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TRANSPARENT LEAF AREA1 Encodes a Secreted Proteolipid Required for Anther Maturation, Morphogenesis, and Differentiation during Leaf Development in Maize

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We report the identification and functional analysis of *TRANSPARENT LEAF AREA1* (*TLA1*), a maize (*Zea mays*) gene representing a novel class of secreted, extremely hydrophobic peptides (proteolipids) with a C-terminal Caax box–like motif. *ZmTLA1* encodes 27 amino acid residues and is most strongly expressed in the egg cell and microspores. Lower transcript amounts were detected during vegetative development. Transgenic maize expressing an antisense transcript displayed a variety of phenotypes. The most visible phenotypes were dwarfism and transparent leaf areas resulting from defective morphogenesis of mesophyll, bundle sheath, stomatal, and epidermal cells during leaf development. Incomplete cell walls were observed, indicating a defect of cytokinesis. The accumulation of gerontoplasts was probably a secondary effect caused by defects of leaf cell morphogenesis. A defect of anther maturation was observed in ~30% of the plants displaying the *tla* phenotype. Male sterility was mainly caused by incomplete disintegration of the tapetal cell layers and tetrad callose as 90% of the microspores developed into functional pollen. Overexpression of *ZmTLA1* seemed to have a lethal effect both in maize and *Arabidopsis thaliana*. Development of primary roots, root hairs, primary leaves, and chloroplasts was suppressed in Arabidopsis seedlings expressing an inducible ZmTLA1–green fluorescent protein (GFP) fusion protein. GFP signals were exclusively detected in cell walls. Based on our observations, we suggest that the ZmTLA1 peptide represents a class of novel plant morphogens required for the development and maturation of leaf and reproductive tissues.

INTRODUCTION

Sexual reproduction and vegetative development require elaborate communication between cells. In the budding yeast *Saccharomyces cerevisiae*, for example, two haploid cell types, known as MATα and MAT**a**, each secrete small peptide pheromones (α- and **a**-factors) that signal cells of the opposite mating type to stop proliferation and to prepare for mating (Marsh et al., 1991; Bölker and Kahmann, 1993). Peptide pheromones are also secreted by many animal reproductive cells. Sea urchin egg cells, for example, each secrete a species-specific oligopeptide to exclusively attract sperm cells of the same species (Kirkman-Brown et al., 2003). In multicellular organisms, hundreds of signaling molecules have been discovered both in invertebrates and vertebrates. The majority of these molecules exhibit multiple functions in numerous biological processes, including cell growth and death, cell morphogenesis and differentiation, embryonic and postembryonic development, immune, inflammatory, and stress responses, and organismal homeostasis, and in animals, sexual behavior, learning, and memory (Dresselhaus and Sprunck, 2003). Like in yeast, these communication events are mediated by diffusable or membrane-bound signaling molecules (ligands), triggering signaling cascades in the receiving cells (Posas et al., 1998). Among the signaling molecules, secreted oligopeptides are the most commonly used, probably because of their diversity.

In contrast with the numerous signaling peptides described in fungi and animals, our knowledge about oligopeptides, peptide hormones, and peptide mediating signaling in plants is still in its infancy (Ryan et al., 2002). Less than 10 plant peptide families are currently regarded as signaling molecules regulating processes such as cell growth, development, defense response, pollen tube guidance, self-incompatibility, root nodulation, and senescence (reviewed in Matsubayashi et al., 2001; Lindsey et al., 2002; Ryan et al., 2002; Dresselhaus and Sprunck, 2003; Sanchez et al., 2004). This small number of plant peptide hormones is in strong contrast with the hundreds of peptide hormones described in animals and fungi and also with the numerous putative plant membrane bound receptor-like kinases (RLKs) (>400 genes in Arabidopsis [*Arabidopsis thaliana*] and >700 in rice [*Oryza sativa*]) (Shiu and Bleecker, 2001; Shiu et al., 2004). Most RLKs are predicted to activate signaling cascades after binding extracellular peptides or small proteins (Cock et al., 2002; Dresselhaus

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and Sprunck, 2003). In plants, ligands for <10 plant RLKs are known. With the exception of brassinosteroids, they are small proteins or oligopeptides (Gómez-Gómez and Boller, 2000; Kachroo et al., 2001; Matsubayashi et al., 2002; Rivas et al., 2002; Rojo et al., 2002; Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003). Thus, it seems likely that the number of plant signaling peptides is enormous, and the majority still awaits discovery.

We are studying cell–cell communication of the haploid female gametophyte (embryo sac) of maize (*Zea mays*) and wheat (*Triticum aestivum*). In cereals, the embryo sac is deeply embedded in several maternal cell layers of the ovule and harbors the two female gametes, egg and central cell. During double fertilization, a unique feature of flowering plants (angiosperms), one sperm cell fuses with the egg cell generating the zygote, while the second sperm cell fertilizes the central cell to generate the nutritive endosperm precursor cell (Weterings and Russell, 2004). In maize, the embryo sac lacks symplastic connections with sporophytic tissues (Diboll and Larson, 1966). Therefore, communications between these two structures before or after fertilization, as well as attraction of the male gametophyte (pollen tube), prevention of polyspermy, and embryo/endosperm development, are probably mediated by secreted molecules. A cDNA library of maize egg cells (Dresselhaus et al., 1994) was used to identify genes that encode putative secreted small proteins or oligopeptides and are expressed specifically in the female gamete. Here, we report on one of the most abundant transcripts of the maize egg cell encoding a plant oligopeptide containing a Caax box–like motif, the main characteristic of the majority of fungal peptide pheromones. Maize *TRANSPARENT LEAF AREA1* (*ZmTLA1*) encodes an extremely hydrophobic oligopeptide of 27 amino acid residues and thus represents a proteolipid. Subcellular localization of a ZmTLA1–green fluorescent protein (GFP) fusion peptide was detected in the apoplast. Pleiotropic transgenic phenotypes were analyzed in detail, indicating that ZmTLA1 is a candidate signaling molecule required for anther maturation and leaf development.

RESULTS

ZmTLA1, an Intronless Single Copy Gene in Maize Encodes a Novel Class of Putative Proteolipds Containing a C-Terminal Caax Box–Like Motif

We performed differential plaque screening and random sequencing from a maize egg cell cDNA library (Dresselhaus et al., 1994) to identify maize genes having both high expression and egg cell specificity. Double plaque lifts from 29,000 plaque-forming units were hybridized with egg cell cDNAs and with cDNAs from in vitro zygotes or seedling tissues. The 250 plaques were selected and further analyzed using reverse RNA gel blots. Seventy clones displaying strong signals with the egg cell cDNA population and weak or lack of signals with other tissues were fully sequenced. One gene was identified as having seven transcripts out of the 70. The same gene was also identified as the largest EST cluster (45 or 4.6% of 988 ESTs) derived from the egg cell cDNA library. We later named this gene *ZmTLA1* (see below). Analyses of the 52 transcripts isolated showed that most *ZmTLA1* transcripts were close to 310 nucleotides in length, although some extended to 502 nucleotides as a result of the usage of different poly(A) signal sites (Figure 1A). Seven putative poly(A) signal sites were identified in the 3′ untranslated region, explaining the variation in transcript length (Hunt, 1994). A fragment corresponding to the 869 bp 5′ of the putative transcription start codon was isolated as the *ZmTLA1* promoter. A Gramineae TATA-box (TACAAAT) was identified 28 to 34 bp upstream of the 5′ end of the majority of *ZmTLA1* transcripts, a distance typical for monocotyledonous plant species. A canonical basic promoter CAAT-box was also identified 76 bp upstream of the transcription start point of the longest *ZmTLA1* transcripts. Other putative promoter elements, such as an auxin-responsive element and a MSA-box for cell cycle dependent regulation, were identified further upstream of the transcription start point.

ZmTLA1 encodes an extremely hydrophobic peptide of 27 amino acid residues (21 amino acids are hydrophobic; Figure 1B) predicted to be secreted to the apoplast. Secondary protein structure predictions suggest an α-helix for the whole peptide. Neither cDNA nor peptide sequence showed any significant homology to known sequences, but a nontypical Caax box–like motif was identified at the far C-terminal end of the protein. The typical Caax box is generally located at the protein C terminus extremity and consists of a Cys (C) residue followed by two aliphatic amino acids (aa) and one of many amino acids (x). The nontypical Caax box motif at the very C terminus of ZmTLA1 is composed of a Cys followed by an aromatic, an aliphatic, and a basic amino acid residue (CWAK).

ZmTLA represents a single copy gene as shown on the genomic DNA gel blot (Figure 1C). Probing DNA from other cereals as well as Arabidopsis indicated that homologous genes exist in *Tripsacum dactyloides*, rice, and barley (*Hordeum vulgare*) (Figure 1D). Because of low signal intensity, sequence identity might be minor and probably occurs only within the open reading frames (ORFs) (a region of only 81 bp in maize) of *TLA1*-like genes. A signal was not detected in Arabidopsis, which is not surprising because signals with other cereals were weak and codon usage in Arabidopsis is different from monocots. *ZmTLA1* was localized to the long arm of chromosome 2 between the molecular markers *chi2* (at 181.2 centimorgan [cM]) and *bnl5.61B* (at 185.0 cM) close to the β-amylase locus (*amy4*) (at 184.1 cM). This chromosomal region is duplicated in allotetraploid maize, as alleles of molecular markers of this region, *bnl8.21*, *ast*, and *bnl5.61*, also map to the long arm of chromosome 7 (Figure 1C).

ZmTLA1 Is Strongly Expressed in Egg Cells and Microspores but Weakly Expressed in Some Vegetative Tissues

ZmTLA1 transcripts were detected in all egg cells and in two of the four synergids tested (Figure 2A). Hybridization signals were weaker in synergids than in eggs cells, and transcripts could not be detected in the other cells of the embryo sac (central cells and antipodals) (Figure 2A). Nine of 10 in vitro–fertilized zygotes analyzed at different stages of development (from shortly after karyogamy, which occurs ~1 h after gamete fusion, up to the two-celled stage, 68 h after in vitro fertilization) showed transcript

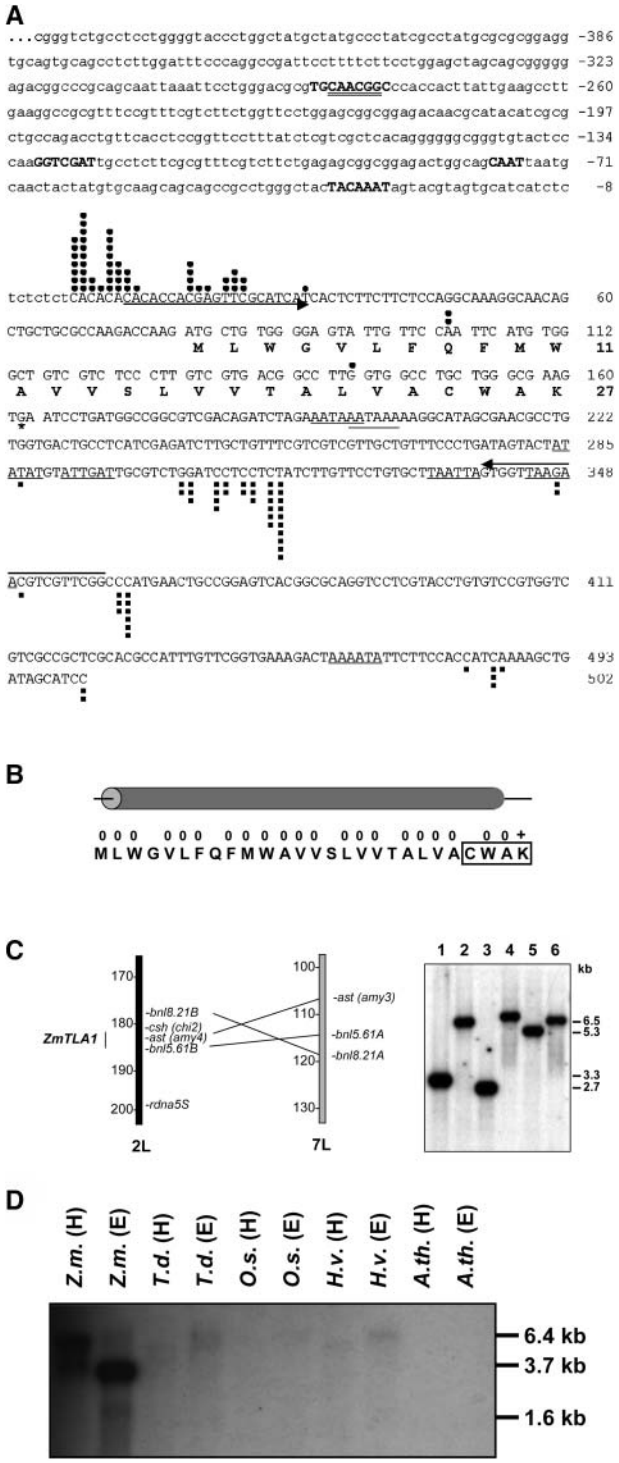

Figure 1. The *ZmTLA1* Gene Is Highly Expressed in Egg Cells and Encodes a Small Hydrophobic Peptide.

(A) A total of 52 *ZmTLA1* cDNAs were isolated out of 988 egg cell ESTs and after differential screening of an egg cell cDNA library of maize. Black dots mark transcription start points of individual transcripts, and black squares indicate poly(A) sites. Seven putative poly(A) signal sites are underlined by a single line. The asterisk marks the putative stop codon. Upstream (869 bp) of the most frequently used transcription start points C and A at positions +1 and +2, respectively, were cloned as the *ZmTLA1* promoter (445 bp of the promoter sequences are shown in lower-case letters). Putative TATA- (TACAAAT), CAAT- (gCAATt), an auxin responsive element (GGTCGAT), and an MSA-box (TGCAACGGC) are marked by bold capital letters within the *ZmTLA1* promoter sequence, and a putative Myb Hv1 binding element is double underlined. Arrows mark primers sites that were used for single cell RT-PCR and genomic amplifications.

(B) The ZmTLA1 predicted peptide consists of 27 mainly hydrophobic amino acid residues (0) with one positively charged amino acid (+) at the very C terminus. The Caax-like motif is boxed, and the whole peptide is predicted to form an α-helix.

(C) The intronless *ZmTLA1* gene maps to the long arm of chromosome 2 (2L) between the molecular markers *csh* (*chi2*) at position 181.2 and *bnl5.61B* at position 185.0. This region of chromosome 2 is known to be duplicated on chromosome 7 (left). Despite this duplication, *ZmTLA1* represents a single copy gene as indicated by the genomic DNA gel blot hybridization (right). Genomic DNA of the maize inbred line A188 was separated in a 0.7% agarose gel after digestion with the following restriction enzymes: *Xho*I (1), *Hin*dIII (2), *Eco*RI (3), *Eco*RV (4), *Dra*I (5), and *Nde*I (6). Exposure at −70°C was performed for 3 d using intensifier screens.

(D) Orthologous *TLA1* genes were detected in maize (*Z.m.*), *Tripsacum dactyloides* (*T.d.*), rice (*O.s.*), and barley (*H.v.*) but not in Arabidopsis (*A.th*). Genomic DNA was restricted with *Hin*dIII (H) and *Eco*RI (E), separated in a 1.2% agarose gel, blotted, hybridized, and washed at high stringency conditions. Exposure at −70°C was performed for 14 d using intensifier screens.

amounts similar to the unfertilized egg cells. *ZmTLA1* expression could not be detected at later stages of embryogenesis (12 to 40 d after pollination) nor in tissue culture material showing a low embryogenic capacity, nucellus, or differentiated leaf mesophyll cells (Figure 2B). By contrast, *ZmTLA1* mRNAs were present in microspores from immature tassels (see also Figure 4C). Expression in sporogenous tissue of the anther was not detected. At later stages during microgametogenesis, *ZmTLA1* transcript was no longer detectable and was absent in pollen at maturity (Figure 2B). A strong signal was detected in microspore-derived highly embryogenic cell suspensions, whereas nonembryogenic cultures showed almost no expression (Figure 2C). As shown in Figure 2C, much weaker expression of *ZmTLA1* was observed during vegetative development. The RNA gel blot shown was exposed for 3 weeks to display weak signals. Minor signals were detected in internodes, the meristematic leaf base, immature ears, and seedlings at 5 as well as 10 d after germination (dag). A stronger signal was observed at yellow-greenish inner leaflet areas of plantlets ~21 dag. This signal intensity decreased during leaf development and was absent in mature leaves.

Transgenic Maize with a *ZmTLA1* Antisense Transcript Displayed Pleiotropic Phenotypes

To investigate *ZmTLA1* function, we searched 20,000 Mutator transposon tagged maize plants (Südwestdeutsche Saatzucht, Rastatt, Germany), but an insertion was not identified in the small *ZmTLA1* gene. We therefore generated antisense transgenic maize plants to downregulate *ZmTLA1* activity using the strong

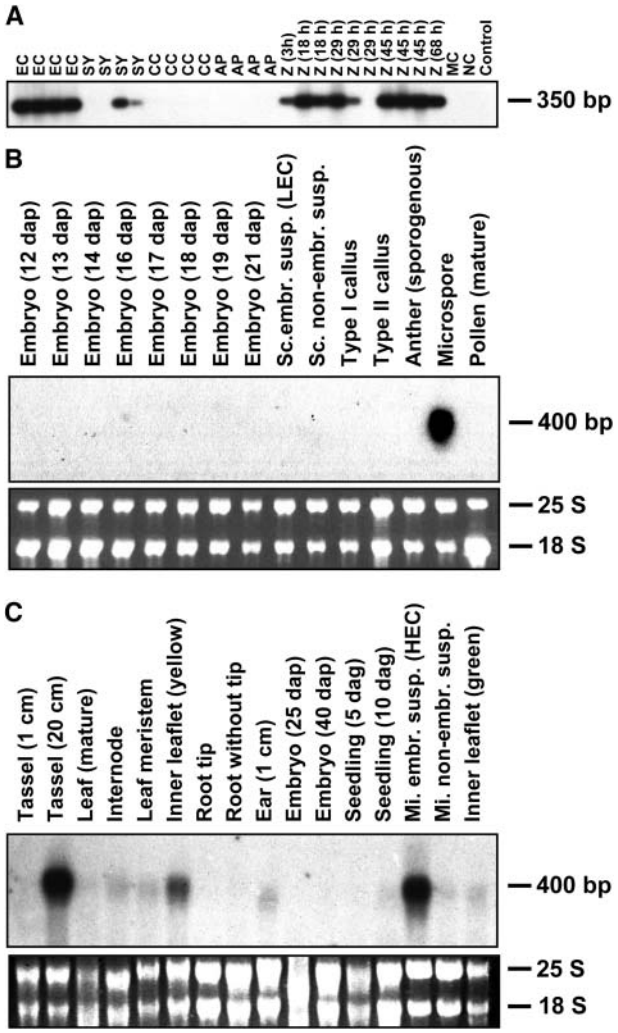

Figure 2. *ZmTLA1* Transcripts Are Present in Unfertilized and Fertilized Egg Cells, in Microspores, and during Leaf Morphogenesis.

(A) Single cell RT-PCR with egg cells (EC), synergids (SY), central cells (CC), antipodal cells (AP), zygotes (Z) at different times after in vitro fertilization (IVF), differentiated mesophyll cells (MC), nucellus cells (NC), and washing buffer (Control).

(B) RNA gel blot hybridization (6 μg total RNA, 2-d exposure).

(C) RNA gel blot hybridization (10 μg total RNA, 21-d exposure). The size of the full-length *ZmTLA1* transcript is between 300 and 500 nucleotides. 18S and 25S rRNA are shown as quantitative controls. HEC, high embryonic capacity; LEC, low embryonic capacity; Mi., microspore, Sc., scutellum.

and constitutive ubiquitin promoter of maize (Christensen and Quail, 1996). Immature embryos (1703) were bombarded with an antisense construct containing a full-length copy of the *ZmTLA1* cDNA. Thirty-two transgenic plants (T0 generation; Table 1) were generated containing one to several copies of the transgene, of which 12 showed transgene expression in mature leaves (Figure 3A shows nine expressing transgenic plants). Some plants displayed identical transgene integration patterns, indicating that they originated from the same transgene integration events (data not shown). In total, 13 different transgenic lines were generated, six of which showed significant expression of the antisense gene. Surprisingly, plants with the identical transgene integration pattern displayed varying amounts of the antisense transcript, which was especially prominent for lines A5 (plants A5a and A5b) and A7 (plants A7a to A7e) (Figure 3A). Progeny of the six expressing independent T0 lines (A3, A4, A5, A6, A7, and A12) showed transmission of the functional transgene (Table 1). Expression of *ZmTLA1* antisense transcripts was investigated in the T1 progeny of most transgenic lines. From ~200 plants analyzed, 68 plants originating from lines A3, A4, A5b, A6b, A7a-e, and A12 showed varying levels of transcript amounts. As already indicated for the T0 generation, a strong divergence of relative transcript amounts was also observed in the progeny generations. Figure 3B shows relative antisense transcript amounts in the leaves of the T2 progeny of three different lines (A3, A4, and A7c) as an example. Transcript levels of A3 progeny varied between 38 and 64% relative to the strongest expressing line (A7c-7-4: 100%), between 4.5 and 95% within the progeny of line A4, and from 3 to 100% within line A7c progeny.

Deviation from wild-type phenotypes was exclusively observed in plants expressing the transgene. The most obvious phenotypes of transgenic lines were dwarfism and/or yellow or transparent leaf areas (*tla* phenotype), which were observed in 76 plants (Figure 3). The severity of the dwarfism observed also correlated with severity of abnormal sexual development. Plants showing a severe dwarfism phenotype (Figure 3C) did not develop reproductive organs. Moderate dwarf plants (Figure 3D) often only produced a tassel and immature (sometimes mature) anthers (Figure 3H), whereas a cob either did not develop or was immature. The *tla* phenotype was visible on most plants showing high transgene expression. Transparent leaf regions appeared at the seedling stage on growing leaves and remained relatively unchanged during leaf maturation and senescence. There were no obvious signs of necrosis, suggesting the transparent area arose from a developmental defect during leaf morphogenesis. Four plants showed large nontransparent yellow to white leaf areas that became green after ~2 weeks. Some plants showing a *tla* phenotype were completely or partly male-sterile (31 plants) and/or female-sterile (35 plants; of these 12 plants developed immature cobs). Yield or seed set was also affected by the *TLA* antisense transcript (Figure 3G, right). Selfing of plant A3-3-1, which contained high levels of transgene mRNA, resulted in significantly lower seed number than plant A2-6-1, which lacked transgene expression (Figure 3G, left). Male sterility was mainly caused by insufficient maturation of anthers (Figure 3H). Often anther pores remained closed. A detailed investigation of the observed phenotypes is reported below.

ZmTLA1 Activity Is Required for Leaf Morphogenesis during Vegetative Development

A wild-type maize leaf showed the typical Kranz anatomy of a C4 plant. The layer of mesophyll cells can be seen surrounding the layer of bundle sheath cells enclosing leaf veins. Chloroplasts were dimorphic, and those of the mesophyll cells contained thylakoid stacks and showed intense red fluorescence (Figures 4A and 4E). Green leaf areas of antisense transgenic plants had

Table 1. Phenotypes of Transgenic Maize Plants Containing a *ZmTLA1* cDNA in Sense and Antisense Orientation Each under the Control of the Constitutive Maize Ubiquitin Promoter

Generation[a]	Number of Plants	Expressing Plants	*tla* Phenotype[b]	Male Sterility[c]	Female Sterility[d]
Wild type (A188)	>100	–	–	–	–
T0 AS	32 (13)[e]	12 (6)	n.d.[f]	n.d.	n.d.
T1 AS	>100	68 (6)	23	16	11
T2 AS	>100	78 (6)	53	15	24
T0 SE	4 (2)	–	–	–	–
T1 SE	7 (1)	–	–	–	–

Transformation efficiency was 1.9% (antisense) and 0.5% (sense), respectively.
[a] T0 are direct regenerants; T1 and T2 are progeny generations. AS, antisense; SE, sense.
[b] *tla* phenotype as shown in Figures 3D to 3F.
[c] Male sterility as shown in Figure 3H or undeveloped male inflorescences (in T0 and T1 only).
[d] Low seed set as shown in Figure 3G (right) or lack of anthesis as shown in Figure 5L.
[e] Number in parentheses indicates number of independent transgenic lines with the same transgene integration pattern.
[f] Not determined.

a stongly affected morphology (Figures 4B and 4F). Mesophyll and bundle sheath cells could no longer be distinguished, and chloroplast number as well as chlorophyll content appeared reduced in those tissues. Yellow to transparent leaf areas of the same plants were characterized by collapsed or not fully developed parenchymal cells (Figures 4C and 4D), whereas only the epidermis and vascular tissue appeared intact in fully transparent regions. Many fluorescent inclusion bodies or droplets were observed by confocal laser scanning microscopy (CLSM) in yellow leaf regions of transgenic plants (Figures 4G and 4H) as well as in collapsed cells of more transparent leaf regions (arrows in Figure 4H). The fluorescence might have resulted from the accumulation of phenolic compounds produced during degeneration of cells. Observations using electron microscopy showed that cytokinesis, cell wall formation, and chloroplast development were distorted, even in green areas of transgenic plants. Wild-type plants had dimorphic chloroplasts in both mesophyll and bundle sheath cells (Figure 4J). Mesophyll chloroplasts in green regions of transgenic plants lacked thylakoid stacks, whereas chloroplasts from the bundle sheath cells were less membranous and lacked starch granules (Figures 4K and 4L). Many plastoglobuli and a prolamellar body, features of a typical gerontoplast, were visible within mesophyll chloroplasts (Figure 4L). Figure 4M shows a collapsed cell of a transparent leaf area with thick cell walls. The cell contained amorphous material that appeared green using CLSM. Compared with wild-type stomata, cell walls of transgenic stomatal cells were less structured, the cytoplasm appeared disorganized, and stomata were likely to be nonfunctional in antisense plants (Figures 4N to 4P). Cell wall formation of epidermal cells was irregular in transparent leaf regions. Interrupted cell walls were observed, especially in transparent leaf regions, indicating a defect of cytokinesis (Figures 4P and 4Q).

ZmTLA1 Activity Is Required for Anther Maturation during Reproductive Development

Endogenous *TLA* as well as transgene expression levels were quantified at different developmental stages in anthers of wild-type plants and transgenic plants using real-time quantitative RT-PCR. *GAPDH* was used as an internal control to normalize *ZmTLA1* transcript amounts. As shown in Figure 5A, the highest transcript amounts were present in wild-type anthers containing microspores, confirming the expression shown in Figure 2B. Relative to microspores, expression levels dropped to 5% at the quartet stage (shortly after meiosis) and 0% at the bicellular to tricellular stage as well as in mature pollen, indicating that *ZmTLA1* is expressed within a small developmental window during microgametogenesis. To compare antisense transcript levels with the occurrence of the various phenotypes, anthers of antisense expressing progeny plants from two different transgenic lines (A4 and A7c) containing microspores were excised and *ZmTLA1* antisense transcript amounts determined after normalization of transcript levels using *GAPDH* and *ZmTLA1* sense transcript amounts. Antisense transcript levels varied between 180 and 1700% relative to the highest sense transcript levels of wild-type microspore containing anthers. Transgenic plants (A4-27 and A7c-7-11) containing only about double the amount of antisense transcript relative to endogenous sense transcript amounts in anthers did not display a phenotype (Figure 5A). The highest expressing lines (A4-28 and A7c-7-12) displayed all described phenotypes, whereas most other plants showed a moderate or strong *tla* phenotype and partly male and/or female sterility. Wild-type plants did not show any of these phenotypes.

Single cell quantitative RT-PCR was used to quantify downregulation of *ZmTLA1* transcript levels in mature egg cells. These cells displayed high *ZmTLA1* transcript amounts, and cells of similar developmental stages could be isolated. Relative *ZmTLA1* transcript amounts in egg cells were determined after normalization with *GAPDH* transcript levels. As shown in Figure 5B, *ZmTLA1* transcript amounts in wild-type egg cells varied from 40 to 189% of the mean expression level of 100%. Egg cells from two transgenic lines (A4-6-12 and A4-9-6) containing high antisense transcript amounts (data not shown) and that displayed both a strong *tla* and *ms* phenotype were excised and used to measure downregulation of relative *ZmTLA1* expression levels. *ZmTLA1* transcript amounts were significantly reduced to

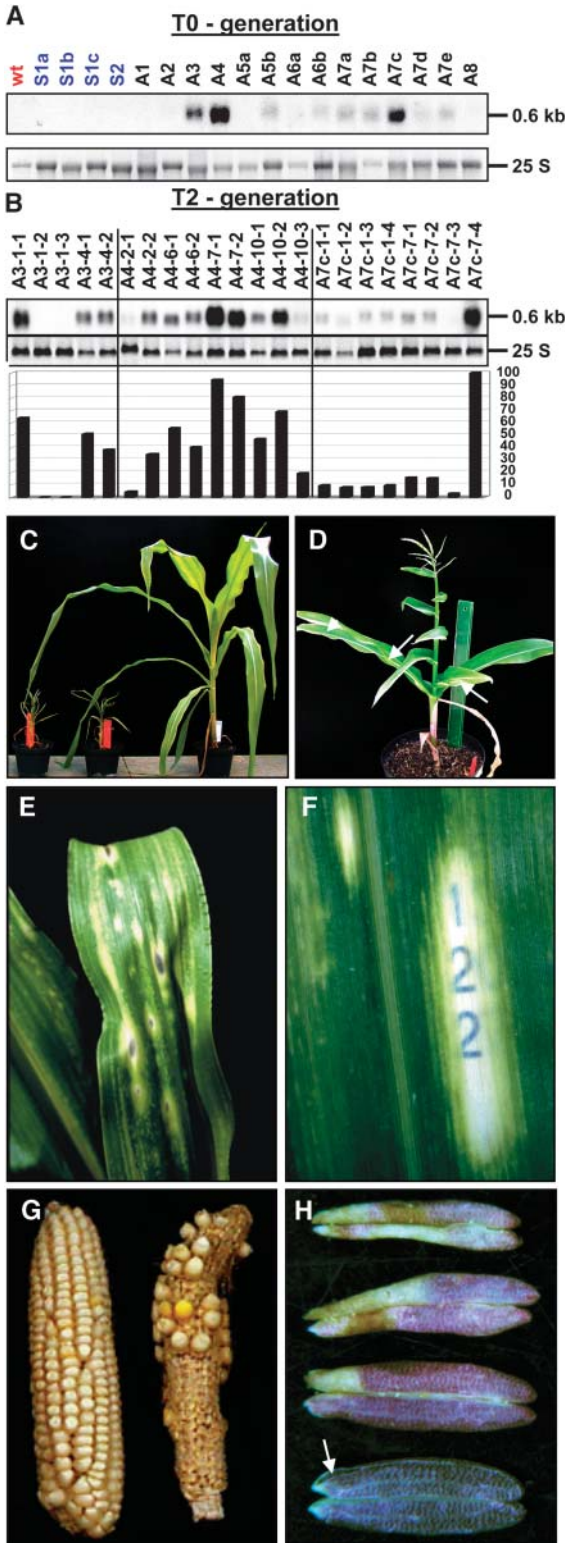

Figure 3. Transgenic Maize Expressing *ZmTLA1* Antisense Transcripts Displayed a Variety of Phenotypes, Including Dwarfism, *tla*, and Male and Female Sterility.

(A) Expression levels of *ZmTLA1* sense (Sx; blue) and antisense transgenes (Ax; black) in leaves of T0 generation plants. Lower-case letters indicate clonal lines.

(B) Relative expression levels in leaves of T2 progeny plants originating from three strongly expressing T0 antisense plants (A3, A4, and A7c).

(C) Five-week-old dwarf T2 transgenic antisense plants (plants A6b-7-2 and A6b-7-3) compared with a wild-type plant (A188) of the same age.

(D) Antisense T2 transgenic plant (A7c-7-4) at anthesis. The plant was small (45 cm) and showed transparent stripes (arrows) and transparent spots (arrowhead). The tassel was fertile, but a cob did not develop.

(E) Transparent leaf areas appeared at the seedling stage primarily on secondary leaves and remained unchanged until senescence (plant A5b-6-2).

(F) Enlargement of transparent leaf spots of plant A4-7-2. Transparent regions were clear and lacked signs of necrosis.

(G) Fertility of the T2 A2-6-1 plant lacking antisense transgene expression (left) compared with T2 A3-3-1 expressing relatively high antisense transcript amounts (right).

(H) Anthers of the T2 antisense plant A4-7-1 were partly immature but contained pollen grains (8 d after anthesis) compared with a wild-type anther 5 d after anthesis (arrow). The arrow also points toward an open pore at the wild-type anther tip through which the pollen escaped.

5 to 22% compared with the mean expression level in wild-type egg cells, showing that downregulation of ZmTLA1 transcript amounts was achieved using antisense technology.

Figures 5C to 5P show the phenotypes of reproductive structures of transgenic plants compared with the wild-type. Pairs of sessile and pedicellate spikelets of wild-type tassel inflorescences dehisced and displayed all 12 anthers 4 d after anthesis (Figure 5C). Anthers were mature, pores at the anther tip opened, and shedding of pollen was complete. Anthers of spikelet pairs from a transgenic line 7 d after anthesis were only partly mature (A4-22 in Figure 5D). Anthers of branch flowers were not fully developed and appeared shriveled. Surprisingly, transgenic anthers, phenotypically indistinguishable from wild-type anthers (Figure 5E), contained pollen grains as much as 7 d after anthesis. Cross sections through wild-type anthers 4 d after anthesis showed the completion of locule fusion and release of pollen (Figure 5F). By contrast, transgenic anthers showed the presence of pollen grains within the cavity of fused locules 7 d after anthesis (Figure 5G). Often mature pollen grains were attached in a group of four probably because of incomplete digestion of callose, which surrounded tetrads after meiosis (Figure 5H). Staining with 4′,6-diamidino-2-phenylindole (DAPI) further revealed that more than 90% transgenic pollen was fully developed as they contained a large vegetative nucleus and two sperm cells (Figure 5J). Pollen germination tests showed viability and germination capability of most pollen grains (data not shown). Cross sections through immature and carved anther regions (arrowhead in Figure 5D) showed that fusion of a locule pair did not occur, and tapetal cell layers were incompletely disintegrated. Locules still contained microspores, the majority of which did not develop properly; many were arrested at the unicellular to bicellular stage (Figure 5K). Interestingly, non-carved immature regions of the same anther displayed fusion of locules and contained a majority of mature pollen grains as shown in Figure 5G.

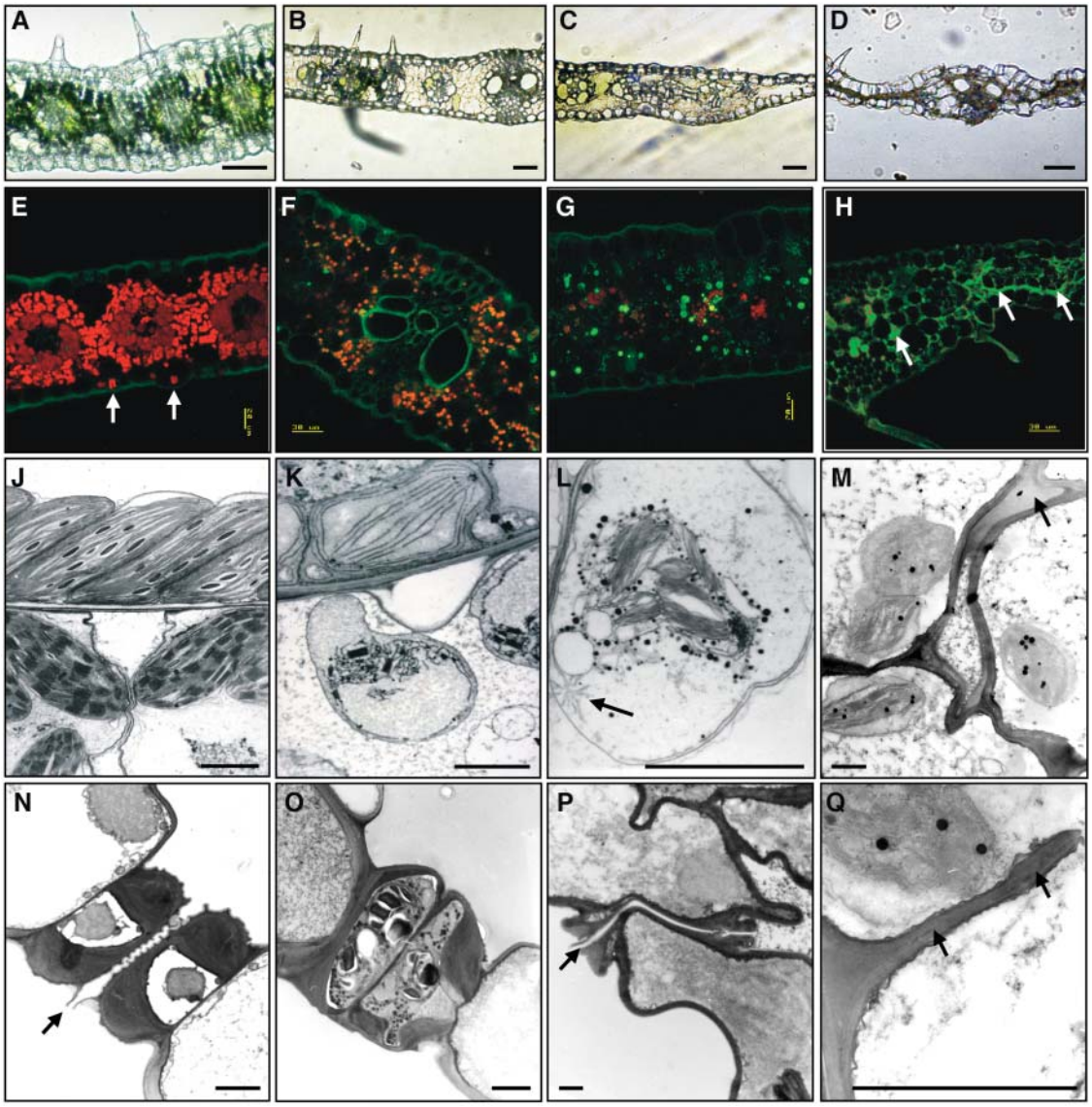

Figure 4. Cytological and Cellular Analyses of the *tla* Phenotype.

(A) to **(H)** Transverse section of leaves in light microscopy (**[A]** to **[D]**) and laser scanning microscopy (**[E]** to **[H]**).
(A) A wild-type mature leaf. Mesophyll cells are dark green, and bundle sheath cells are light green.
(B) Transgenic leaf blade lacking an obvious morphological phenotype. Cell morphogenesis appeared normal, but mesophyll cells were less green.
(C) Transition from a yellow to a transparent area (left to right) of a mature transgenic leaf. Gradient observed within the tissue structure. From morphologically intact mesophyll cells in the yellow area of the leaf to collapsed or missing cells within the transparent region.
(D) Typical transparent leaf area on an antisense transgenic line. Epidermal cells and xylem appeared intact, whereas most other cells seemed to be collapsed or not developed.
(E) Wild-type mature leaf. Mesophyll and bundle sheath cells can be easily distinguished because of differences in red fluorescence. Arrows point toward stomatal cells.
(F) Green leaf area of a transgenic plant. Mesophyll and bundle sheath cell chloroplasts are reduced in number and do not show cell dimorphism.
(G) Yellow leaf area of mature transgenic leaf. Chloroplasts were rare, and large green fluorescent bodies were present.
(H) Transition area from yellow to transparent of an antisense transgenic leaf (left to right). Many cells seemed collapsed (arrows).
(J) to **(Q)** Transmission electron microscopy.
(J) Border between a bundle sheath (top) and mesophyll cell (bottom) of a wild-type mature leaf. Note the chloroplast dimorphism.
(K) Border between a leaf bundle sheath (top) and mesophyll cell (bottom) of a transgenic plant. Both chloroplast types were visible, and their biogenesis seemed arrested.
(L) A rudimentary prolamellar body (arrow) and many plastoglobuli were visible within a mesophyll chloroplast of a transgenic plant.
(M) Collapsed cell of a transparent leaf area of a transgenic plant with thick cell walls enclosing amorphous material (arrow).

Transgenic plants showing a strong *tla* and dwarfism phenotype often generated cobs with short silks, as shown in Figure 5L, which remained enclosed within hull leaves and thus could not be pollinated. The short silks appear to be the only source of sterility because ovules and embryo sacs of such dwarf plants were phenotypically wild type (Figures 5M and 5N). Plants of a normal size containing high antisense transcript amounts and that displayed both a strong *tla* and *ms* phenotype were pollinated, and embryo sacs were isolated after 7 d. Of ~80 ovules analyzed, all contained both embryo and endosperm, indicating that fertilization and seed development were not affected (Figures 5O and 5P). The shape of both embryo and endosperm was comparable to that of the wild type of the same age, but eventually development was slightly delayed in the transgenic plants.

ZmTLA1 Is a Secreted Peptide Bypassing the Secretory Pathway

Transcient expression in epidermal onion (*Allium cepa*) cells of a ZmTLA1-GFP chimeric protein displayed fluorescence only in the apoplasts and diffused within the cell walls of neighboring cells (Figure 6A). A control experiment using GFP protein alone allowed detection in both nucleus and cytoplasm (Figure 6B). A similar picture was observed in the hypocotyl of transgenic Arabidopsis seedlings expressing an inducible ZmTLA1-GFP fusion protein (Figure 7Q). In contrast with the control (the cytoplasmic *mGFP5-ER*; Figure 7H), the short ORF of *ZmTLA1* was sufficient for translocation of GFP in the extracellular space. Compared with the control *mGFP5-ER* (Figure 7H), the ZmTLA1-GFP fusion protein never accumulated in the endoplasmic reticulum, Golgi apparatus, or any vesicles. For comparison, Figure 6C shows a ZmES4-GFP fusion protein bombarded into an onion epidermal cell. In contrast with the ZmTLA1-GFP chimeric protein, ZmES4-GFP was secreted to the extracellular space after passing the secretory pathway and was visible in the endoplasmic reticulum around the nucleus, in the Golgi apparatus, and within secretory vesicles. These results indicate that the small ORF of ZmTLA1 is functional and sufficient for secretion to the extracellular space bypassing the classical secretory pathway.

Ectopic Expression of *ZmTLA1* Suppressed Growth and Differentiation in Arabidopsis

We were unable to regenerate transgenic maize plants that would ectopically express the TLA peptide (*pUbi*:*ZmTLA1*). The very few plants arising from these studies (4 out of 850 bombarded embryos) contained nonfunctional transgenes (Figure 3A). Considering the low transformation efficiency (0.5% compared with 1.9% of the antisense approach) and the observation that none of the few obtained transgenic plants harbored functional transgenes indicates that ectopic expression of *ZmTLA1* in maize might be lethal.

Similar results were obtained by overexpressing *ZmTLA1* in Arabidopsis. Of ~500,000 kanamycin-selected progeny seedlings derived from transformed Arabidopsis plants, none survived the primary leaf stage. Most seedlings died at an early cotyledonous stage. Figure 7A shows a wild-type (left) and a dying transgenic plantlet (right), both 32 dag. Thus, a dexamethasone-inducible system consisting of the ORF of *ZmTLA1* fused to GFP was used to monitor localization of the chimeric protein and to investigate the cause of lethality. For comparison, a transgenic Arabidopsis line was used, expressing GFP exclusively in the cytoplasm (endoplasmic reticulum) of seedling cells (Figures 7B to 7H). Control seedlings showed the formation of root hairs soon after germination (Figures 7B and 7C) and elongation of the primary root before proplastids differentiated into chloroplasts (Figures 7D and 7E). Elongation of hypocotyl and greening of cotyledons occurred some 5 d after germination initiation (Figures 7F and 7G). The same developmental scheme was observed when wild-type seeds were germinated in induction medium containing dexamethasone. By contrast, seedlings expressing a ZmTLA1-GFP chimeric protein exhibited a developmental arrest 6 to 10 d after germination initiation in induction medium (Figures 7J to 7Q). The majority of seedlings displayed GFP signals throughout the seedling as shown in Figure 7J. A few independent transgenic seedlings showed GFP signals in different compartments, possibly because of the different chromosomal location of the transgene (position effects). A developmental arrest was clearly correlated with GFP localization. Elongation of hypocotyl cells, development of the primary root, and root hair differentiation were suppressed in seedlings displaying strong GFP signals in the hypocotyl and the seedling root. Cotyledons remained small and differentiation of chloroplasts was suppressed in lines displaying strong GFP signals in cotyledons (Figures 7K and 7L). A remarkable transgenic line is shown in Figures 7M and 7N. Only a few cells of one cotyledon expressed the fusion protein. This cotyledon is smaller than the second one, but most cells (except GFP expressing cells) were able to develop differentiated chloroplasts. This line showed strong GFP signals in the basal part of the hypocotyl. In this region, neither initiation of root hair differentiation nor development of the primary root took place. In summary, overexpression of ZmTLA1 in Arabidopsis confirmed the extracellular localization of a GFP fusion protein and supported the conclusion that ZmTLA1 is involved in various processes of cell differentiation and morphogenesis because high fusion protein amounts suppressed differentiation processes in Arabidopsis seedlings.

Figure 4. (continued).

(N) Wild-type stomatal cells.

(O) Stomatal cells of a light-green area of a transgenic plant.

(P) Stomatal and epidermal cells of a transparent region of a transgenic plant. The arrow points toward the stomatal opening.

(Q) Incomplete cell walls (arrows) were frequently observed in transparent leaf areas, indicating a defect of cytokinesis.

Bars = 50 μm in **(A)** to **(D)**, 20 μm in **(E)** and **(G)**, 30 μm in **(F)** and **(H)**, and 2 μm in **(J)** to **(Q)**.

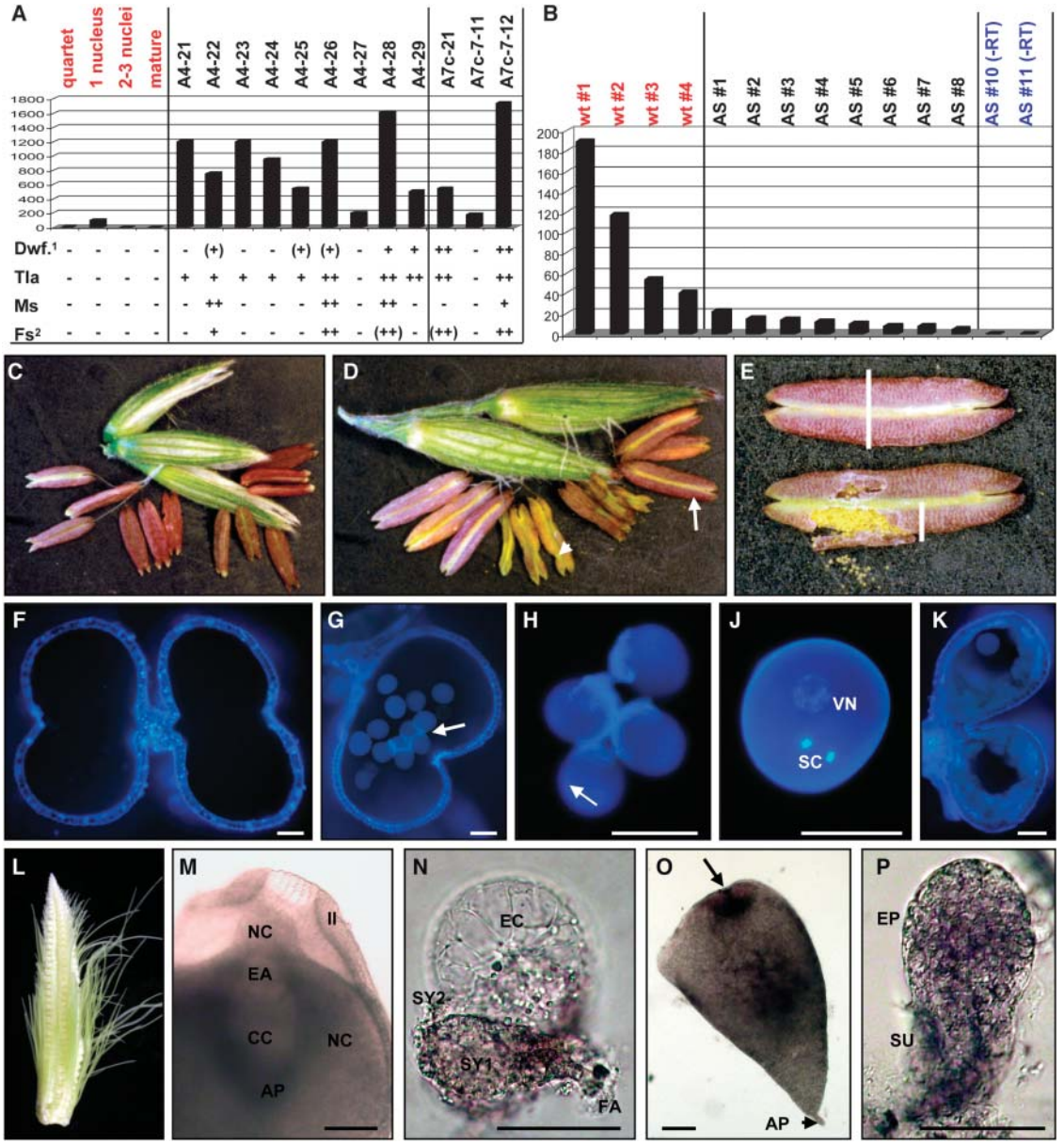

Figure 5. Relative Quantification of *ZmTLA1* and Antisense mRNA Levels in Wild-Type and Transgenic Tissues Correlated with Observed Phenotypes.

(A) Expression levels of *ZmTLA1* transcript in wild-type anthers (red) containing pollen at different developmental stages (quartet to mature). Relative expression in anthers containing microspores was set as 100. Antisense transcript levels of anthers containing microspores of T1 and T2 progenies from strongest antisense expressing T0 plants (A4 and A7c) are shown in the middle and at the right. The occurrence of different mutant phenotypes (Dwf., dwarfism; Tla, transparent leaf areas; Ms, male sterility; Fs, female sterility) is indicated.

(B) *ZmTLA1* transcript levels in egg cells from wild-type (A188; red) and antisense plants. The mean value of four wild-type egg cells shown at the left was 100. Transcript amounts of transgenic egg cells relative to the mean value of wild-type egg cells are shown in the middle, and genomic amplifications from two transgenic egg cells (PCR without RT reaction; blue) are shown at the right.

(C) Anthers from a pair of wild-type sessile and pedicellate spikelets 4 d after anthesis. Anthers were mature and shed their pollen.

(D) A pair of transgenic spikelets 7 d after anthesis. Anthers of branch flowers (arrowhead) were less developed than anthers of terminal flowers (arrow), which appeared wild type. All anthers still contained pollen grains.

(E) Anther indicated by an arrow in **(D)** was opened to show pollen grains. A comparable wild-type anther is shown above the mutated anther.

(F) Transverse section of an empty wild-type anther 4 d after anthesis as indicated by the bar in **(E)**.

(G) Transverse section of a transgenic anther 7 d after anthesis as indicated by the bar in **(E)**. The anther contained pollen, which were partly embedded in callosic cell wall material (arrow).

(H) Detailed view of callose embedded pollen.

DISCUSSION

We have described here the molecular isolation and functional characterization of the *TLA1* gene of maize, which encodes a novel class of plant peptides. Because of the small size of the peptide, conventional and specific database searches for short sequences were unsuccessful in identifying homologs of ZmTLA1. To date, short ORFs smaller than 50 amino acid residues are unannotated; thus, tBLASTn (translated Basic Local Alignment Search Tool of nucleotide sequences) searches were performed using the whole rice and Arabidopsis genomes, respectively. Such searches have been successfully conducted with the 77 amino acids of the INFLORESCENT DEFICIENT IN ABSCISSION peptide of Arabidopsis and identified four additional unannotated homologous proteins of <100 amino acids in the genome (Butenko et al., 2003). However, using the 27 amino acids of the TLA1 peptide failed to identify homologous peptides. In general, sequence identity is low among signaling peptide family members within one species and even lower to homologs of other species (e.g., Cock and McCormick, 2001; Vanoosthuyse et al., 2001; Yang et al., 2001; Butenko et al., 2003; Mergaert et al., 2003; Ryan and Pearce, 2003; Márton et al., 2005), providing another explanation why *TLA1*-like genes could not be identified in other plants. Nevertheless, a genomic DNA gel blot indicated that homologs exist in rice and other cereals, but because the 27 amino acids of ZmTLA1 might represent a propeptide that could be further processed, the sequence identity was too low to identify the corresponding homologs. The accuracy of gene annotation and prediction in rice is currently a matter of debate (Jabbari et al., 2004) and might be another argument that hindered the identification of a *ZmTLA1* homolog in this species. Arabidopsis employs different codon usage from that of cereals (Wong et al., 2002); therefore, hybridization signals were not expected in a genomic DNA gel blot.

The predicted ZmTLA1 peptide has an α-helical structure, is extremely hydrophobic, and possesses a C-terminal Caax box–like motif. Thus, according to Folch and Lees (1951), ZmTLA1 represents a proteolipid, a class of extremely hydrophobic proteins. Only a few plant proteolipids are known (Capel et al., 1997; Pearce et al., 1998), and functions have not been identified for any of them. One proteolipid family highly conserved in eukaryotes (including plants) was predicted to contain two α-helical structures. The corresponding plant genes were reported to be induced by low temperature, salt stress, and abscisic acid. More recently, the Arabidopsis family gene members were reported as being strongly induced during the first stages of seed development and germination (Medina et al., 2001). A yeast homolog, PLASMA MEMBRANE PROTEOLIPID3 (PMP3), was hypothesized to be involved in regulating the membrane potential (Navarre and Goffeau, 2000). Although ZmTLA1 lacks any homology to these conserved proteins, it is noteworthy that the size of the ZmTLA1 peptide is exactly 50% of the length of PMP3 and contains one predicted α-helix instead of two. Genes encoding small hydrophobic signaling peptides have been mainly described in microorganisms. *Staphylococcus epidermis*, for example, secretes a mixture of proteolipids consisting of hydrophobic peptides of 22–, 25–, and 44–amino acid residues, respectively (Mehlin et al., 1999). These peptides stimulate the inflammatory response mediated by mammalian Toll-like cell surface receptors

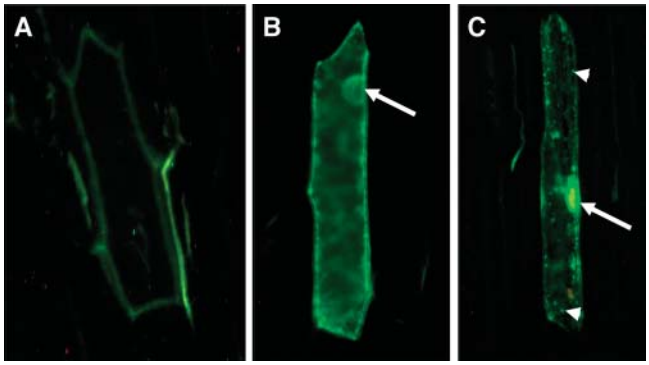

Figure 6. A ZmTLA1-GFP Fusion Protein Was Secreted to the Apoplast Bypassing the Secretory Pathway.

(A) Onion epidermal cell transiently expressing an inducible ZmTLA1-GFP chimeric protein. GFP fluorescence was detected exclusively in the extracellular space and was diffusing within few cell rows.
(B) Onion epidermal cell bombarded with a *pUbi*:*GFP* construct. Fluorescence was visible in cytoplasm and nucleus (arrow).
(C) Onion epidermal cells bombarded with a *pUbi*:*ZmES4-GFP* construct showed secretion of this extracellular peptide via the secretory pathway. Arrow points toward nucleus surrounded by endoplasmic reticulum and arrowheads toward secretory vesicles.

Figure 5. (continued).

(J) The majority of pollen from transgenic lines was fully differentiated, containing a vegetative nucleus (VN) and two sperm cells (SC).
(K) Transverse section of a tassel branch anther 7 d after anthesis (arrowhead in **[D]**). The two loculi were unfused, and tapetal cells as well as mother cell wall material were incompletely disintegrated. Few pollen within this anther region reached maturity.
(L) Cob lacking silk emergence of a dwarf and male sterile *tla* transgenic plant 2 weeks after male anthesis. Husk leaves were removed to show incomplete development of silk.
(M) Embryo sac from nonsilking cob **(L)** was similar to the wild type containing an egg apparatus (EA), central cell (CC), and antipodals (AP). Surrounding maternal nucellar (NC) tissues and inner integument (II) were fully differentiated. The integuments were lifted to display the micropylar opening of the ovule.
(N) A microdissected egg apparatus of a nonsilking cob containing the egg cell (EC), two synergids (SY), and the filiform apparatus (FA).
(O) Isolated embryo sac 7 d after pollination of a tall transgenic plant, which showed both the *tla* phenotype and anther maturation defects. The position of the embryo (arrow) and antipodals (AP; arrowhead) is shown.
(P) An isolated embryo from the fertilized embryo sac shown in **(M)** appeared normal, consisting of the embryo proper (EP) and suspensor (SU).
Images shown in **(D)** to **(H)** were stained with 4′,6-diamidino-2-phenylindole. Bars = 100 μm in **(D)** to **(G)**, **(K)**, **(M)**, and **(N)** and 50 μm in **(H)** and **(L)**.

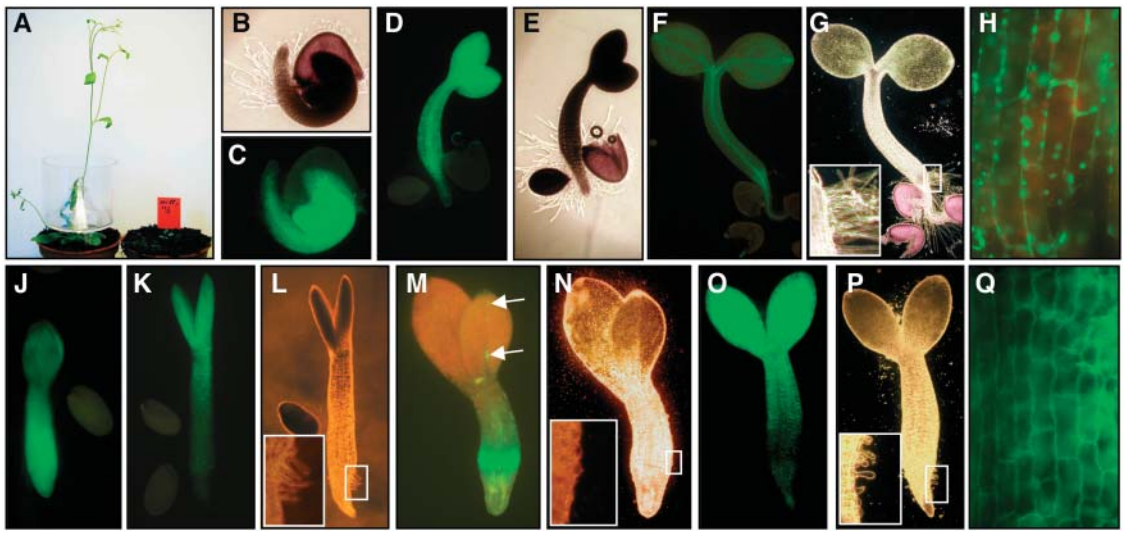

Figure 7. Ectopic Expression of *ZmTLA1* in Arabidopsis Suppressed Developmental Processes, Including Cell Morphogenesis.

(A) Wild-type (ecotype Wassilewskija) and kanamycin-selected transgenic Arabidopsis plants 32 dag.
(B) to **(H)** Germination of Arabidopsis seedlings containing a cytoplasmic GFP (*p35S:mGFP5-ER*) as a control.
(B) and **(C)** Light- and UV-microscopic images of a seedling shortly after germination initiation showed morphogenesis of root hairs.
(D) and **(E)** UV- and light-microscopic images of a seedling 3 dag showed root elongation.
(F) and **(G)** UV- and light-microscopic images of a seedling 5 dag showed further elongation of primary root and hypocotyl, greening, and, thus, biogenesis of chloroplast in the upper part of the hypocotyl and cotyledons. Inset in **(G)** shows an enlargement of the root hair zone.
(H) Enlargement of a hypocotyl region showed large cells with GFP signals in Golgi and Golgi-derived vesicles but not in the cell wall. Note the red fluorescence from mature chloroplasts.
(J) to **(Q)** Arabidopsis seedlings of different transgenic lines expressing an inducible ZmTLA1-GFP fusion protein. Development of seedlings was arrested at 6 to 10 dag in induction medium.
(J) UV microscopy of a seedling displaying strong GFP signals in the hypocotyl and root apex. Elongation of the hypocotyl and differentiation of root hairs and primary root were suppressed.
(K) and **(L)** UV and light microscopy of a seedling displaying strong GFP signals in the upper part of the hypocotyl and cotyledons. Inset in **(L)** shows an arrest of root hair development. Note that cotyledons displaying strong GFP signals remained small.
(M) and **(N)** UV and light microscopy of a seedling displaying strong GFP signals only in the lower part of the hypocotyl. Arrows point toward GFP expressing cells in one cotyledon, which remained smaller than the second cotyledon. Inset in **(N)** shows the lack of root hair differentiation in the zone displaying strong GFP signals.
(O) and **(P)** UV and light microscopy of a seedling displaying strong GFP signals only in the upper part of the hypocotyl and cotyledons. Inset in **(P)** shows an arrest of root hair development.
(Q) Enlargement of a hypocotyl region of **(O)** showing less elongated hypocotyl cells with GFP signals exclusively in the cell wall.

(Hajjar et al., 2001). The 25–amino acid peptide is also known as the haemolytical δ-toxin because it produces pores in blood cells (Mehlin et al., 1999). In summary, proteolipids seem to have pleiotropic functions, including signaling. ZmTLA1 has features of such a signaling molecule, and a fusion protein was localized in the apoplast instead of the plasma membrane and was even able to diffuse into the cell walls of neighboring cell rows. A mature and eventually modified smaller ZmTLA1 oligopeptide might diffuse over longer distances.

The other feature of ZmTLA1 is the C-terminal Caax box–like motif. This 4–amino acid motif consists of ''C'' for Cys, an ''a'' for an aliphatic amino acid, and an ''x'' for any amino acid and specifies proteolytic cleavage of the last three amino acids as well as prenylation and carboxymethylation of the Cys residue (Schafer and Rine, 1992; Ashby, 1998; Yalovsky et al., 1999). Because one aliphatic amino acid residue in ZmTLA1 is replaced by an aromatic amino acid residue, we can only speculate on whether the ZmTLA1 oligopeptide is processed and modified in a similar manner. However, the size of ZmTLA1 (27 amino acids) is in the range of fungal Caax box peptide precursors (25 to 40 amino acids), which represent a class of highly specific pheromones lacking sequence homology to each other except for the Caax box (Bölker and Kahmann, 1993; Brown and Casselton, 2001). Haploid cells of many fungi secrete Caax box peptides as chemi-attractants to communicate with compatible mating types (Leberer et al., 1997), but they were also shown to be necessary for proceeding through meiosis in *Schizosaccharomyces pombe* and *Schizophyllum commune* (Leupold et al., 1989; Fowler et al., 1999). However, most Caax box peptides are also involved in the regulation of vegetative functions (Leberer et al., 1997; Casselton, 2002). In *Cryptococcus neoformans*, for example, a Caax box peptide induces hyphal growth (Moore and Edman, 1993), and in *Ustilago maydis*, another one is required for inducing and maintaining the filamentous state (Bölker and

Kahmann, 1993). Similarly, *ZmTLA1* is most strongly expressed in haploid reproductive cells and to a lesser extent in diploid vegetative cells and displays pleiotropic functions during both sexual and vegetative development similar to fungal Caax box peptides.

Besides its structural similarities with known Caax box propeptides, our results suggest strongly that ZmTLA1 is a secreted hormone-like peptide in planta. Transient and transgenic expression of chimeric ZmTLA-GFP proteins showed that ZmTLA1 is localized in the apoplast. The observation of phenotypes in non-ZmTLA expressing tissues, where ZmTLA activity is potentially reduced, provides another indication that TLA functions as a signaling molecule. One such phenotype is the male sterility caused by insufficient disintegration of tapetal cell layers and, especially, dissolution of the callose walls surrounding the microsporogenous cells. Callase is secreted from tapetal cell layers (Hird et al., 1993), and ZmTLA1 is not expressed in the tapetum but in the adjacent microspores. Because of this timely and very specific expression, ZmTLA1 may thus be involved in signaling the induction of callase secretion as well as tapetal cell disintegration, a programmed cell death process known to be initiated at an early stage of pollen development (Ku et al., 2003).

ZmTLA is highly expressed in the wild-type egg cell (based on the EST analysis of the egg cell library, *ZmTLA1* is even the highest expressed gene); however, no clear female-specific phenotype could be observed in transgenic egg cells showing significantly reduced *ZmTLA1* transcript amounts. We assume that the remaining *ZmTLA1* transcript amounts were sufficient for ZmTLA1 function. Concerning a possible function of ZmTLA1 related to the induction of degradation processes, one may speculate that ZmTLA may also be necessary to induce, for example, degeneration of nucellar tissue that occurs after fertilization (Domínguez et al., 2001).

ZmTLA1 transcript levels in vegetative tissues were much lower than in egg cells and microspores. This could explain the more consistent phenotype observed during the vegetative development of most plants with high amounts of antisense transcript. Morphological studies revealed that downregulation of ZmTLA1 activity disrupted morphogenesis of all leaf cells except the vascular tissue. Plastid biogenesis in both bundle sheath and mesophyll cells was severely affected. Whether the arrest of chloroplast biogenesis and the transformation of chloroplasts into gerontoplast is a cause or consequence of arrested cell morphogenesis and maturation is unclear. However, the ZmTLA1 peptide was not localized in plastids, and because leaf cell morphogenesis is coordinated with chloroplast biogenesis (Biswal et al., 2003), it is likely to be a consequence of defective cell differentiation and maturation rather than a cause. TLA1 appears to be required relatively late during leaf development, after leaf primordia have been established and veins have been formed. During maize leaf development, the two photosynthetic tissues are organized in relation to leaf vasculature and develop dimorphic chloroplasts (Nelson and Langdale, 1992; Nelson and Dengler, 1997). ZmTLA1 might represent a signal that induces and maintains this differentiation process. Cells that express *ZmTLA1* during leaf development might now be identified using the putative promoter sequence described in this report.

Recently, Clay and Nelson (2002) reported a mutant in Arabidopsis that influences leaf cell patterning. The provascular/procambial cell-specific gene *VASCULAR HIGHWAY1* (*VH1*) is required to form the venation pattern in leaves. Similar to the *ZmTLA1* mutation, *vh1* affects leaf thickness. Moreover, leaves are thinned in some regions to a single epidermal cell layer, similar to the *tla* phenotype. *VH1* encodes a Leu-rich repeat RLK, and it was suggested that this cell surface receptor might transduce extracellular signals into downstream cell differentiation responses in leaves. The extracellular ligand might be a peptide, as peptides are the main ligands of Leu-rich repeat RLKs (Kobe and Deisenhofer, 1994). Whether ZmTLA1 is a player in the VH1 or a similar signaling pathway remains to be shown, but the finding that misexpression of *ZmTLA1* in Arabidopsis suppressed differentiation processes suggests that similar or orthologous peptides may exist.

The phenotype produced by *ZmTLA1* overexpression in Arabidopsis might also have a different cause. The high amounts of ZmTLA1-GFP produced in the transgenic cells could, for example, have plugged the secretion system used to transport the peptide into the apoplast. It is interesting to note here that many fungal Caax box peptides, including the yeast **a**-factor, are secreted using a specialized transport system involving, for example, ABC transporters (ATP binding cassette proteins), that, like ZmTLA1, bypass the secretory pathway (Chen et al., 1997). On the other hand, unnaturally high amounts of the hydrophobic proteolipid might interfere with the plasma membrane in overexpressing plants, or ZmTLA1 might bind unspecifically to cell wall and membrane proteins, blocking one or more differentiation processes. Weaker promoters and cell type–specific promoters should be used in future experiments to study an overexpressing phenotype. Nevertheless, we could identify one Arabidopsis line where just a few cells expressed a ZmTLA1-GFP fusion protein in one cotyledon, but morphogenesis and maturation of the whole cotyledon was affected. This finding implies a cell nonautonomous effect as expected for a secreted signaling peptide. Unfortunately, the egg cell function of ZmTLA1 remained unknown. An insertion line or inducible knockout exclusively in the egg cell will now be required to achieve complete downregulation or knockout of the *ZmTLA1* gene to elucidate its function in the egg cell and/or zygote.

METHODS

Plant Material, Isolation of Cells from the Unfertilized Embryo Sac of Maize, in Vitro Fertilization, and Maize in Vitro Cultures

Maize (*Zea mays*) inbred lines A188 (Green and Phillips, 1975) and H99 (D'Halluin et al., 1992) as well as transgenic lines *Tripsacum dactyloids* and rice (*Oryza sativa*) were grown under standard greenhouse conditions at 26°C with 16 h of light and a relative air humidity of 60%. Barley (*Hordeum vulgare*) was grown at 18°C with 16 h of light, and *Arabidopsis thaliana* plants were grown in growth chambers under high light intensity at a daylength of 16 h, relative humidity of 60%, and temperature of 22°C during the day and 20°C at night.

Cells of the maize embryo sac were isolated according to Kranz et al. (1991). In vitro zygotes were generated as described (Kranz and Lörz, 1993). Microspore-derived suspension cultures were obtained from B. Krautwig (described in Krautwig and Lörz, 1995), scutellum-derived suspension cultures, embryogenic type I and type II callus, as well as

anther callus were from S. Hansen (described in Heuer et al., 2001), and microspores prepared after Heuer et al. (2000).

Differential Plaque Screening, Promoter Isolation, DNA Sequencing, and Bioinformatic Analysis

A cDNA library of isolated maize egg cells (Dresselhaus et al., 1994) was screened by differential plaque screening (Dresselhaus et al., 1996) for candidates highly expressed in egg cells. Double plaque lifts were prepared from 15-cm plates of the egg cell library at a density of 500 plaque-forming units. The filters were hybridized with PCR-amplified [^{32}P]-cDNA from the egg cell and with either [^{32}P]-cDNA of in vitro zygotes (Dresselhaus et al., 1996) or with cDNAs from aerial parts of seedlings, 10 dag. Candidate cDNA clones were further screened by reverse RNA gel blot hybridization (Dresselhaus et al., 1999). All identified putative egg cell–specific cDNA clones were fully sequenced. In addition, ESTs of more than 1000 random clones were generated from the same egg cell cDNA library. DNA sequencing was conducted at the AGOWA sequencing service (Berlin, Germany). The putative *ZmTLA1* promoter was isolated from maize inbred line A188 using the Universal GenomeWalker kit (Clontech, Palo Alto, CA). Successful promoter PCR amplifications were obtained from a *Dra*I genomic library with the outer adapter primer AP1 (5′-CTAATACGACTCACTATAGGGC-3′) and outer *ZmTLA1*-specific primer PTLA1 (5′-CGACGACGAAACAGCAAGATCTCGATGAG-3′). The nested adapter primer NP1 (5′-TCGAGCGGCCGCCCGGGCAGGT-3′) and the nested *ZmTLA1*-specific primer PTLA2 (5′-GCCTTTGC-CTGGAGAAGAAGAGTGATGATG-3′) were used for a secondary PCR. PCR products were cloned and sequenced. The whole *ZmTLA1* gene was amplified from genomic DNA of the maize inbred line A188 with the primers PTLA5 (5′-AGGCTGGATCAAAGGCACTTTAAG-3′) and PTLA6 (5′-TCACCGAACAAATGGCGTGCGAG-3′) using proofreading *Pfu* DNA polymerase. DNA and amino acid sequence data were processed using the DNASTAR program software package (Lasergene, Madison, WI). EST clustering was performed using CAP3 (Huang and Madan, 1999). Sequence data were compiled and compared online with EMBL, GenBank, DDBJ, SwissProt, Protein Information Resource, and Protein Research Foundation databases with FASTA and BLAST algorithms (Pearson, 1990). Prediction of protein localization sites was performed online using PSORT (http://psort.nibb.ac.jp), iPSORT (http://www.HypothesisCreator.net/iPSORT), and SignalP V2.0 (http://www.cbs.dtu.dk/services/SignalP-2.0). Secondary structure prediction was performed online at http://insulin.bio.warwick.ac.uk.

Hybridization and Gene Mapping

Extraction of genomic DNA from plant tissues was performed according to Dellaporta et al. (1983). Capillary DNA gel blots were performed as described (Sambrook et al., 1989) onto Hybond N$^+$ membranes (Amersham Pharmacia Biotech, Uppsala, Sweden) with 0.4 M NaOH. After transfer, membranes were hybridized overnight with radioactive probes prepared using the Prime-It random primer labeling kit (Stratagene, La Jolla, CA) in QuickHyb (Stratagene) or Church buffer (7% SDS, 0.5 M NaH_2PO_4, pH 7.2, and 1 mM EDTA) containing 100 μg/mL of salmon sperm DNA. Membranes were then washed with decreasing concentrations of SSC with a final wash at 65°C in 0.2× SSC/0.1% SDS. Filters were exposed at −70°C to Kodak X-Omat AR or Hyperfim MP (Amersham Pharmacia Biotech) films using intensifier screens. The 350 bp of the *ZmTLA1* cDNA was amplified by PCR (primers TLAfor (5′-CACACCAC-GAGTTCGCATCAT-3′) and TLArev (5′-CCGAACGACGTTCTTAAC-CAC-3′) described in Figure 1A and used as a radioactive probe for genomic DNA gel blots and to identify polymorphism between the parents of the inbred mapping populations CO159 × TX303 and CM37 × T232A (Burr and Burr, 1991). The resulting polymorphisms were scored within the corresponding loci and placed on the Brookhaven National Laboratory map (available at http://demeter.bio.bnl.gov) using the MapMaker program (Burr and Burr, 1991).

Generation of Constructs and Plant Transformation

To generate sense and antisense constructs of *ZmTLA1*, a 350-bp *ZmTLA1* cDNA was excised from the vector (pBluescript SK−) with the restriction enzymes *Sma*I and *Asp*718 (sense) as well as *Asp*718 and *Sac*I (antisense) and ligated into the vector *pUbi.Cas* (Christensen and Quail, 1996). Cotransformation was performed using the plasmid *p35S*:*PAT* carrying the selectable marker PAT for glufosinate ammonium resistance. The maize inbred lines A188 and H99 as well as their reciprocal hybrids were used for transformation by particle bombardment according to Brettschneider et al. (1997).

The TLA-GFP construct was generated as follows: the GFP gene was PCR amplified from the GFP expression vector pMon30049 (Pang et al., 1996) with the primers GFPBam (5′-GGATCCGGCCGATGGGCAA-GGGC-3′) and g-Spe (5′-ACTAGTCACTTGTAGAGTTCATCC-3′) and the resulting fragment digested with *Bam*HI. The ORF of *ZmTLA1* was amplified with the primers z-Xho (5′-CTCGAGTTCGCATCATCACTC-3′) and z-Bgl (5′-AGATCTTCGCCCAGCAGGC-3′) and the resulting fragment was cut with *Bgl*II. The two DNA fragments were ligated over their *Bam*HI/*Bgl*II sites and PCR amplified with the primers g-Spe and z-Xho. The resulting TLA-GFP fragment was restricted with *Xho*I and *Spe*I and cloned into the appropriate interfaces of the inducible vector pTA7001 (Aoyama and Chua, 1997). The final vector was sequenced. Plasmid DNA containing the TLA-GFP fusion protein were bombarded to epidermal onion cell layers (*Allium cepa*) using a pneumatic particle gun (PDS-1000/He; Bio-Rad, Hercules, CA) according to the procedure described by Scott et al. (1999), except that inner epidermal peels (2 × 2.5 cm) were placed inside up on 0.5% agar plates. The condition of bombardment was 1100 psi rupture discs under a vacuum of 28 mm Hg with 6-cm target distance using 0.3- to 1.1-μm gold particles. After bombardment, tissues were incubated on 0.5% agar plates containing dexamethasone (Sigma-Aldrich, St. Louis, MO) to a final concentration of 10 μM and placed in the dark for 18 to 24 h at 26°C. Samples were observed directly or transferred to glass slides. As controls, a construct containing p*35S*:*GFP* (Pang et al., 1996) and p*Ubi*:*ZmES4-GFP* (fusion of the ORF of the maize *ZmES4* gene [Cordts et al., 2001] to GFP using the vector *pUbi.Cas* [Christensen and Quail, 1996]) was bombarded at epidermal onion cells and incubated on 0.5% agar plates containing the same dexamethasone concentration as used in experiments with the ZmTLA-GFP construct.

The *ZmTLA1-GFP* construct described above was also used to transform Arabidopsis. In addition, an overexpression construct was generated by inserting the full-length cDNA of *ZmTLA1* with *Bam*HI and *Xho*I into the binary vector *pB515.3*. This vector is a derivative of *pB121I* (Clontech), carrying a duplicated 35S promoter of *Cauliflower mosaic virus* and the NOS terminator. Vacuum infiltration transformation was performed with the Arabidopsis ecotype Wassilewkija according to the method described by Bechtold and Pelletier (1993) using 0.01% Silwett L-77. Transgenic seedlings were either obtained after plating surface-sterilized T1 seeds on plates containing MS salts, 0.75% agar, and 50 mg/L of kanamycin for 2 d at 4°C dark and for 1 week at 22°C with 16-h light and a relative humidity of 60% or after seeds were germinated in soil (3000 seeds/flat) and sprayed twice with a 1-d interval with 100 mg/L of kanamycin in 0.1% Triton X-100. After 1 d, a second spray test followed with 200 mg/L of kanamycin in 0.1% Triton X-100. After spraying, seedlings were covered for 24 h with a plastic dome to prevent excessive dehydration as a result of the application of the Triton X-100. For monitoring expression of the ZmTLA-GFP fusion protein, Arabidopsis seeds were sterilized with 96% ethanol for 2 min and rinsed with sterile water three to four times before placing them on medium with 0.5× MS

salts (Sigma-Aldrich) and 1× Gamborg's B5-Vitamin (Gamborg et al., 1968) without sucrose and agar. Gene expression was induced as described above.

Expression Analyses by RNA Gel Blots, Single Cell RT-PCR, and Quantitative RT-PCR

Plant material for RNA gel blot analyses was either collected in the greenhouse from different tissues and organs of the maize inbred line A188 or from in vitro cultures. RNA was extracted from all samples with TRIzol (Gibco BRL, Cleveland, OH) according to the manufacturer's specification. RNA was separated in 1.5% denaturing agarose gels and transferred with 10× SSC onto Amersham Hybond N^+ membranes. RNA was cross-linked to membranes with 300 mJ of radiation in a UV Stratalinker 1800 (Stratagene). Hybridization, washing, and exposure were performed according to the procedure described for DNA gel blots. Single cell RT-PCR analysis was performed as described by Cordts et al. (2001) using the primers TLAfor and TLArev described above. Thirty-five PCR cycles were conducted, and 10 μL of the PCR products was separated in an agarose gel, blotted, and hybridized as described above.

Quantitative RT-PCR analyses were performed as follows: poly(A)$^+$ mRNA was extracted from four to six anthers using the Dynabeads mRNA DIRECT Micro kit (Dynal Biotech, Oslo, Norway). Immediately after isolation, poly(A)$^+$ mRNA was used for first-strand cDNA synthesis for 75 min at 50°C, using an oligo(dT)$_{18}$ primer, RevertAid H^- reverse transcriptase, and RNase inhibitor (all MBI-Fermentas, St. Leon-Rot, Germany), according to the manufacturer's instructions. Two separate real-time PCRs were performed on each cDNA using either the *ZmTLA1*-specific primers TLA1 (5′-CTCTTCTTCTCCAGGCAAAGG-3′) and TLA2 (5′-CTATCAGGGAAACAGCAACGAC-3′; 400 nM each primer) or *GAPDH*-specific primers Gap1 and Gap2 (400 nM each primer; Richert et al., 1996) using an iCycler iQ machine and iQ SYBR Green Supermix (both Bio-Rad), according to the manufacturer's recommendations. PCR results were controlled by agarose gel electrophoresis and processed with the iCycler iQ real-time detection system software, version 3.0, for Windows (Bio-Rad). *GAPDH*-specific PCR products were used to normalize *ZmTLA1* transcript amounts. Single cell quantitative RT-PCR analysis was performed with isolated egg cells. Poly(A)$^+$ mRNA extraction and cDNA synthesis were performed with single cells as described above, with the exception that the *ZmTLA1*-specific primer TLA2 and *GAPDH*-specific primer Gap2 were used in the same RT reaction, according to the protocol described by Richert et al. (1996). RT products were divided and separately amplified by real-time PCR as described above. Egg cells without RT reaction and water controls were always used to control amplification from genomic DNA or contaminations. PCR results were always controlled by agarose gel electrophoresis. Samples showing both *ZmEA1*- and *GAPDH*-specific amplifications were further processed as described above.

Microscopy

Leaf and anther samples were hand-sectioned using a razor blade and analyzed by light microscopy (Olympus IMT-2; Tokyo, Japan), fluorescence microscopy (Zeiss Axiovert 35M or 200; Jena, Germany), and CLSM (TCS40 Leica-Laser-Technology' Wetzlar, Germany), the latter as described by Knebel et al. (1990). Samples were excited with UV light produced by an HBO 50/Ac lamp using Zeiss filter set 38 (excitation, band-pass 470/40 nm; emission, band-pass 525/50 nm) at the Axiovert microscope for GFP measurements, a fluorescein isothiocyanate filter (Zeiss; excitation at 450 to 490 nm and emission at >515) was used to visualize GFP and chloroplast autofluorescence simultaneously, and a 4′,6-diamidino-2-phenylindole filter (Zeiss; excitation at 359 to 371 nm and emission >397 nm) to visualize DNA and cell wall material. For fluorescence analysis using the CLSM, samples were excited by 488 nm with an argon laser. Meterial for transmission electron microscopy was prepared as described by Robinson et al. (1987).

Nucleotide sequence data from this article have been deposited with the EMBL/GenBank/DDBJ data libraries under the following accession numbers: AY211982 (*ZmTLA1* cDNA) and AY736279 (*ZmTLA1* gene).

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