seed oil content compared to their corresponding null segregates 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 days. D, Transgenic and null plants at 3-4 leaf stage. Seed was planted in soil mixture and grown in greenhouse. Picture showed a typical line seven days after planting.

Figure 2. ZmLEC1 activates expression of GUS driven by a maize WRI1 promoter in maize BMS culture cells. ZmWRI1 Pro::GUS reporter or ZmWRI1 pro::GUS reporter/ Ubi Pro:ZmLEC1 was introduced into maize BMS culture cells via Agrobacteria transformation. Expression of GUS from ZmWRI1 promoter was monitored 24, 48, 72, 96 and 120 hours after transformation. Picture showed GUS staining of culture cells after 120 hours post transformation.

Figure 3. Transgenic over-expression of ZmWRI1 in maize. A, Effects of over-expression of ZmWRI1 under OLE promoter on embryo oil concentration and seed oil content. Each point represents an average of 10 null seeds (open triangle) and 10 transgenic seeds (closed triangle) from each transgenic line. Total 15 transgenic lines were analyzed. B, Seed oil content of T3 homozygous transgenic lines (closed) and their corresponding null segregates (open). For each transgenic line, 10 seeds per ear, 8 homozygous transgenic or null ears were analyzed. Data are shown as mean ± SD. All 5 transgenic lines showed a significant increase in seed oil content compared to null segregates as determine by Student’s t-test (P<0.01). C, Warm germination test of
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Figure 4. Protein and starch content of ZmWRI1 transgenic embryos. The data represent mean ± SD of 3 replicate samples. Each sample was run in triplet in starch and protein assays. All 3 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).

Figure 5. Early stand count and plant height of transgenic ZmWRI1 plants in field. Six rows of transgenic and 3 rows of null were planted in field for each transgenic line. Early stand count % was calculated by number of plants divided by total seeds planted at 3 weeks after planting. Plant height of 10 plants in the middle of each row was measured. Data are shown as mean ± SD. All 5 transgenic lines showed no significant difference from null as determined by Student’s t-test (p>0.1).

Figure 6. Yield test of transgenic ZmWRI1 hybrid. Total 5 transgenic lines and their corresponding nulls were crossed to a male tester, PH1B5, to produce hybrid seeds. Yield was tested in 8 locations in the United States with 3 repeats for each line at each location in 2007. Error bars are 95% confidence interval. All 5 transgenic lines showed no significant difference from nulls as determined by Student’s t-test (p>0.10).
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