GOLDEN 2: A Novel Transcriptional Regulator of Cellular Differentiation in the Maize Leaf

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The differentiation of distinct cell types within the leaf is essential for normal plant development. We characterized previously a transposon-induced mutant of maize (bundle sheath defective1) that disrupts the differentiation of a single photosynthetic cell type in the leaf. In this study, we show that this mutation is allelic to golden2 (g2), a lesion first reported 70 years ago. We cloned G2 by using Suppressor-mutator as a molecular tag. The gene encodes a 2.2-kb transcript that is present throughout the wild-type leaf but is most abundant in C4 leaf blade tissue. Gene sequence data showed the existence of a bipartite nuclear localization signal encoded by the first exon, and we determined that G2 reporter gene fusions are targeted to the nucleus in onion epidermal cells. Further sequence analysis indicated the presence of a novel motif within the deduced protein sequence that shares features with TEA DNA binding domains. Therefore, we propose that G2 acts as a novel transcriptional regulator of cellular differentiation in the maize leaf.

INTRODUCTION

Efficient leaf function is dependent on the differentiation of multiple cell types in a defined pattern. In monocot grasses, this pattern is particularly uniform in that leaves are strap shaped and cells are arranged in longitudinal files. At maturity, a typical grass leaf consists of blade and sheath regions that are delimited by a ligule (Brown, 1958; Freeling, 1992). The entire leaf is divided longitudinally by a series of parallel veins. The most prominent of these is the large central midrib (Esau, 1942; Sharman, 1942). Surrounding these veins are rings of bundle sheath cells, which are themselves surrounded by mesophyll cells. In the more commonly found C3 grasses such as wheat, barley, and rice, the mesophyll cells are photosynthetic, whereas the bundle sheath cells are conducting or supporting cells. However, in C4 grasses, such as maize, both cell types are photosynthetic (reviewed in Edwards and Walker, 1983).

In all C4 plants, bundle sheath and mesophyll cells are morphologically and functionally distinct. In the model C4 plant maize, bundle sheath cells have agranal chloroplasts that are arranged centrifugally within the cell, whereas mesophyll cells have granal chloroplasts that are randomly arranged (reviewed in Nelson and Langdale, 1992). Each of the two cell types accumulates a distinct complement of photosynthetic enzymes. Ribulose bisphosphate carboxylase (RuBPCase) and NADP-malic enzyme (NADP-ME) function in the bundle sheath cells, whereas phosphoenolpyruvate carboxylase (PEPCase), pyruvate phosphate dikinase (PPdK), and NADP-malate dehydrogenase (NADP-MDH) function in the mesophyll cells (Edwards and Huber, 1979). Therefore, the differentiation of dimorphic bundle sheath and mesophyll cells requires (1) the development of distinct cellular morphologies, (2) cell type-specific chloroplast development, and (3) cell type-specific photosynthetic gene expression.

Current evidence suggests that maize develops a C3 pattern of cell type differentiation by default (RuBPCase in all photosynthetic cells) and that C4 specialization is achieved through the interpretation of a light-induced signal that emanates from the leaf vasculature (Langdale et al., 1988; Langdale and Nelson, 1991). Perception and interpretation of this signal vary, depending on the distance of a cell from the vein. Mesophyll cells within a two-cell radius of a vein are adjacent to bundle sheath cells and differentiate appropriately as C4 mesophyll. However, mesophyll cells located farther than two cells away from the vein (and thus not in contact with bundle sheath cells) accumulate RuBPCase instead of mesophyll cell-specific enzymes and photosynthesize by using the C3 pathway (Langdale et al., 1988). As a result, mature leaf blades that show a vein-BS-M-M-BS-vein pattern (where BS is bundle sheath and M is mesophyll) perform C4 photosynthesis, and leaflike organs, such as leaf sheaths and husk leaves, that exhibit wider vein spacing perform C3 photosynthesis (summarized in Figure 1). In dark-grown tissues, a C3 pattern of photosynthetic differentiation is observed because both bundle sheath and mesophyll cells accumulate RuBPCase. However, etiolated leaves are nonphotosynthetic (Sheen and Bogorad, 1986; Langdale et al., 1988).

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To dissect the pathways leading to dimorphic photosynthetic cell type differentiation, we have isolated mutations that disrupt maize leaf development. The majority of these mutants exhibited perturbed development of both bundle sheath and mesophyll cells; however, nine mutations that specifically disrupted bundle sheath cell differentiation were identified previously (Langdale and Kidner, 1994; Langdale et al., 1995; Roth et al., 1996). In bundle sheath defective (bsd) mutants, normal cellular morphology has been observed, but bundle sheath cells failed to differentiate photosynthetically. The bsd1-mutable1 (bsd1-m1) mutation was identified by using transposon mutagenesis. Characterization of the bsd1-m1 mutant phenotype revealed that disrupted Bsd1 gene function results in both biochemical and morphological perturbations to bundle sheath cell development (Langdale and Kidner, 1994). In bsd1-m1 leaf blades, mesophyll cells develop normally, but bundle sheath chloroplast morphology is aberrant, and bundle sheath cell-specific C4 gene products fail to accumulate to wild-type levels. bsd1-m1 plants also display perturbed development of C4 differentiated tissue (i.e., etiolated leaf blades and leaflike organs with more than four cells between each vein) because RuBPCase does not accumulate. These observations led to the hypothesis that Bsd1 acts to promote cellular differentiation in both C3 and C4 tissues but that in C4 tissues it plays a pivotal role in bundle sheath cell development (Langdale and Kidner, 1994). Because cellular morphology and the relative arrangement of cells in bsd1-m1 mutant leaves are normal, Bsd1 plays no role in directing cell division patterns or in regulating cell shape.

In this study, we show that bsd1-m1 is allelic to golden2 (g2), a mutation first reported 70 years ago (Jenkins, 1927). As such, the bsd1-m1 allele has been renamed g2-bsd1-m1, and the wild-type gene is now referred to as G2. We report here the cloning and characterization of the G2 gene. Our findings suggest that G2 acts as a novel transcriptional regulator of bundle sheath cell chloroplast development in both C3 and C4 maize tissues.

**RESULTS**

**g2 Mutant Alleles**

bsd1-m1 is a recessive mutation that arose in a maize line carrying the transposable element Suppressor-mutator (Spm; Langdale and Kidner, 1994). Mutant plants have pale green leaf blades with white leaf sheaths (Figure 2A) and exhibit dark green clonal sectors of revertant tissue (Figure 2B). The bsd1-m1 mutation was mapped genetically to the distal half of chromosome arm 3S by using B-A chromosome translocation stocks (Beckett, 1994). The most distal genetic marker on 3S is g2, a pale green recessive mutation.

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*Figure 1. Schematic Representation of the Differentiation State of Bundle Sheath and Mesophyll Cells in the Maize Leaf Blade and Sheath.*

BS, bundle sheath; M, mesophyll; MDH, NADP-dependent malate dehydrogenase; ME, NADP-dependent malic enzyme; PEPCase, phosphoenolpyruvate carboxylase; PPdK, pyruvate phosphate dikinase; RubPCase, ribulose bisphosphate carboxylase.
that is similar in many respects to bsd1-m1 (Jenkins, 1927). Test crosses between bsd1-m1 and g2 yielded all mutant progeny, indicating that bsd1-m1 and g2 are allelic. As such, bsd1-m1 was renamed g2-bsd1-m1. A stable mutant allele (g2-bsd1-stable1 [g2-bsd1-s1]) was derived from g2-bsd1-m1 when plants were outcrossed to the inbred line W23. Crosses between g2-bsd1-s1 and g2-bsd1-m1 plants yielded progeny that were all mutant, indicating that both mutations represent alleles of the G2 gene. g2-bsd1-s1 seedlings have pale green leaf blades and white leaf sheaths but do not exhibit revertant sectors in the absence of autonomous Spm elements. Genetic crosses to lines containing autonomous Spm elements demonstrated that the g2-bsd1-s1 allele contains a defective Spm (dSpm) element.

g2 has been shown previously to be allelic to pg14, another Spm-induced allele (Peterson, 1953). Therefore, four mutant alleles of the G2 gene have been identified: g2-reference (g2-R), g2-bsd1-m1, g2-bsd1-s1, and g2-pg14.

**Isolation of the G2 Gene by Using Spm as a Molecular Tag**

Families segregating g2-bsd1-s1 mutant plants were used to clone the G2 gene. When genomic DNA from individuals within these families was digested with Haell and hybridized with Spm, a 4.5-kb fragment was found to segregate with the mutant allele (Figure 3A). The fragment was absent from both progenitors of the mutagenesis (opaque2 [o2] and o2-m20), from W23, and from homozygous wild-type siblings. This Haell fragment was cloned, and G2 DNA flanking the dSpm element was amplified by using polymerase chain reaction. The G2 gene fragment hybridized with the 4.5-kb Haell fragment in DNA from g2-bsd1-s1 plants and with a 2.0-kb fragment in DNA from heterozygous (G2/g2-bsd1-s1) and homozygous G2 plants (Figure 3B). The 2.0-kb fragment represents the allele derived from the inbred line W23. To test for linkage between the cloned G2 fragment and the g2-bsd1-m1 allele, hybridizations were performed with DNA isolated from individuals within families segregating g2-bsd1-m1 mutants. In DNA isolated from homozygous g2-bsd1-m1 plants, a 10.2-kb fragment hybridized with the cloned gene (Figure 3C). The existence of a 10.2-kb fragment in DNA isolated from g2-bsd1-m1 plants indicated that the Spm residing in the g2-bsd1-m1 allele is larger than that present in g2-bsd1-s1. This suggests that g2-bsd1-s1 arose as a deletion derivative of the g2-bsd1-m1 allele.

To confirm that the cloned DNA fragment represented the G2 gene rather than a tightly linked locus, gel blot analysis was performed with DNA extracted from mutant tissue (pale green) and dark green revertant sectors of individual g2-bsd1-m1 leaves. Hybridization with the Spm probe revealed the presence of the 10.2-kb fragment in DNA isolated from both mutant and dark green samples (Figure 3D). However, the relative fragment copy number in the mutant sample was nearly twice that seen in the dark green sample. Subsequent hybridization of the same DNA blot with the putative G2 gene probe identified a 2.3-kb fragment present in DNA from dark green but not mutant tissue (Figure 3D). This confirmed that we had cloned the G2 gene because loss of the mutant phenotype (due to excision of the Spm element from one of the two g2-bsd1-m1 alleles present) was correlated with the appearance of the 2.3-kb fragment corresponding to the progenitor wild-type allele. The map location of the G2 gene was confirmed subsequently by mapping the

![Figure 2. g2-bsd1-m1 Mutant Phenotype.](image_url)

(A) Whole-plant phenotype showing pale green leaf blades and white leaf sheaths. Revertant sectors are visible throughout the plant.

(B) Close-up of mutant leaf blade, showing early (large) and late (small) revertant sectors.
cloned gene fragment to chromosome 3S 0 by using a maize recombinant inbred population (Burr et al., 1987).

The G2 Gene Encodes a Putative Novel Transcription Factor and Is Targeted to the Nucleus

To isolate the wild-type G2 gene, we screened a genomic library with sequences flanking the dSpm insertion. A clone with a 12-kb insert (a1-Bsd1) was isolated and mapped with restriction enzymes. A cDNA clone was identified subsequently by using a subfragment of this genomic clone (pLNH10) to probe a maize leaf cDNA library. A comparison of sequence data derived from cDNA and genomic clones elucidated the G2 gene structure (Figure 4A). The G2 gene product is encoded by five exons that are interspersed with introns of ~2.5 kb. Sequence data derived from genomic clones of the g2-bsd1-s1 allele (data not shown) showed that the dSpm is inserted in the second intron (Figure 4A). Analysis of the G2 cDNA sequence indicates that the G2 transcript encodes a protein of 461 amino acids (Figure 4B). Alignment of the cDNA sequence with partial sequence from the genomic clone indicates that the cDNA clone is likely to be full length, with the first available translation initiation codon at 412 bp. Characteristic features of eukaryotic promoters are present further upstream within the genomic sequence (Figure 4B). A putative bipartite nuclear localization signal was identified within the protein of the type identified in Xenopus nucleoplasmin (Figure 4B; Robbins et al., 1991). In addition, DNA and protein homology searches identified a small class of Arabidopsis and rice expressed sequence tags with similarity to the G2 sequence encoded

**Figure 3.** Gel Blot Analysis of Genomic DNA from Plants Segregating g2-bsd1 Mutant Alleles.

(A) HaeII-digested DNA isolated from plants segregating the g2-bsd1-s1 allele hybridized with Spm. A 4.5-kb fragment segregated with the mutant allele (indicated by an arrow).

(B) Same DNA gel blot as shown in (A) hybridized with the G2 gene fragment generated by polymerase chain reaction. The arrow points to the 4.5-kb fragment that segregated with the mutant allele. Wild-type alleles were represented by 2.3-kb (o2 and o2-m20 progenitors) or 2.0-kb (W23) hybridizing fragments.

(C) HaeII-digested DNA isolated from plants segregating the g2-bsd1-m1 allele hybridized with the G2 gene fragment. A 10.2-kb fragment segregated with the mutant allele (indicated by the arrow at right), and a 2.3-kb fragment segregated with the wild-type progenitor allele (shown by the arrow at left).

(D) Hybridization of HaeII-digested DNA isolated from mutant and revertant sectors of a g2-bsd1-m1 plant. The gel at left shows hybridization with Spm, and the arrow identifies a 10.2-kb fragment present in two copies in DNA from mutant tissue and one copy in DNA from revertant tissue. The gel at right shows the same DNA blot hybridized with the G2 gene fragment. The pattern of hybridization to the 10.2-kb fragment is the same as with Spm (shown by the arrow at left). However, the G2 gene fragment also hybridizes with the 2.3-kb wild-type fragment in the revertant sample (shown by the position of the right-hand arrow).
by the second exon (Figure 4C). Further analysis of this sequence indicated that the region shares features with TEA DNA binding domains (Figure 4D; Bürglin, 1991). In addition, limited homology also was identified with a potato Myb-like protein that has been shown to bind DNA (data not shown; Baranowskija et al., 1994).

To determine whether the putative nuclear localization signal in G2 is functional, we fused the entire G2 coding region downstream of the β-glucuronidase (GUS) gene. Fusion constructs were introduced into onion epidermal cells by microprojectile bombardment, and transformed cells were assayed for GUS localization. As shown in Figure 5, the G2 sequence targeted GUS to the nucleus. This observation suggests that G2 acts within the nucleus.

Levels of the G2 Transcript Distinguish C3 and C4 Leaf Tissues

In wild-type plants, the dark green leaf blade displays a characteristic pattern of C4 photosynthetic differentiation whereby photosynthetic enzymes accumulate in specific cell types. In contrast, the pale green leaf sheath differentiates as C3 photosynthetic tissue in that RuBPCase accumulates in all photosynthetic cells (Langdale et al., 1988; Figure 1). Because g2-bsd1-m1 plants exhibit mutant leaf sheaths and blades, the G2 gene probably plays a role in both C3 and C4 photosynthetic differentiation. To determine whether G2 gene expression patterns support this suggestion, we first identified a sequence in the 3′ untranslated region of our cDNA clone that hybridized specifically with G2 (data not shown). Transcript accumulation patterns in different maize tissues were then determined by RNA gel blot analysis. The G2 gene–specific probe detected a 2.2-kb transcript that was present throughout wild-type leaf development but was absent from roots. Significantly, G2 transcripts were most abundant in leaf blade tissue (C4) and were detectable at lower levels in leaf primordia and leaf sheath tissue (C3; Figure 6). These results suggest that the abundance of G2 transcripts is correlated with the physiological state (i.e., C4 versus C3) of the tissue. Because leaf blades are more photosynthetically active than leaf sheaths (Langdale et al., 1988), we cannot exclude the possibility that transcript abundance is correlated with actual photosynthetic competence.

In g2-bsd1-s1 mutant plants, G2 transcripts were not detected in leaf primordia and leaf sheath tissue (Figure 6). However, in g2-bsd1-s1 leaf blades, two different transcripts of ~2.1 and 5 kb were detected. Because the larger transcript also hybridizes with 5Spm (data not shown), it probably represents an aberrantly spliced transcript that has retained the majority of the 5Spm insertion. Notably, the ribulose bisphosphate carboxylase small subunit (RbcS) transcripts accumulate in g2-bsd1-s1 mutant leaf blades (Figure 6), whereas they are absent from mutant sectors in g2-bsd1-m1 leaf blades (Langdale and Kidner, 1994). This suggests that one or both of the G2 transcripts detected in g2-bsd1-s1 leaf blades encode at least a partially functional gene product.

G2 Is Expressed in Both Dark- and Light-Grown Leaves

Because photosynthetic function is light dependent, we examined G2 transcript accumulation profiles in etiolated and light-shifted wild-type seedlings. Consistent with the fact that g2-bsd1 plants exhibit a mutant phenotype when grown in the dark (RuBPCase does not accumulate), G2 transcripts were detected in etiolated tissue (Figure 7). However, higher transcript levels were observed in light-shifted tissue. These data support the notion that steady state levels of G2 transcripts are light enhanced; however, relative to RbcS and other photosynthetic genes, this enhancement is slight.

G2 Is Expressed in Both Bundle Sheath and Mesophyll Cells

To examine the cellular distribution of G2 transcripts, RNA was isolated from purified bundle sheath and mesophyll cells. The purity of cell preparations was confirmed by hybridizing RNA gel blots with mesophyll cell–specific phosphoenolpyruvate carboxylase (Ppc1) and bundle sheath cell–specific RbcS radiolabeled probes (Figure 8A). Even after prolonged exposure of blots to film, no evidence of cellular cross-contamination was found. When G2 was hybridized with the same RNA samples, transcripts were detected in both bundle sheath and mesophyll cells (Figure 8B). This suggests that any cell-specific aspects of G2 function are mediated by post-transcriptional events. Although levels of transcripts appeared higher in bundle sheath than mesophyll cells, we believe that this difference is not significant because similar differences were noted between total leaf samples that were harvested intact and those that were incubated in mesophyll cell isolation buffer (minus cellulase) alongside mesophyll cell preparations. Hence, the difference in G2 transcript levels observed between bundle sheath and mesophyll cell samples probably reflects the different isolation procedures (mechanical and rapid for bundle sheath; enzymatic and slow for mesophyll) rather than cell type differences. In situ hybridization data are needed to confirm these expression data, but current methods are not sensitive enough to detect the very low levels of G2 transcripts that accumulate in leaf tissue.

DISCUSSION

g2-bsd1-m1 mutants have been shown previously to exhibit perturbed bundle sheath cell development because bundle sheath cell–specific photosynthetic enzymes fail to accumulate, and bundle sheath cell chloroplasts do not develop (Langdale and Kidner, 1994). In addition to this aspect of the
Figure 4. G2 Gene Sequence.

(A) Intron/exon structure of the G2 gene, showing the insertion site of Spm in the g2-bsd1-s1 allele, location of the nuclear localization signal (NLS), and location of the TEA-like domain. Numbers indicate exons.
phenotype, g2-bsd1-m1 mutants also exhibit perturbed differentiation in organs that develop a C₃ pattern of differentiation. For example, in etiolated leaves, leaf sheaths, and husk leaves, RuBPCase is absent from both bundle sheath and mesophyll cells, even though perturbations to etioplast structure are bundle sheath cell specific (Langdale and Kidner, 1994; R. Roth and J.A. Langdale, unpublished data). Interestingly, the g2-bsd1-s1 phenotype is less severe than the g2-bsd1-m1 phenotype. Although leaf sheaths are always white in g2-bsd1-s1 plants, leaf blades are greener than g2-bsd1-m1 counterparts, and mutant plants will often flower and set seed, despite the absence of revertant sectors.

Data presented here suggest that g2-bsd1-s1 is a deletion derivative of g2-bsd1-m1, with the deletion within the Spm element. Genomic sequence data suggest that in both alleles, the transposon resides within the second intron, with transcription of the transposon occurring in the opposite direction to that of G2 (L.N. Hall and J.A. Langdale, unpublished data). In g2-bsd1-s1, however, it appears that sequence within the dSpm acts as an alternative splice site. From the size of the partially spliced transcript (Figure 6), we propose that the aberrant splicing event utilizes a cryptic splice site at the 5’ end of the dSpm element, resulting in a transcript that retains most of the dSpm element. Such dSpm-induced splicing aberrations have been reported previously (Kim et al., 1987; Raboy et al., 1989). Notably, the proposed splicing event would leave the nuclear localization signal and the DNA binding domain regions intact. As such, it is conceivable that the resulting protein product retains some function. These data could explain why the g2-bsd1-s1 phenotype is less severe than is the g2-bsd1-m1 phenotype. Preliminary RNA gel blot analysis of G2-R and g2-pg14 leaves further suggests a negative correlation between G2 transcript accumulation levels and the severity of the mutant phenotype. However, because the four alleles are not yet isogenic, we cannot determine whether phenotypic severity is correlated with allelic differences within the G2 gene or with the action of unlinked modifiers.

Although all of the expression studies reported in this study have utilized a G2 gene-specific probe, the full-length G2 cDNA hybridized with multiple genomic sequences on DNA gel blots (data not shown). Preliminary data also suggest that a small gene family is present in the C₃ grass rice (L. Cribb and J.A. Langdale, unpublished data). The existence of a G2 gene family in maize is of particular interest because phenotypic recovery is observed toward the tip of the leaf in g2-bsd1-m1 mutants (other alleles have not been tested for this feature). We originally proposed several possible explanations for this event, one of which was that a related gene functions at later stages of development (Langdale and Kidner, 1994). The existence of G2-like family members makes this suggestion feasible. Another explanation was that the g2-bsd1-m1 mutation causes G2 transcripts to accumulate at later developmental stages than normal. We can now discount this possibility because we were unable to detect an increase in G2 transcript levels in g2-bsd1-m1 leaves that exhibited phenotypic recovery (data not shown).

The amino acid sequence encoded by the second exon of G2 resembles a class of TEA DNA binding domains found in a number of genes that regulate developmental processes in diverse species (Bürglin, 1991; Figure 4D). In Aspergillus nidulans, the abaA gene is required for the terminal stages of conidiophore development (Andrianopoulos and Timberlake, 1994); in Saccharomyces cerevisiae, the TEC1 gene regulates Ty1 activity (Laloux et al., 1990), and in humans, TEF-1 acts as a transcriptional activator (Xiao et al., 1991). Notably, in Drosophila the scalloped gene regulates cell-specific gene expression during differentiation of the nervous system (Campbell et al., 1992). To date, no TEA domain encoding genes have been reported in plants.

The similarity observed between exon 2 of G2 and the TEA domain is limited; however, the spacing pattern of regions that can form α helices is maintained. In TEA domains, three α helices are predicted (Bürglin, 1991; Figure 4D). The induction of mutations in either helix 1 or 3 abolishes DNA binding, but mutation of helix 2 does not (Hwang et al., 1993). Significantly, G2 is predicted to form only two helices that share sequence similarity with helices 1 and 3 of the TEA domain. In combination with the fact that G2 will target

Figure 4. (continued).

(B) G2 gene sequence obtained from genomic (in italics; –400 to –1 bp) and cDNA (1 to 2191 bp) clones. Putative CAAT (–3 and –59 bp), TAATA (–66 bp), and GC (–77 bp) boxes are underlined. The NLS is boxed, and the TEA-like domain is underlined (970 to 1113 bp). Other features of the sequence include an upstream leader sequence length of 411 bp, a 3’ untranslated sequence of 397 bp, the start codon at 412 bp, and the stop codon at 1795 bp. Multiple poly(A) signals exist at 2044 and 2108 to 2144 bp.

(C) Alignment of G2 amino acid sequence (amino acids 170 to 252) with rice and Arabidopsis expressed sequence tag (EST) amino acid sequences. Conserved amino acids are boxed. EMBL (E) and GenBank (G) accession numbers of ESTs are as follows: EST.1, Z18410 (E); EST.2, Z37493 (E); EST.3, T41875 (G); EST.4, N38232 (G); EST.5, N38325 (G); EST.6, T41999 (G); EST.7, T45758 (G); EST.8, H76190 (G); and rice, D15824 (G).

(D) Alignment of the amino acid sequence encoded by G2 with known TEA DNA binding domains. Conserved amino acids are boxed; helix breaking residues are indicated by dots; solid bars indicate putative α helices; the dashed bar indicates the extent to which putative helical regions extend into exon 3 and are conserved in rice; asterisks indicate a gap of 16 amino acids; and dashes indicate a space inserted for alignment.
The reporter protein GUS to the nucleus (Figure 5), these observations support the hypothesis that G2 acts as a transcription factor. Furthermore, it is likely that the DNA binding domain represents a novel class of binding domain in plants that is conserved at least in maize, rice, and Arabidopsis (Figure 4C). Seventy-five percent sequence conservation between exon 5 of G2 and the 3′ end of a rice G2-like gene further suggests that this proline-rich region could act as a transcriptional activation domain (L. Cribb and J. A. Langdale, unpublished data). It is not yet possible to deduce the direct targets of G2 gene action; however, both genetic and molecular approaches can be used to pursue this further.

Existing models propose that maize leaves differentiate a C3 pattern of gene expression by default and that the switch to C4 development is mediated by a light-enhanced interaction between bundle sheath and mesophyll cells, whereby RuBPCase becomes repressed in mesophyll cells close to a vein (Langdale et al., 1988; Langdale and Nelson, 1991). Cell-specific C4 differentiation occurs concomitantly with this repression. Both the accumulation patterns of G2 transcripts in wild-type plants and the g2-bsd1 mutant phenotype suggest that the G2 gene has a role in the differentiation of both C3 and C4 tissues. Specifically, G2 transcripts accumulate in etiolated leaves (C3), light-grown leaf sheaths (C3), and light-grown leaf blades (C4). Mutant phenotypes suggest a role for G2 in both bundle sheath and mesophyll cells of C3 tissue but only in bundle sheath cells of C4 tissue. These observations are consistent with the proposal that G2 acts as a transcription factor to promote the accumulation of RuBPCase or other photosynthetic enzymes that are associated with the Calvin cycle. These enzymes accumulate in both bundle sheath and mesophyll cells of C3 tissue but are restricted to bundle sheath cells in C4 tissue.

For a number of reasons, however, we do not believe that G2 has a direct influence on RuBPCase accumulation patterns. First, both RbcS transcripts (Figure 6) and RuBPCase (data not shown) have been detected in g2-bsd1 mutant tissue in the absence of G2 transcripts. Second, the accumulation profile of G2 transcripts does not reflect that of RbcS because RbcS transcript levels are lowest at the tip of the leaf blade where G2 transcript levels are highest (Figure 6). Finally, work with the bsd2-m1 mutant of maize has shown that if RuBPCase fails to accumulate, etioplasts and chloroplasts develop normally until photooxidation destroys chloro-
roplast structure (Roth et al., 1996). This is in contrast to the phenotype exhibited by g2-bsd1 mutant plants in which etioplasts in bundle sheath cells are aberrant and a general block in bundle sheath cell chloroplast development is observed (Langdale and Kidner, 1994).

Thus, we propose that the primary effect of the g2-bsd1 mutation is specifically to perturb bundle sheath cell plastid biogenesis in a light-independent manner. Perturbations to RuBPCase must therefore occur indirectly either as a result of intracellular mechanisms in both bundle sheath and mesophyll cells or as a result of intercellular communication. For example, perturbations to etioplast development in bundle sheath cells or to bundle sheath chloroplast development in C3 tissues could lead not only to a downregulation of RuBPCase in bundle sheath cells but also to a downregulation in neighboring mesophyll cells. Similar cross-talk between bundle sheath and mesophyll cells with respect to RuBPCase gene regulation is observed in bsd2-m1 mutants (Roth et al., 1996). In the absence of further experimentation, however, alternative explanations are also possible.

If G2 functions specifically to regulate plastid biogenesis in bundle sheath cells, why do G2 transcripts accumulate in both bundle sheath and mesophyll cells? Clearly, in the absence of a true null allele, we cannot discount the possibility that G2 performs a similar function in both bundle sheath and mesophyll cells and that the bundle sheath cell–specific phenotype in existing mutants results from the fact that bundle sheath cells are more sensitive than mesophyll cells to reduced G2 function. However, because all g2 mutants examined to date display a bundle sheath cell–specific defect in light-grown leaf blades, even in the absence of detectable G2 transcripts, we do not believe that G2 regulates photosynthetic development of mesophyll cells in the light. Thus, we propose that post-transcriptional processes are responsible for the cell specificity of gene action. For example, the G2 protein may accumulate specifically in bundle sheath cells or may be post-translationally activated only in bundle sheath cells. Alternatively, G2 function may depend on interactions with a bundle sheath cell–specific partner. Further studies clearly are needed to determine which of these alternatives is correct and to ascertain how G2 and its downstream targets interact with the putative vein-emanating signal that induces C4 development.

METHODS

Plant Material

o2, o2-m20, and g2-bsd1-m1 (bsd1-m1) maize stocks were as previously described (Langdale and Kidner, 1994). A family segregating
the g2-bsd1-s1 allele was recovered after crossing and backcrossing g2-bsd1-m1 into the inbred line W23. This segregating population was used for DNA analysis. Homozygous mutant (g2-bsd1-s1) and homozygous wild-type (G2) stocks were generated for RNA gel blot analysis. Inbred line W23 was obtained from the Maize Genetics Stock Center (Urbana, IL), and inbred line B73 was a gift from B. Lowe (Dekalb, Mystic, CT). Onion bulbs were purchased from a local grocery store.

**Growth Conditions**

Seedlings were grown in soil in a glasshouse (average daytime temperature of 28°C; average nighttime temperature of 16°C) with a 16-hr-light (500 μmol m⁻² sec⁻¹) and 8-hr-dark cycle. Plastochrons 1 to 5 (P1 to P5) leaf primordia were harvested 6 days after planting, when seedlings were 1.5 to 2 cm tall and all leaves were still enclosed in the coleoptile. The coleoptile was removed, and the young shoots were excised 2 to 3 mm above the shoot apical meristem. Third leaves and roots from light-grown seedlings were harvested 21 to 25 days after sowing, as the fifth leaf was emerging. Leaf sheaths were harvested intact, and leaf blades were divided into basal and distal halves. Mesophyll cells were isolated after cellule digestion of the third leaves, as previously described (Sheen and Bogorad, 1985). Bundle sheath cells were isolated by mechanical disruption of third leaves, as described by Westhoff et al. (1991).

Etiolated seedlings were grown in vermiculite in complete darkness at 28°C for 6 days. For RNA gel blot analysis, whole seedlings were harvested above the coleoptilar ridge. Light-shifted (greening) seedlings were grown in complete darkness, as described above, and then shifted to a growth chamber. Seedlings were shifted at the beginning of a 16-hr-light and 8-hr-dark cycle (131 μmol m⁻² sec⁻¹). Whole seedlings were harvested after 24 hr.

**Preparation of DNA and Gel Blot Analysis**

DNA was isolated from leaf tissue, according to Chen and DellaPorta (1994). DNA gel blot analysis was performed as described in Langdale et al. (1991). The Suppressor-mutator (Spm) clone pBx1 (which contains a 457-bp sequence from the 5' end of the element) was a gift from B. Lowe (Dekalb, Mystic, CT).

**G2 Cloning**

Genomic DNA was isolated from g2-bsd1-s1 seedlings, digested with the restriction enzyme HaeII, and size fractionated through a 0.7% agarose gel. The 4- to 5-kb size fraction was excised, and DNA was extracted from the gel by electroelution (Sambrook et al., 1989). DNA was purified further using an Elutip column (Schleicher & Schuell, Dassel, Germany). All subsequent cloning procedures were performed according to Sambrook et al. (1989). Before ligation into the Smal site of the plasmid pGEM7ZF (Promega, Madison, WI), the 3' overhang generated by HaeII was removed by using the exonuclease activity of DNA polymerase I (the Klenow fragment). Ligated plasmid was introduced into competent Escherichia coli XL1Blue MRF' (Stratagene, La Jolla, CA) by electroporation. Recombinant bacterial colonies (25,000) were screened by hybridization with Spm. Four independent clones were identified (pbsd1.1, pbsd1.2, pbsd1.3, and pbsd1.4).

Wild-type genomic and cDNA clones were isolated from libraries prepared from B73 maize tissue. The cDNA library was a gift from A. Barkan (University of Oregon, Eugene), and the genomic library was purchased from Clontech (Palo Alto, CA).

**Amplification of DNA Fragments by the Polymerase Chain Reaction**

Polymerase chain reaction was used to generate a G2 gene fragment from pbsd1.1. Primers used were homologous to the T7 promoter of pGEM7ZF and the 3' end of Spm. The primer derived from Spm was a gift from B. Lowe.

**DNA Sequencing**

Plasmid subclones of both the genomic and cDNA clones were sequenced fully on both strands by using a Sequenase kit (Amersham, UK). Sequence data were analyzed by using Genejockey II (Biosoft, Cambridge, UK), pSORT (http://psort.nibb.ac.jp/), BLAST (http://kiwi.imgen.bcm.tmc.edu:B088/search-launcher/launcher.html), and BLOCKS (http://www.blocks.fhcrc.org/blocks_search.html) programs. A comparison of cDNA and genomic sequence elucidated the intron and exon boundaries.
Nuclear Localization Assays

A G2 gene fragment comprising the whole coding region was ligated 3’ to β-glucuronidase (GUS) in the plant expression vector pRTL2-GUS (Restrepo et al., 1990), according to standard protocols (Sambrook et al., 1989). Sequence of the fusion junctions of this clone was verified by dyeoxy sequencing of double-stranded DNA templates by using a Sequenase kit. The GUS (pRTL2-GUS) and GUS-Nla (pRTL2-GUS-Nla) constructs were obtained from V. Irish (Yale University, New Haven, CT). DNA for bombardment was prepared by using a plasmid midiprep kit (Qiagen, Hilden, Germany) and quantitated spectrophotometrically. Inner epidermal peels from onion bulbs were placed on solid Chu medium (Sigma) and then incubated for 2 hr at 37°C in the following solution: 100 mM sodium phosphate buffer, pH 7, 1 mM EDTA, 1% Triton X-100, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc). Cells were then stained in 2 μg mL⁻¹ 4′,6-diamidino-2-phenylindole solution in 0.05 M Tris-HCl, pH 7, and finally mounted on microscope slides. Photographs were taken using an Axiohot microscope (Zeiss, Welwyn Garden City, UK) and Ektachrome film (Kodak).

Isolation of RNA and Gel Blot Analysis

RNA was isolated, electrophoresed, blotted, and hybridized as described in Langdale et al. (1988). The ribulose bisphosphate carboxylase small subunit (RbcS) and phosphoenolpyruvate carboxylase (Ppc1) clones were as previously described (Langdale et al., 1988). The ubiquitin clone pSKUB1 was a gift from P. Quail (Plant Gene Expression Center, Albany, CA; Christensen et al., 1992).

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