The *Etched1* gene of *Zea mays* (L.) encodes a zinc ribbon protein that belongs to the transcriptionally active chromosome (TAC) of plastids and is similar to the transcription factor TFIIIS

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Summary

*Etched1* (*et1*) is a pleiotropic, recessive mutation of maize that causes fissured and cracked mature kernels and virecent seedlings. Microscopic examinations of the *et1* phenotype revealed an aberrant plastid development in mutant kernels and mutant leaves. Here, we report on the cloning of the *et1* gene by transposon tagging, the localization of the gene product in chloroplasts, and its putative function in the plastid transcriptional apparatus. Several alleles of *Mutator (Mu)*-induced *et1* mutants, the *et1-reference (et1-R)* mutant, and *Et1* wild-type were cloned and analyzed at the molecular level. Northern analyses with wild-type plants revealed that *Et1* transcripts are present in kernels, leaves, and other types of tissue, and no *Et1* expression could be detected in the *et1* mutants analyzed. The ET1 protein is imported by chloroplasts and has been immunologically detected in transcriptionally active chromosome (TAC) fractions derived from chloroplasts. Accordingly, the relative transcriptional activity of TAC fractions was significantly reduced in chloroplasts of *et1-R* plants. ET1 is the first zinc ribbon (ZR) protein shown to be targeted to plastids. With regard to its localization and its striking structural similarity to the eukaryotic transcription elongation factor TFIIIS, it is feasible that ET1 functions in plastid transcription elongation by reactivation of arrested RNA polymerases.

Keywords: *etched1*, plastid nucleoids, transcriptional active chromosome, transposon tagging, plastid transcription, TFIIIS.

Introduction

The analysis of genes involved in the development of the maize endosperm is of particular interest in order to understand the structural and regulatory features of seed growth. In maize, many mutants and a number of genes affecting tissue and organ development have been isolated. Numerous mutants have been described, which exhibit abnormal endosperm development (for review: Coe et al., 1988). Among these, a few are pleiotropic and thus of particular interest because they affect the development of different tissue types. *Etched1* (*et1*) is one such mutation, which affects the development of kernels as well as of seedlings. *Etched1* is a recessive mutation that was first identified and described by Stadler (1940). The mutant reference allele (*et1-reference (et1-R)*) was isolated from the progeny of a population of maize plants pollinated with X-ray irradiated pollen. Kernels homozygous for the *et1-R* allele are fissured because of depressions and crevices on the endosperm surface (Figure 1a–c). Prior biochemical and
structural analyses of the et1-R kernels revealed that starch synthesis is reduced and starchless endosperm cells are present around the cracks and scars in the kernels (Figure 1; Sangeetha and Reddy, 1991). The et1 kernel phenotype becomes visible approximately 15 days after pollination (DAP). The degree of etching differs among the kernels on an ear and can vary from a weak to a very severe phenotype. This phenotypic variation is apparently not correlated with any specific genetic background (Sangeetha and Reddy, 1991).

et1 mutant seedlings exhibit a virescent phenotype, i.e. their leaves are initially pale green (Figure 1d,g) but become fully green approximately 2 weeks after germination. The effect of the et1 mutation on seedling growth primarily concerns chloroplast development. During initial leaf growth, the morphogenesis of chloroplasts in mutant seedlings is impaired or delayed (Figure 1k-m). Carotenoids, chlorophyll pigments, and chlorophyll-protein complexes accumulate at reduced levels in the et1 leaves (Ramesh et al., 1984; Sangeetha et al., 1986). However, after approximately 2 weeks, a shift to normal chloroplast development occurs and the yellow to pale-green leaves turn fully green. Further growth and development of the plant is normal and does not appear to be influenced by the mutation (Sangeetha and Reddy, 1991). This suggests that the ET1 protein is required for a fundamental process during plastid differentiation early in seedling and kernel development.

Additional et1 mutants (et1-M) have been isolated from active Mutator (Mu) lines (Scanlon et al., 1994; this report). Among these mutants, some exhibit a phenotype distinct from that of the et1-R plants. The homozygous kernels for these et1 alleles are shrunken, severely etched, and develop either no or only small embryos. These differences in phenotypes appear to be the result of allelic differences among the mutants. Scanlon et al. (1994) have shown that crosses of these et1-M mutants with the reference allele weaken the severe phenotype in an allele dosage-dependent manner.

In this report, we describe the isolation and characterization of the ET1 gene using a forward gene-tagging approach. We further report on the identification of the ET1 protein as part of the transcriptionally active chromosome (TAC) derived from plastid nucleoids (Hallick et al., 1976; Krause and Krupinska, 2000; Suck et al., 1996). The sequence of the ET1 protein exhibits significant similarity to the zinc ribbon (ZR) domain of the eukaryotic transcription factor TFIIIS. TFIIIS (also known as SII) is capable of reactivating arrested RNA polymerase II (Pol II) by inducing the intrinsic ribonuclease activity of the polymerase. After removal of displaced nucleotides at the 3′ end of the nascent RNA, polymerization is re-started (Kettenberger et al., 2003; Opalka et al., 2003).

ET1 is the first identified member of a new family of plastid localized ZR proteins. Based on our data, we hypothesize that the ET1 protein plays a role in transcript elongation in chloroplasts similar to the role of TFIIIS in RNA Pol II transcription. This function is in agreement with the retarded chloroplast development observed in leaves of the et1 mutant.

Results

Isolation of the et1 gene from the Mu-induced allele, et1-M3

Several Mu-induced et1 mutants were used for the isolation of the gene locus by a transposon-tagging strategy (review: Chomet, 1994; Wienes et al., 1982). Cloning of the et1 gene was conducted using et1-M3, one of the Mu transposon-induced mutant alleles available (Scanlon et al., 1994). A modified amplification of insertion mutagenized sites (AIMS) procedure (Frey et al., 1998; Lauert et al., 1999) was used to identify and isolate genomic fragments that co-segregated with the etched phenotype. A 171-bp HpaII/G PCR fragment was identified from populations segregating for mutant (et1-R/et1-M3) and wild-type (et1-R/Et1) phenotypes (AIMS-1; Figure 2). This fragment could only be amplified from genomic DNA isolated from mutant plants (21 individuals analyzed) and was not detectable in wild-type plants (15 individuals analyzed). In a more detailed AIMS analysis using this mutant, two further PCR fragments, an 83-bp Msel/C and a 157-bp HincII/G fragment (AIMS-2 and

Figure 1. etched1 phenotype.
(a-c) Stereomicroscopic view of mature, desiccated kernels homozygous for et1-R, et1-M3, and et1-M16, respectively. The fissures observed on the kernel surface are a characteristic phenotype of et1 mutants (blue arrow).
(d, g) Virescent seedlings of et1-M15/et1-R and et1-R genotypes, respectively.
(e, f) Thin transverse hand sections of 30 DAP et1-R kernels. (e) Transverse section stained with IKI. The starch endosperm cells are colored deep violet. Starchless cells can be observed as colorless regions radiating out from the center up to the periphery (white arrows). (f) Enlarged stereo-microscopic view (5 ×) of the endosperm showing starchless (si) sectors and a depression (blue arrow).
(h) Enlarged stereo-microscopic view (20 ×) of the peripheral region of an si sector. Note the typical surface depression and a colored and intact aleurone (al) layer, which is pulled away from the multilayered pericarp (pc).
(i) Longitudinal cross-section of a mature, desiccated et1-M3/et1-R kernel photographed under UV-epifluorescent light. The arrow points to a fissure as shown in (a) and (h). Note the presence of intact al cells and collapsed walls of the si endosperm cells beneath the fissure (e = endosperm).
(j) Longitudinal cross-section of a mature, desiccated LC (wild-type) kernel photographed under UV-epifluorescent light. Note the well-structured al layer and etissue.
(k, l) Ultrastructure of et1-M3/et1-R chloroplasts. Sections were prepared from leaves of virescent seedlings. Note the lack of organized thylakoid and grana structures (arrows).
(m) Ultrastructure of a wild-type chloroplast isolated from LC with highly organized thylakoid structures (bars = 1 μm).

AIMS-3, Figure 3a), were identified to co-segregate with the et1-M phenotype.

The 171-bp HpaI/G fragment was used to isolate putative Et1 genomic clones from a lambda library derived from plants with a heterozygous et1-M3/Et1-B73 genotype, from which clones representing wild-type (Et1) or mutant alleles (et1) could be expected. Four different clones were isolated from this screen. One of them, λet1-M3-c5, that contains a Mu8 element insert (Figure 3a) represents the mutant allele. Two other clones, λEt1-c11 and λEt1-c14 that lack Mu insertions, represent wild-type allele Et1-B73 (Figure 3b). A 2.5-kbp HindIII/Xhol fragment (Frag 3 in Figure 3b) from clone λEt1-c11 was used for RFLP mapping. This clone mapped to the long arm of chromosome 3 (3.09), between markers cdo962B and npi425A, i.e. approximately to the same position to which the et1 gene has been mapped (Neuffer et al., 1997).

Analysis of additional et1 mutant alleles

Further evidence of having cloned the Et1 gene came from the analysis of additional et1-M alleles as well as of the reference allele et1-R. Single-copy sequences from clones λet1-c11 and λet1-c14 were used to clone and isolate corresponding sequences from the mutant alleles et1-M12 (provided by P. Stinar), et1-M15, and et1-M16 (both isolated by a directed tagging approach). The sequence of et1-M10 (Scanlon et al., 1994) was isolated via PCR amplification experiments.

A detailed analysis of the et1 locus from these et1-M mutants revealed a Mu insertion in all these alleles, either at the same position or in the vicinity of the Mu8 insertion found in et1-M3 (Figure 4a). Hybridization analysis of the et1-R genomic clone using different wild-type genomic fragments as probes revealed deletions and rearrangements around the locus (Figure 3c,d). In contrast to the mutant, wild-type alleles from the inbred genotypes Line C (LC) and B73 (the progenitor of et1-M3) contain no Mu element insertions in the corresponding sequences. These analyses strongly indicated that the cloned sequences represent the et1 locus.

Sequence analysis of Et1 and et1 alleles

The Et1 sequences from wild-type clones mentioned above were used to isolate a full-length Et1 cDNA (cEt1-9) from a
lambda cDNA library prepared from wild-type LC-developing kernels 13–28 DAP. This cDNA is 772 bp in length and contains an open-reading frame (ORF) of 163 amino acids (aa; Figure 4a). The structure of the gene was elucidated by comparison of this cDNA sequence to wild-type genomic clones. The Et1 gene contains four exons of length 164, 88, 129, and 281 bp, and three introns of length 75, 670, and 145 bp (data not shown).

By comparison of wild-type Et1 gene structure with the et1-M mutant alleles analyzed, the positions of the Mu element insertions were identified. In the alleles et1-M3, et1-M10, et1-M12, and et1-M15, a Mu8 element was present in the 5′ untranslated region (UTR) of the et1 gene. Interestingly, the position of insertion and orientation of the Mu element was identical for et1-M10 and et1-M12 (in 5′ to 3′ orientation), as well as for et1-M3 and et1-M15 (in 3′ to 5′ orientation). This is not rather surprising as independent Mu-insertion sites at identical positions have also been described for glossy and bronze genes (Dietrich et al., 2002). Moreover, these two positions of insertion were in close proximity to each other (Figure 4a).

In the case of the et1-M16 allele, a Mu1 element was present in the first exon within the ORF in 5′ to 3′ orientation. In the case of et1-R, nearly the complete et1 gene was

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**Figure 3.** The et1 genomic locus and a comparison between the wild-type, et1-M3, and et1-R alleles.

(a) Restriction enzyme map of the et1-M3 mutant genomic λ-clone et1-M3-c5. The position of a Mu8 element insertion at the et1 locus is shown as a filled triangle. This region is shown enlarged above the clone with the positions of the isolated AIMS fragments (which co-segregate with the et1-M3 mutation) relative to the position of the Mu8 insertion (AIMS-1: 171-bp HpalI/G fragment; AIMS-2: 83-bp MseI/C fragment; and AIMS-3: 157-bp HpiII/G fragment).

(b) Restriction enzyme map based on two Et1 wild-type genomic λ-clones, Et1-c14 and Et1-c11. For comparison, the position of the Mu8 element insertion in the et1-M3 mutant allele (see a) is shown as a dashed triangle. Subfragments of the two wild-type clones (Fig 1–Fig 3) were used for further hybridization experiments (description below).

(c) Restriction enzyme map of the et1-R genomic λ-clone et1-R-c3. The regions hybridizing with wild-type Et1 probes Frag 1–Frag 3 are shown as open rectangles. The dotted lines symbolize that the exact location is not known.

(d) Schematic comparison between the sequences derived from Et1 Frag 3 and et1-R Frag 4. Note that the sequence identity does not encompass the transcribed region of the Et1 gene. The |−| marks the position of the first nucleotide of the cDNA clone cEt1-9. A dashed triangle indicates the location of the Mu8 insertion in the et1-M3 mutant allele.

The open circles in (a,c) represent the arms of the lambda vector. B, BamHI; H, HindIII; E, EcoRI; and X, Xhol.
found to be deleted (Figure 3d), which is consistent with the finding that X-ray treatment usually causes DNA deletions.

To further prove the correct cloning of the et1 gene, a green revertant sector of a young and otherwise pale et1-M3 seedling leaves was analyzed via PCR experiments. These experiments revealed that in the revertant sector, the Mu8 element was absent from its position of insertion in the et1-M3 allele. However, an imperfect target-site duplication, typically left behind by transposons after excision, was observed (Figure 4b). As this Mu8 element was present in the 5' UTR of the gene, the transposon's excision footprint did not disturb the ORF, resulting in normal chloroplast development and wild-type phenotype in the revertant sector of the seedling leaf.

Expression of the Et1 gene

Northern experiments were performed with total RNA from different tissue types of wild-type as well as of mutant plants using the 3' UTR of the Et1 gene as a probe. No Et1 expression was observed in any of the analyzed et1 mutants (Figure 5a,b). The hybridizing mRNA observed in wild-type plants corresponded to a transcript length of approximately 750 nucleotides, as expected from the length of the cloned cDNA (Figure 4). The strongest hybridization signals were observed with RNAs from leaves at different stages of development, and from developing kernels (Figure 5a). In other parts of the plant, e.g. stems and silks, lower amounts of Et1-hybridizing mRNA accumulated (data not shown). After separating the embryo from 20 DAP kernels, Et1 mRNA was almost exclusively detected in the remaining kernel tissue and not in the embryos (data not shown). This indicates that in the wild type, Et1 is predominantly expressed in those types of tissue that exhibit the et1 phenotype in the mutants.

To determine whether the expression of Et1 in leaves is stimulated by light, RNA isolated from dark-grown etiolated wild-type seedlings was used for Northern analysis. These plants had a highly reduced Et1 expression when compared to seedlings grown under normal light conditions (Figure 5b), indicating that light is a factor triggering Et1 expression in this tissue.

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ET1 protein structure

The putative ET1 protein is 163 aa in length and has a deduced molecular weight of 19.6 kDa. Computational analyses of the ET1 protein sequence predicted an N-terminal transit sequence for plastid import. After cleavage of the transit sequence, the predicted mature protein would contain 99 aa with a predicted molecular weight of 11.9 kDa (Figure 4a). Database searches revealed a significant sequence similarity of the mature ET1 protein to the ZR domain (domain III) of the eukaryotic transcription factor TFIIIS (also known as SII) from yeast, fission yeast, and human (Figure 6a,b). The ET1 sequence has lower similarity to other ZR-containing proteins, e.g. RNA polymerase II subunit 9 (Rpb9) (Figure 6a; Hemming and Edwards, 2000; Wang et al., 1998). Based on tertiary structure models of yeast TFIIIS and the TFIIIS/RNA Pol II complex, the ZR domain is characterized by a three-stranded β-sheet structure, possessing four cysteine residues, which are held together by a zinc atom and an acidic hairpin loop containing two invariant acidic residues, D and E, essential for TFIIIS activity. The TFIIIS ZR domain inserts deeply into the RNA Pol II pore and positions the two acidic residues in the enzyme’s active site. In eukaryotes, TFIIIS is essential for reactivating arrested RNA Pol II complexes. This is achieved by a conformational change in the active site of the enzyme that enhances the intrinsic ribonuclease activity. Ribonucleolytic cleavage of displaced 3’ ends of nascent RNA is required for progression of RNA polymerization (Awrey et al., 1998; Conaway et al., 2003; Kettenberger et al., 2003 and citations therein; Opalka et al., 2003 and citations therein). Thereby, TFIIIS mitigates pausing of the RNA polymerase and increases the overall elongation rate.

Among the conserved amino acids shared between TFIIIS and ET1 are four putative cysteine ligands of the zinc atom and one of the two acidic residues (aa E116; Figure 6a). The secondary structure of the ET1 protein was deduced using different in silico methods. Most of these methods predicted a three-stranded β-sheet structure strikingly similar to that of TFIIIS. In addition, two α-helices in the interdomain linker and in the C-terminal region of domain II from TFIIIS might also be conserved in ET1 (Figure 6a). Tertiary structure modeling of the ET1 ZR domain using the yeast TFIIIS domain as template resulted in a comparable ZR structure for ET1 (Figure 6d). As is the case with residue E291 in TFIIIS (Kettenberger et al., 2003), the acidic residue E116 of ET1 is predicted to be exposed at the tip of the hairpin loop.

Furtheron, a number of non-annotated putative plant proteins with a high sequence similarity to ET1 were identified (Figure 6a,c). For none of these proteins a function has been assigned yet. The region of similarity is mainly restricted to the ZR domain. In maize, a very close ET1 homolog, Zea mays zinc ribbon 1 (ZmZR1; 87% similarity at the protein level) was cloned from the et1-R line (unpublished data). A phylogenetic analysis of ZR1 proteins showed two distinct subgroups for monocotyledonous and dicotyledonous plants, respectively. A putative ZR protein from the fern Ceratopteris richardii is more related to the dicotyledonous group (Figure 6c).

Computational analyses of the deduced ZmET1 and ZmZR1 proteins predicted N-terminal plastid target sequences for both (Figures 4 and 6b and data not shown). All other putative ZR1 plant proteins were also predicted to contain N-terminal plastid import sequences (data not shown).
The ET1 protein is imported into chloroplasts

To verify that ET1 is a plastid protein, chloroplast import experiments were conducted using in vitro translated and radioactively labeled ET1 protein and spinach chloroplasts. These experiments (Figure 7) showed that the ET1 protein is imported into the stroma of chloroplasts and that it contains an N-terminal transit peptide, which is cleaved off after transport, resulting in a reduced size of the mature protein in the stroma fraction. The size of the processed protein is in agreement with the prediction (Figure 4a). The absence of the ET1 protein from membrane fractions either treated with protease or untreated indicates that it is not associated with thylakoid membranes (Figure 7).

To determine if ET1 is imported into chloroplasts through the general import pathway (Soll and Tien, 1998), an unlabeled competitor protein, known to be transported through this pathway, was included in these experiments. With increasing amounts of the competitor protein, decreasing amounts of ET1 were detected in the chloroplast stroma.
fraction (Figure 7b), clearly showing that the ET1 protein is imported into the stroma of chloroplasts via the general import pathway.

**The ET1 protein is a component of the TAC of chloroplasts**

Computational analysis of ET1 and its similarity to TFIIIS suggested a putative function of the protein in the transcriptional apparatus of plastids. Upon lysis of plastids, transcriptional activity is found in the soluble fraction and in the membrane fraction (Igloi and Kössel, 1992). The latter fraction is tightly bound to the plastid DNA and is capable of elongating transcripts that have already been initiated in vivo (Halicck et al., 1976). Compared to the original crude TAC fraction (Halicck et al., 1976) the specific activity of this TAC may be significantly enhanced by different purification steps (Krause and Krupinska, 2000).

A crude fraction (TACI) and a highly purified fraction (TACII) were prepared from chloroplasts of the wild type (ET1) and of non-virent et1 mutant (et1-R) leaves collected from about 4-week-old plants, respectively. Transcriptional activity was determined by incorporation of $^{32}$P-labeled nucleotides into transcripts initiated before preparation of TAC (Krupinska and Falk, 1994). Maize TACII fractions had a fourfold higher specific activity than TACI fractions. The relative activity of both et1-R-derived TAC fractions was less than half when compared to the relative activity of the corresponding TAC fractions from wild-type leaves (Figure 8a). These differences in the overall transcriptional activity are not reflected by qualitative changes in the composition of transcripts. The latter has been shown by hybridization of TAC-derived transcripts with 22 selected plastid genes represented on a DNA-dot-blot filter (provided as Supplementary Material). The filter carried probes of genes with either plastid-encoded RNA

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polymerase (PEP; psaA, psbA, psbC–psbF, rbcL, and ndhH) or nuclear-encoded phage-type RNA polymerase (NEP) promoter (rpoA, and rpoB/C operon) and of genes possessing both types of promoters (rrn operon, atpB). Relative transcriptional activities among the selected genes in both cases were similar to those observed in mature chloroplasts derived from fully expanded primary foliage leaves of barley (Krupinska and Falk, 1994). Taken together, these data suggest that the lack of ET1 protein leads to a reduced transcription rate without affecting the gene specificity of the transcriptional apparatus.

To investigate whether the ET1 protein belongs to the TAC fraction, TAC proteins derived from wild-type and et1-R mutant chloroplasts were analyzed immunologically with a polyclonal antibody raised against a deduced ET1 peptide sequence. The specificity of the antibody was shown before by precipitation of the in vitro translated ET1 pre-protein (data not shown). Western analyses were performed with total plastid proteins and with proteins derived from crude (TACI) and highly purified (TACII) TAC fractions, respectively (Figure 8b). As shown before for the purified TAC fraction prepared from spinach (Krause and Krupinska, 2000) in the TACII fraction derived from maize wild-type and mutant chloroplasts, 30–40 polypeptides were detectable by silver staining. TACI and TACII samples loaded onto the gel had similar protein contents but different transcriptional activities, respectively. While the amounts of TACI corresponded to 5000 U relative transcriptional activity, the amounts of TACII corresponded to 20 000 U of relative transcriptional activity (Figure 8a,b). Immunological analyses clearly revealed the presence of a protein of 12 kDa in TAC fractions derived from wild-type chloroplasts. The expected molecular weight of the processed ET1 protein is 12 kDa. The protein is highly enriched with the TAC fractions as it is not detectable in unfractonated protein extracts from chloroplasts (Figure 8b; arrow). Because of its higher relative abundance in TACII compared to TACI, the abundance of the 12 kDa protein in wild-type chloroplasts rather correlates with the relative transcriptional activity than with the amount of loaded protein, which is equal for TACI and TACII (Figure 8b). In comparison to the TAC fractions from wild-type chloroplasts, TAC fractions from the et1-R mutant plastids gave only very faint signals after immunodetection, although higher protein amounts had been loaded on the gel because of the reduced transcriptional activity of et1-R TAC fractions (Figure 8a,b). As the peptide antibody is directed against a region present in both, ET1 and its homolog ZmZR1, it is conceivable that the weak detected signal is caused by a cross-reaction of the antibody with the ZmZR1 protein.

These results suggest that ET1 is a constituent of the TAC and that its abundance correlates with the rate of overall transcriptional activity in this fraction.

**Discussion**

**Isolation of the et1 gene**

The cloning of genes via transposon tagging is a well-established procedure in maize (review: Chomet, 1994;
Wienand et al., 1982). To clone the affected gene of a Mu-induced mutant, a genomic fragment containing a transposon-specific sequence co-segregating with the mutant phenotype had to be identified. Several independent Mu-induced et1 alleles were used for the isolation and investigation of the et1 locus. The et1 gene was characterized through the molecular analysis of six different et1 mutants. The presence of a Mu8 element in the 5′ UTR of four alleles, viz et1-M3, et1-M10, et1-M12, and et1-M15, and a Mu1 element within the ORF in exon1 of the et1-M16 allele was identified (Figure 4a). No Mu inserts were detected in wild-type Et1 genes.

Germlinal revertants or somatic revertant sectors can provide further evidence of the successful cloning of a gene. As germlinal revertants are rarely observed for Mu-induced mutants, somatic revertant sectors were often used to show that the excision of the element from the respective locus restores the gene activity and is responsible for wild-type phenotype in these sectors. This was shown for et1-M3 by analyzing a revertant leaf sector. The Mu8 element was found to be missing from the et1 locus in the green revertant sectors of et1-M3 leaves; however, duplicated target sites of insertion (footprints) were still present (Figure 4b). As the element had integrated into the 5′ UTR of the gene, its excision did not interfere with the transcription and translation of the gene.

The et1 phenotype is observed in kernels and also during the first 2 weeks after seedling germination. Expression of the Et1 gene was detected during endosperm development in wild-type plants at 10, 15 and 20 DAP. Generally, this period comprises the stages of endosperm differentiation and kernel desiccation, which begin after complete cellularization, taking place at 4 DAP (Becraft and Asuncion-Crabb, 2000; Kranz et al., 1998). Starch synthesis also takes place about 2 weeks or less after fertilization (Kiesselbach, 1949). In contrast to the wild type, in all the analyzed mutants, no Et1 expression was observed during this developmental period. Thus, the absence of Et1 transcripts in the mutants very likely leads to the aberrant development of the endosperm during this stage of development. In combination, the presented data demonstrate the successful cloning of the Et1 gene.

The et1-R mutant allele was derived from an X-ray-treated population. Molecular analysis of genomic clones in et1-R indicated that rearrangements and deletions of the locus apparently occurred during the mutation event in this line (Figure 3). The irradiation apparently deleted the entire coding region of the et1 gene, leaving only the promoter region intact. Hence, et1-R is expected to be a null allele. This is surprising given that the et1-R allele conditions a ‘weaker’ mutant phenotype than the et1-M alleles. One possible explanation takes into account the fact that the et1 mutants originated from different genetic backgrounds. Differential activities because of allelic variations of an et1 homolog might be responsible for the phenotypic variations in these lines. A high degree of allelic divergence and a differential gene expression because of line-dependent allelic variation is a characteristic feature of maize (Fu and Dooner, 2002; Kim and Krishnan, 2003; Song and Messing, 2002). It could also be that in phenotypically severe et1 mutant lines, Zmzr1 expression is repressed, whereas in only some (e.g. et1-R) weak expression is going on. Such an observation has been made in the analysis of the anthocyanin biosynthesis repressor gene intensifier1 (Burr et al., 1996).

Microscopic examinations of the developing endosperm of et1-R mutant kernels (Figure 1) revealed that the effect of the et1 mutation appears to be restricted mainly to small dispersed radial sectors of endosperm radiating out from the center up to the periphery (Figure 1e,f,h). The leaves of the et1 mutant show a temporary phenotype that depends on the developmental stage of the plant. Therefore, it is also conceivable that another gene or a set of genes might take over the role of Et1 and restore normal plastid development during later stages of leaf development. A specific spatial or temporal expression of these genes could also be responsible for the observed phenotype in kernels. Indeed, such a close Et1 homolog was identified in the et1-R line (Zmzr1; Figure 6). The transcribed regions of Et1 and Zmzr1 are very similar, whereas the promoter sequences of both genes are highly variable pointing to a different control of gene expression for both genes (data not shown). Expression of the Et1 gene in seedlings was repressed in the dark (Figure 5b), which is in accordance with a function of the gene product during chloroplast development. Although the Zmzr1 cDNA was cloned from a cDNA library of et1-R kernels, several attempts to analyze the Zmzr1 expression by Northern analysis failed so far, most likely because of a very low abundance of the corresponding mRNA. Accordingly, it was only possible to amplify Zmzr1 corresponding cDNA fragments by two successive rounds of RT-PCR from the et1-R cDNA library (data not shown). As expression of the Zmzr1 gene is not detectable in total RNA of either light- or dark-grown et1-R seedlings, the level of the ET1 homolog in the et1 mutants may be not sufficient to fully compensate for the function of ET1 during chloroplast development. In agreement with these findings, only a faint 12-kDa protein was detected in TAC fractions derived from et1-R plastids by the antibody, which is specific for a peptide sequence included in both ET1 and Zm2R1.

Analogously to et1, it has been shown that mutations in the gene of the maize chloroplast membrane protein high chlorophyll fluorescence 106 (HCF106) caused by the insertion of Mu elements could partially be compensated by the closely related homolog HCF106c. This homolog has been mapped to a region that is part of the most recent set of segmental duplications in the maize genome (Settles et al., 2001). It is conceivable that Et1 and Zmzr1 might be
connected in a similar evolutionary way. This is further supported by a comparison between the identified putative ZR1 proteins, which suggest that in most plants only one ZR1 gene is present, e.g. AtZR1 in Arabidopsis, whereas more than one putative ZR1 protein could only be found in maize (ET1 and ZmZR1) and in the polyploid plant wheat (TaZR1a–TaZR1c; Figure 6c).

Role of the ET1 protein in the plastids

The pleiotropic effect of the et1 mutation, affecting both amyloplast and chloroplast development, led to the hypothesis that the gene might be involved in an essential process common to development of amyloplasts as well as chloroplasts. Chloroplast import experiments demonstrated that the ET1 protein is indeed targeted to the stroma of plastids. Therefore, ET1 is the first described ZR-containing protein in this organelle.

Comparison of the ET1 protein to online databases and its in silico structural analysis revealed that it exhibits some similarity to C-terminal ZR domains of proteins interacting with nucleic acids, e.g. that of the eukaryotic RNA Pol II subunit Rpb9 (Figure 6a) and the transcription factor TFII B (Chen et al., 2000; Hemming et al., 2000; data not shown). Highest similarity was found to the ZR domain of the eukaryotic transcription elongation factor TFII S (Figure 6a). This factor is composed of an N-terminal domain I, a central domain II, and the C-terminal ZR (domain III) that contains a protruding acidic β-hairpin turn (Figure 6b). The ZR domain is an ubiquitous motif in the archaeeal as well as in the eukaryotic transcription machinery (Chen et al., 2000).

Furthermore, Männistö et al. (2003) showed that a ZR containing transcriptional regulator is encoded by the bacteriophage PM2 genome and is capable of regulating the eubacterial RNA polymerase. TFII S in eukaryotes and its functional counterpart GreB in bacteria are the only known transcription factors that are capable of restarting arrested RNA polymerases, thereby ensuring efficient mRNA synthesis (Conaway et al., 2003; Uptain et al., 1997; Wind and Reines, 2000). RNA elongation is blocked when the RNA transcript loses contact with the DNA template (Erie, 2002; Shilatifard et al., 2003). TFII S mediates the restart of the RNA Pol II by a conformational change in the active center of Pol II, leading to the activation of its intrinsic RNA nuclease activity. During the conformational change, the acidic residues of the ZR β-hairpin are positioned into the active site (Kettenberger et al., 2003). Because of the TFII S-promoted cleavage of a displaced 3’ end of the RNA, a new 3’ end that is properly base paired with the DNA is produced and can be elongated by the polymerase (Uptain et al., 1997; Wind and Reines, 2000).

Plastid transcriptional activity can be detected in two fractions, a soluble and a membrane fraction (Iglói and Kössel, 1992). In the latter fraction, which is called TAC, the transcriptional apparatus is tightly associated with the plastid DNA and is capable of elongating transcripts that have been already initiated in vivo (Hallick et al., 1976). Western analysis demonstrated that the ET1 protein belongs to the TAC fraction and that the abundance of ET1 correlates with the relative transcriptional activity of the complex (Figure 8b). In the et1-R mutant line, the relative transcriptional activity of both a crude and a highly purified TAC fraction is significantly reduced compared to wild-type plants (Figure 8a). As the TAC fractions were prepared from non-virescent green leaves of the mutant, the differences in plastid transcriptional activity are not likely to be caused by secondary effects of the et1 mutation.

Despite the differences in the rate of overall transcription, the relative transcriptional activities of different plastid genes in this fraction are similar as investigated by hybridization of in vitro elongated TAC transcripts with selected genes (data not shown). These results indicate that the ET1 protein stimulates overall transcriptional activity and does not affect the relative transcription of specific genes. Based on the structural model that has been published for the interaction of TFII S with RNA Pol II in yeast (Kettenberger et al., 2003) and GreB with the eubacterial RNA polymerase (Opalka et al., 2003), a similar mechanism of interaction with a plastid RNA polymerase might be considered for the ZR domain of ET1. Although the ZR domain of ET1 shows the highest similarity to that of TFII S when compared to other ZR containing transcriptional regulators from the database, it could not be excluded that ET1 function in plastids might differ from that of TFII S.

Plastids possess at least two different types of RNA polymerases (Billig et al., 2000; Hettké et al., 2000; Maliga, 1998; review: Hess and Böörner, 1999). If ET1 is influencing plastid transcription, it could do so by acting as a regulatory subunit of at least one of these RNA polymerases. So far, it has been shown that the TAC fraction contains the PEP of the eubacterial type (Suck et al., 1996). Transcription of housekeeping genes by the TAC-associated polymerase(s) (Iglói and Kössel, 1992; Krupinska and Falk, 1994) however suggests that the NEP is included in the fraction, too. ZR-like proteins have been found to be part of eukaryotic as well as of archaeal and prokaryotic transcriptional complexes (Chen et al., 2000). Even a bacteriophage encodes a ZR-containing factor that has been shown to regulate transcription by the eubacterial RNA polymerase (Männistö et al., 2003).

Based on the sequence comparison of ET1 with other proteins that contain ZR domains and its high sequence similarity to the different identified plant proteins, it appears that ET1 is the first described member of a family of small ZR-containing plastid proteins.

The phenotype observed in leaves of et1-R mutants and the light-dependent expression of the Et1 gene is in accordance with the hypothesis that ET1 functions similar to
TFII S and GreB as a regulator of transcription elongation. It is interesting that besides chloroplast development in the leaves also endosperm development is impaired in the et1-R mutants. This suggests that plastid gene expression is required for development of functional amyloplasts.

As already shown by Baumgartner et al. (1989) with barley primary foliage leaves, proper chloroplast development in the light is associated with a tremendous increase in the level of plastid gene expression. This is achieved by an increase of both the copy number of plastid DNA and the rate of transcription. Timing and level of plastid gene expression is also impaired in the abc1 mutant of Arabidopsis thaliana, which has a pale-green leaf phenotype resembling the phenotype of the et1-R mutant (Shirano et al., 2000). In case of the abc1 mutant, the phenotype is caused by a mutation in the gene encoding sigma factor SigB. While nuclear-encoded SigB is of prokaryotic origin as its cognate RNA polymerase (PEP), ET1 with its ZR domain is of eukaryotic origin. According to Sato’s model of discontinuous evolution of plastid nucleoids (Sato, 2001), the plastid genetic machinery has lost many prokaryotic proteins and has acquired nuclear-encoded eukaryotic-type proteins, e.g. the PEND protein, which is belonging to the bZIP proteins (Sato, 2001; Sato et al., 1998). Another example for a protein of eukaryotic origin with a function in plastid nucleoids is the coiled-coil DNA-binding protein MF1, which seems to function as a nucleoid anchor at the interface to the thylakoid membranes (Jeong et al., 2003).

Current experiments aim at identifying proteins interacting with the ET1 protein. Moreover overall RNA levels will be compared between the wild type and et1 mutant at different stages of plastid development. In addition, analyses of a zm knockout mutant of A. thaliana are in progress.

Experimental procedures

Basic molecular biology techniques, unless otherwise specified, were conducted according to Sambrook et al. (1989).

Maize stocks

Wild-type Et1 maize inbred lines used in these studies were LC (a color-converted W22 line; Wienand et al., 1986), B73, and H99. The Mu-induced alleles et1-M1–et1-M10 have been described previously by Scanlon et al. (1994). The allele et1-M12 was kindly provided by Phil Stinard (Maize Genetics Cooperation Stock Center, Urbana, IL, USA). The mutants et1-M15 and et1-M16 were isolated as described below. Unless otherwise noted, maize plants were grown in a greenhouse in Hamburg with 16 h of light at 25°C under a relative humidity of 55–95%.

Directed tagging of Et1

The mutants et1-M15 and et1-M16 were isolated from the cross A1 Et1/A1 Et1 Mu × a1 et1-R/a1 et1-R. The female parent (listed first) was an active Mu stock that had been maintained as described by Stinard et al. (1993). Both parents of this cross were planted in an isolation plot. The female parent was manually emasculated prior to anthesis. Colored, etched kernels identified in the progeny of this cross were test-crossed to et1-R to establish that they indeed carried novel et1-M alleles.

Southern and Northern analyses

Maize genomic DNA was isolated following the protocol provided by Dellaporta et al. (1983). Total RNA was isolated from different plant parts as described previously by Weisshaar et al. (1991). Southern and Northern blot hybridizations were carried out under stringent conditions according to Sambrook et al. (1989). Either the complete Et1 cDNA or the 3’ UTR of the gene were used as a probe for Northern blots.

AIMS analysis

A modified version (Lau et al., 1999) of the AIMS analysis (Frey et al., 1998) was used to analyze et1-M3 mutants (et1-R/et1-M3) along with their wild-type siblings (et1-R/et1-R). The experiment was performed using three different four-base restriction cutters individually, i.e. HpaII, Msel, and HinPI.

Construction and screening of genomic and cDNA libraries

Genomic libraries were constructed with lambda Fix II vector (Stratagene, Heidelberg, Germany) following manufacturer’s protocols. DNA was isolated from individuals of the following genotypes for this purpose: LC, et1-M3/et1, et1-R, et1-M18/et1-R, and et1-M15/et1-R. A cDNA library was constructed with the mRNA isolated from LC kernels harvested at 13, 17, 19, 20, 22, and 28 DAP. Equal amounts of total RNA from these probes were mixed prior to the isolation of poly(A)+ mRNA using oligo-dT cellulose. A lambda ZAPIII (Stratagene) vector was used and the library was constructed using an oligo-dT primer for the first-strand synthesis as instructed by the manufacturer. All libraries were screened following standard protocols (Sambrook et al., 1989). A 171-bp HpaII/G fragment, identified in the AIMS analysis to co-segregate with the et1-M3 mutation, was used as a probe to screen the et1-M3/et1 genomic library. A 2.5-kbp HindIII–Xhol genomic fragment (Frag 3 in Figure 3) was then used to screen an et1-R/et1-M16 as well as an LC genomic library. The et1-M15 library was screened with the full-size cDNA clone cEt1-9.

Restriction fragment length polymorphism mapping in recombinant inbreds

The 2.5-kbp HindIII–Xhol fragment (Figure 3) was used as a hybridization probe to map the et1 gene in two recombinant inbred populations Cm37XT232 (46 individuals) and 1x303 × Co159 (41 individuals) (Burr and Burr, 1991; Burr et al., 1988). This probe identified a BamHI polymorphism in these populations.

Cloning of et1-M3 Revertant-1 integration site and PCR analysis

A revertant sector (dark-green on a pale-green background) of a leaf of an et1-M3/et1-R plant was dissected and genomic DNA was
isolated using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and according to the manufacturer’s instructions. The following PCR was performed with the isolated genomic DNA: DNA (100 ng); 10 pmol each of primers Et-PCR1 and Et-PCR2; 0.625 mM dNTPs; and 2.5 U Taq polymerase (Life Technologies, Karlsruhe, Germany) in a total volume of 50 μl. The PCR program was as follows: 94°C for 5 min, 85°C/98°C addition of the primers; 29 × (94°C for 45 sec; 65°C for 45 sec, and 72°C for 1 min); 1 × (94°C for 45 sec, 65°C for 45 sec, and 72°C for 10 min); and 4°C. After completion of the PCR, the products were purified with the QiAquick PCR Purification Kit (Qiagen) as instructed by the manufacturer. The products were then blunt ended with Klenow polymerase, extracted with phenol and chloroform, and run over a Sephadex G-25 column. The resulting purified blunt-ended products were ligated into EcoRV-cut pZero (Invitrogen, Karlsruhe, Germany). Clones carrying the appropriate insert were found in a colony hybridization experiment using cET1-9 as probe. Positive clones were sequenced.

Primers used were: Et-PCR1, 5’-GAAGCGGAGACAATGGAGCC-TCTTG-3’; and Et-PCR2, 5’-TCTTGGGCTCAACGGTGGACAG-3’. The 5’ end of Et-PCR1 is 78 bases upstream of the first nucleotide and Et-PCR2 expands from position 207 to 183 of the cDNA cET1-9, respectively.

Nucleic acid sequencing
Plasmid subclones were sequenced based on the dideoxy-nucleotide chain termination method using the Big Dye Terminator sequencing kit (Perkin Elmer/ Applied Biosystems, Weiterstadt, Germany) and specific primers based on the manufacturer’s protocols, and analyzed on an automated DNA sequencer (model 377; Applied Biosystems).

Sequence analyses and database searches
Sequence alignments were performed by hand using GENE DOC 2.6.001 (Nicholas and Nicholas, 1997). Phylogenetic relationships were calculated using CLUSTALX 1.8.1 (Thompson et al., 1997) and depicted using TREEVIEW 1.6.6 (Page, 1996). Database searches were conducted online with the actual releases of the available protein and nucleic acid databases with the BLAST algorithm (Altschul et al., 1990, 1997). Computational analyses for the prediction of intracellular protein localization signals were conducted using the programs TARGETP, CHLOROP, and PSORT (Emanuelsson et al., 1999, 2000; Nakai and Kanehisa, 1992).

The similarity of ET1 to the ZR domain of TFIIS was identified through the Pfam database of hidden Markov Models (Bateman et al., 1999) available at the PROSITE database. The predicted secondary structure of ET1 was derived from the consensus of calculations using 14 different prediction programs available online at the EXPSy Molecular Biology server (http://www.expasy.ch/tools). Tertiary structure modeling was achieved online using CPHMODELS 2.0 (Lund et al., 2002) and was visualized using RASWIN 2.6 (Sayle and Milner-White, 1995).

Light and electron microscopy
The et1 phenotype of mutant kernels was examined by preparing hand sections of the kernels and viewing under a Zeiss stereomicroscope. Twenty DAP et1-R kernels were examined either unstained or after violet blue staining of starch with IKI (iodide-potassium-iodide) solution. The et1-M3/et1-R kernels were examined by staining with methylene blue and viewing under a Zeiss stereomicroscope using UV epifluorescent light.

To examine the phenotype of young et1-M3/et1-R seedlings, the second youngest leaf of pale seedlings, 120 mm in height, was used. The leaf was cut into five equal (0.2–0.3 cm) broad pieces, fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0), further cut into 1–1.5 mm pieces, post-fixed in 2% OsO4 in 0.05 M cacodylate buffer for 2 h, dehydrated in increasing concentrations of acetone (4°C), washed in 100% acetone (RT), infiltrated with Spurr’s resin:acetone in 1: 3 ratio for 1 h, followed by 1 : 1 ratio of both for 1 h, then in 3 : 1 ratio overnight, and finally with 100% Spurr’s resin for 4 h (Spurr, 1969). The resin with the leaf pieces was allowed to polymerize at 70°C for 24 h. Ultrathin sections (0.05–0.1 μm) of the embedded leaves were viewed under a transmission electron microscope.

Chloroplast import experiments
The complete ET1 cDNA cET1-9 was cloned into an effective in vitro transcription vector pBAT possessing a rabbit β-globin leader sequence at the 5’ end of the site of cloning (Annweiler et al., 1991) and transcribed with T3 polymerase based on Sambrook et al. (1989). The in vitro translation was carried out at 30°C for 90 min using the rabbit reticulocyte lysate kit (Promega, Mannheim, Germany). For this, about 5% of the synthesized mRNA was precipitated and centrifuged down, and the pellet was directly re-suspended in 12.5 μl in vitro translation mix (4.35 μl of Η2Ο; 0.9 μl of 1 M ΚCl; 0.25 μl of 100 mM DTT; 0.25 μl of amino acid mix (minus cysteine); 6.25 μl of reticulocyte lysate; and 0.5 μl of 35S-cysteine). Afterwards, the translation mix was directly used for the chloroplast import experiment conducted using isolated spinach chloroplasts following the protocol provided by Clausmeyer et al. (1993). A competition experiment with the unlabeled 33-kDa protein subunit of the oxygen-evolving complex was performed according to Michl et al. (1994). After the import reaction, the different chloroplast protein fractions were analyzed using a resolving SDS-polyacrylamide gel prepared according to Sambrook et al. (1989).

Preparation of plastids and TAC
Maize was grown in the greenhouse at 22°C with 16 h light and 8 h dark. Plastids were prepared from leaves of about 4-week-old plants as described by Poulsen (1983). For extraction of proteins plastids were solubilized in 62.5 mM Tris–HCl, pH 6.8), 10% (v/v) glycerol, 1% (w/v) SDS, and 5% (v/v) β-mercaptoethanol at 95°C for 5 min. TACs of different purity were prepared as described by Krause and Krupinska (2000). TAC fractions obtained after a single gel filtration step are referred to as TACI. Highly enriched TAC fractions obtained after two subsequent gel filtration steps and ultracentrifugation are referred to as TACII. Before gel filtration, the fractions were precipitated by proteamine sulfate and pellets were re-dissolved by heparin. Transcriptional activity of the TAC fractions was determined as described previously by Krupinska and Falk (1994) and Suck et al. (1996).

Immunological analyses
A polyclonal antibody was raised by Biogenes (Berlin, Germany) against the peptide SDAVPSPEA-C, which was deduced from the putative ET1 protein sequence. This peptide sequence is also present in the sequence of the putative ZmZR1 protein. Plastid proteins were analyzed by SDS-polyacrylamide gel
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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2094/TPJ2094sm.htm

Figure S1. Transcription analysis by hybridization of TAC transcripts to DNA dot blots derived from 4-week-old leaves of wild-type (a) and et1-R (b) maize plants. Two identical filters carrying four concentrations (5 (1×)–320 ng (64×)) of plasmid DNA of recombinant pBluescript clones containing specific barley or maize (+) plastid DNA fragments and original pBluescript as control were used. Hybridization reactions were performed with the same amount of radioactively labeled TAC transcripts, and both autoradiograms were exposed for the same time. Cross-hybridization of both filters delivered identical results (data not shown).

References


