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bundle sheath defective2, a Mutation That Disrupts the Coordinated Development of Bundle Sheath and Mesophyll Cells in the Maize Leaf

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Within the maize leaf primordium, coordinated cell division and differentiation patterns result in the development of two morphologically and biochemically distinct photosynthetic cell types, the bundle sheath and the mesophyll. The *bundle sheath defective2-mutable1* (*bsd2-m1*) mutation specifically disrupts C_4 differentiation in bundle sheath cells in that the levels of bundle sheath cell-specific photosynthetic enzymes are reduced and the bundle sheath chloroplast structure is aberrant. In contrast, mesophyll cell-specific enzymes accumulate to normal levels, and the mesophyll cell chloroplast structure is not perturbed. Throughout mutant leaf development, the large and small subunits of ribulose bisphosphate carboxylase are absent; however, both *rbcL* and *RbcS* transcripts accumulate. Moreover, chloroplast-encoded *rbcL* transcripts accumulate ectopically in mesophyll cells. Although the bundle sheath cell chloroplast structure deteriorates rapidly when plants are exposed to light, this deterioration is most likely a secondary effect resulting from cell-specific photooxidative damage. Therefore, we propose that the *Bsd2* gene plays a direct role in the post-transcriptional control of *rbcL* transcript accumulation and/or translation, both in bundle sheath and mesophyll cells, and an indirect role in the maintenance of bundle sheath cell chloroplast structure.

INTRODUCTION

As leaves develop from undifferentiated leaf primordia into mature functional organs, patterns of cell division and differentiation need to be coordinated. Although current evidence suggests that developmental coordination is achieved through the interpretation of positional information, the signals involved remain elusive. The maize leaf is an excellent system in which to study the regulation of cellular differentiation events because it is well characterized morphologically and functionally. Like the majority of grass leaves, the maize leaf is an elongated strap-shaped structure that is divided into blade and sheath. As a consequence of maturation patterns, a developmental gradient exists along the proximal–distal axis of the leaf, with the most mature cells at the tip (Sharman, 1942; Kirchanski, 1975; Sylvester et al., 1990). Within the internal leaf layers, a series of parallel veins forms the framework around which two photosynthetic cell types, the bundle sheath and the mesophyll, differentiate (Esau, 1942; Russell and Evert, 1985). This Kranz-type anatomy, with concentric rings of bundle sheath (inner) and mesophyll (outer) cells around the vascular system, reflects the physiological requirement for the rapid flux of metabolites between these two cell types (Brown, 1975). In a two-cell shuttle, CO_2 is initially fixed in mesophyll cells by phosphoenolpyruvate carboxylase (PEPCase) into the C_4 acid oxaloacetate. Following conversion of oxaloacetate to malate, the C_4 compound is rapidly transferred to the neighboring bundle sheath cells, where it is decarboxylated by NADP-dependent malic enzyme (NADP-ME). The released CO_2 is then rapidly reassimilated in the Calvin cycle by ribulose bisphosphate carboxylase (RuBPCase), as occurs in C_3 plants (for reviews, see Edwards and Huber, 1979, 1981; Edwards and Walker, 1983). This compartmentation of RuBPCase away from atmospheric O_2 confers a photosynthetic advantage to C_4 plants under conditions of high temperature and light intensity (Hatch, 1978).

The dimorphic bundle sheath and mesophyll cells of C_4 leaves exhibit specific ultrastructural features. In the thin-walled mesophyll cells, randomly arranged chloroplasts contain granal stacks and lack starch grains. In contrast, the thick-walled bundle sheath cells contain large chloroplasts that are characteristically arranged centrifugally in the cell and lack granal stacking. Studies of the ultrastructural development of dimorphic chloroplasts in maize and sugarcane have shown that bundle sheath and mesophyll chloroplasts are morphologically similar early in development in that they both contain granal stacks (Laetsch and Price, 1969; Brangeon, 1973; Laetsch, 1974; Kirchanski, 1975). As the plastids approach maturity, however, bundle sheath chloroplasts "dedifferentiate" into the agranal structures seen in the mature leaf. This loss of granal

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stacking in bundle sheath chloroplasts is generally associated with the loss of polypeptides associated with photosystem II activity (Wrischer, 1989; Meierhoff and Westhoff, 1993).

Bundle sheath and mesophyll cells are also biochemically distinct in that they accumulate specific complements of C_4 photosynthetic enzymes. In bundle sheath cells, RuBPCase and NADP-ME accumulate, whereas PEPCase, pyruvate phosphodikinase (PPDK), and NADP-dependent malate dehydrogenase (NADP-MDH) are present in mesophyll cells (Edwards and Huber, 1979). The development of C_4 photosynthetic gene expression patterns has been well documented and provides an excellent diagnostic tool with which to study cellular differentiation in the maize leaf (for review, see Nelson and Langdale, 1992). Current evidence suggests that light is an important factor in the differentiation of C_4 patterns. In dark-grown maize leaves, low levels of RuBPCase accumulate in both bundle sheath and mesophyll cells, whereas other C_4 enzymes are barely detectable (Sheen and Bogorad, 1985; Langdale et al., 1988b). These observations suggest that dark-grown maize exhibits a C_3 pattern of photosynthetic gene expression whereby RuBPCase accumulates in all photosynthetic cells by default (reviewed in Nelson and Langdale, 1992). Upon greening, RuBPCase accumulation becomes restricted to bundle sheath cells through localized turnover of RuBPCase in mesophyll cells.

The molecular mechanisms that allow bundle sheath cell–specific accumulation of the chloroplast-encoded large subunit and nuclear-encoded small subunit of RuBPCase have been studied in maize, amaranth, and sorghum leaves (Boinski et al., 1993; Kubicki et al., 1994). For example, transcriptional run-on analyses of RuBPCase large subunit (*rbcL*) mRNAs in separated bundle sheath and mesophyll chloroplasts of light-grown amaranth leaves demonstrated that the *rbcL* gene is transcribed in both cell types, even though *rbcL* transcripts and peptides accumulate in a bundle sheath cell–specific manner (Boinski et al., 1993). This suggests that in amaranth, the differential accumulation of *rbcL* mRNAs is controlled post-transcriptionally. Indeed, in a number of species, control of mRNA stability appears to be one of several mechanisms leading to the differential expression of plastid genes (reviewed in Gruissem and Tonkyn, 1993; Mayfield et al., 1995). Differences in RuBPCase small subunit (*RbcS*) transcript levels may also be regulated post-transcriptionally (Sheen, 1990). Run-on transcription analyses of *RbcS* mRNAs in greening mesophyll protoplasts of maize showed *RbcS* transcript initiation, even though mRNAs do not accumulate in mesophyll cells. Furthermore, in vitro experiments involving transient expression assays of a reporter gene under the control of an *RbcS* promoter showed *RbcS* gene activity in both bundle sheath and mesophyll cells of greening leaves (Bansal et al., 1992; Viret et al., 1994). These studies provide cumulative evidence that post-transcriptional mechanisms contribute to the suppression of *RbcS* mRNA levels in mesophyll cells.

Although C_4 differentiation patterns have been characterized extensively (reviewed in Nelson and Langdale, 1992), the exact nature of the cellular interactions involved is unknown. To address this issue further, we are identifying genes that facilitate cell-type differentiation. By isolating mutants that exhibit perturbed differentiation of a single cell type, regulatory hierarchies are being dissected. Thus far, ~100 transposon- and ethyl methanesulfonate–induced pale green mutants have been screened for defective C_4 enzyme accumulation patterns and cell-specific chloroplast disruption (Langdale et al., 1995; L.N. Hall and J.A. Langdale, unpublished data). The majority of mutants exhibit perturbed development of both bundle sheath and mesophyll cells; however, six mutations that specifically disrupt bundle sheath cell differentiation have been identified. One of these, *bundle sheath defective1* (*bsd1*), has been reported previously (Langdale and Kidner, 1994). The *bsd1-mutable1* (*bsd1-m1*) mutation uncouples the tightly coordinated differentiation of bundle sheath and mesophyll cells in that bundle sheath cells fail to differentiate whereas mesophyll cells develop normally. Loss of *Bsd1* gene function results in both biochemical and morphological disruptions in that the development of bundle sheath chloroplasts is disrupted and bundle sheath cell–specific C_4 gene products fail to accumulate.

In this study, we report the characterization of a second mutation that causes a bundle sheath–defective phenotype in the maize leaf. The *bsd2-m1* allele was isolated from a line containing active *Mutator* transposable elements by W.F. Sheridan (University of North Dakota, Grand Forks). In *bsd2-m1* seedlings, bundle sheath cell chloroplasts exhibited aberrant morphology, whereas mesophyll cell chloroplasts were normal. Furthermore, bundle sheath cell-type–specific C_4 enzyme levels were reduced when compared with the wild type, whereas mesophyll cell–specific C_4 proteins accumulated to normal levels. The presence of bundle sheath cell–specific C_4 mRNAs suggests that the *bsd2-m1* mutation disrupts C_4 protein accumulation at the post-transcriptional level. The ectopic accumulation of *rbcL* transcripts in mesophyll cells provides further evidence that the *Bsd2* gene product plays a role in RNA metabolism during normal leaf development.

RESULTS

Bundle Sheath Cell-Specific C_4 Enzyme Levels Are Reduced in *bsd2-m1* Leaves

Segregation analyses indicated that the mutant phenotype associated with *bsd2-m1* is caused by a single recessive nuclear mutation. *bsd2-m1* leaves are pale green and occasionally exhibit sectors of dark green tissue, presumably due to somatic excision or suppression of a transposable element (see Methods). When illuminated with UV light, seedlings display a high chlorophyll fluorescence (*hcf*) phenotype, suggesting that photosynthetic function is defective (Miles, 1994). The inability of *bsd2-m1* seedlings to sustain growth after the depletion of endosperm reserves further suggests that mutants are photosynthetically compromised.

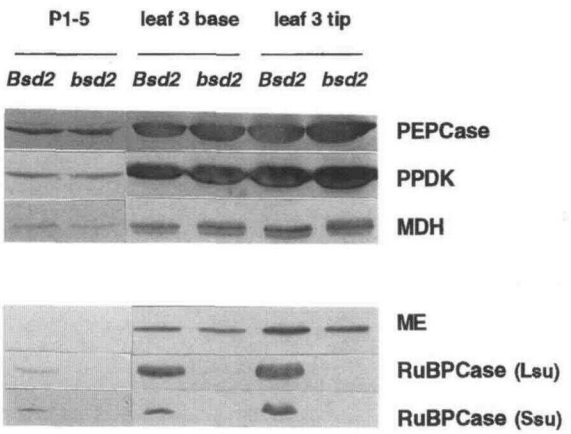

Figure 1. Immunoblot Analysis of Total Protein Extracted from P1–5 Leaf Primordia and Third Leaf Blades (Base and Tip) of Wild-Type (*Bsd2*) and *bsd2-m1* (*bsd2*) Seedlings.

(Top) Mesophyll cell–specific enzymes PEPCase (109 kD), PPDK (95 kD), and NADP-MDH (MDH; 41 kD) are shown.
(Bottom) Bundle sheath cell–specific enzymes NADP-ME (ME; 61 kD), RuBPCase large subunit (Lsu; 53 kD), and RuBPCase small subunit (Ssu; 14 kD) are shown.
Third leaves were 27 cm (wild type) and 24 cm (*bsd2*) from ligule to tip. Protein was isolated using a standard weight of tissue per volume of extraction buffer. Equal sample volumes were loaded in each lane.

To determine the effect of the *bsd2-m1* mutation on C_4 photosynthetic enzyme accumulation patterns, proteins were extracted from young leaf primordia (plastochrons 1 to 5 [P1–5]) and seedling leaves (third leaf base and tip). (A plastochron is the time interval between the initiation of successive leaf primordia.) Immunoblots were reacted with antibodies raised against both mesophyll cell–specific enzymes (PEPCase, PPDK, and NADP-MDH) and bundle sheath cell–specific enzymes (RuBPCase and NADP-ME). As shown in Figure 1, during all developmental stages examined, levels of mesophyll cell–specific enzymes were equal in wild-type (*Bsd2*) and mutant (*bsd2-m1*) seedlings. This suggests that the wild-type *Bsd2* gene product does not control mesophyll cell–specific C_4 accumulation patterns. In contrast, the bundle sheath cell–specific RuBPCase large subunit and small subunit proteins were absent from mutant P1–5 leaf primordia and remained undetectable throughout all stages of *bsd2-m1* leaf development (Figure 1). The *bsd2-m1* mutation did not affect NADP-ME accumulation during the early stages of leaf development, because normal levels accumulated at the base of the third leaf. In comparison with the wild type, however, a minor reduction in NADP-ME protein levels was seen consistently at the tip of *bsd2-m1* third leaves (Figure 1). These results suggest that the *Bsd2* gene product is required throughout leaf development for the accumulation of RuBPCase, but only during later developmental stages to sustain NADP-ME protein levels.

To investigate further the decrease in NADP-ME levels at later stages of *bsd2-m1* leaf development, we examined NADP-ME profiles at the cellular level. Figure 2 provides a comparison of NADP-ME accumulation patterns in *Bsd2* and *bsd2-m1* tissue at the base and at the tip of light-grown third leaves. NADP-ME proteins are appropriately compartmentalized in bundle sheath chloroplasts throughout mutant leaf development. At the leaf tip, however, the distribution of NADP-ME within the chloroplasts appears punctate when compared with the wild type. In addition, the staining of the bundle sheath cell walls in *bsd2-m1* leaf tips suggests that the intracellular compartmentation of ME is lost during later stages of *bsd2-m1* leaf development, possibly as a result of leakage from disrupted bundle sheath chloroplasts. This raises the possibility that the reduction of NADP-ME levels in mutant leaves is an indirect consequence of a breakdown in bundle sheath cell chloroplast structure in mature cells at the leaf tip.

Bundle Sheath Cell-Specific mRNAs Accumulate in *bsd2-m1* Leaves

To determine whether the reduction of bundle sheath cell–specific protein levels in *bsd2-m1* leaves is manifest at a transcriptional or post-transcriptional level, C_4 mRNAs were analyzed on RNA gel blots. Figure 3 (lanes 1 and 2) shows that both bundle sheath and mesophyll cell–specific transcripts accumulated in the third leaf blades of *bsd2-m1* plants. However, in comparison with the wild type, *bsd2-m1* leaves contained reduced *RbcS* transcript levels and elevated *rbcL* transcript levels. These data suggest that bundle sheath cell–specific enzyme levels are reduced in *bsd2-m1* leaves as a result of aberrations in post-transcriptional mechanisms.

To examine the extent to which the *bsd2-m1* mutation affects spatial patterns of C_4 gene expression, RNA was isolated from purified bundle sheath and mesophyll cell types. RNA gel blot analysis showed that all of the nuclear-encoded C_4 mRNAs (*Mod1, RbcS, Ppc1, Ppdk1,* and *Mdh1)* accumulate in the appropriate cell type of *bsd2-m1* mutant leaves (Figure 3A, lanes 3 to 6). This suggests that in light-grown leaves, the *bsd2-m1* mutation does not disrupt the spatial regulation of nuclear-encoded C_4 genes. Furthermore, the absence of *RbcS* and *Mod1* mRNAs from mesophyll cells (Figure 3A, lanes 5 and 6) and *Ppc1* and *Mdh1* transcripts from bundle sheath cells (lanes 3 and 4) showed that negligible levels of nuclear cross-contamination occurred during the preparation of isolated bundle sheath and mesophyll cells.

In contrast to the situation seen with nuclear genes, the chloroplast-encoded *rbcL* mRNA accumulated ectopically in mutant leaves (Figure 3B). Whereas *rbcL* transcripts accumulated in a bundle sheath cell–specific manner in wild-type leaves (Figure 3B, lanes 3 and 5), transcripts accumulated to equivalent levels in both bundle sheath and mesophyll cells of *bsd2-m1* leaves (lanes 4 and 6). The levels observed in each mutant cell type were slightly lower than those seen in wild-type bundle sheath cells. The low level of *rbcL* transcripts

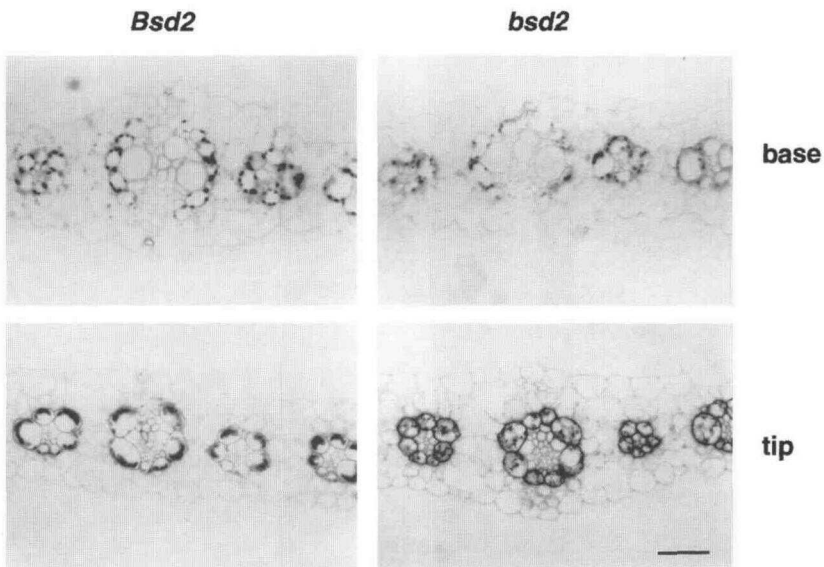

Figure 2. Immunolocalization of NADP-ME Protein in Serial Sections of Wild-Type (*Bsd2*) and *bsd2-m1* (*bsd2*) Third Leaves (Base and Tip). Bar = 45 μm.

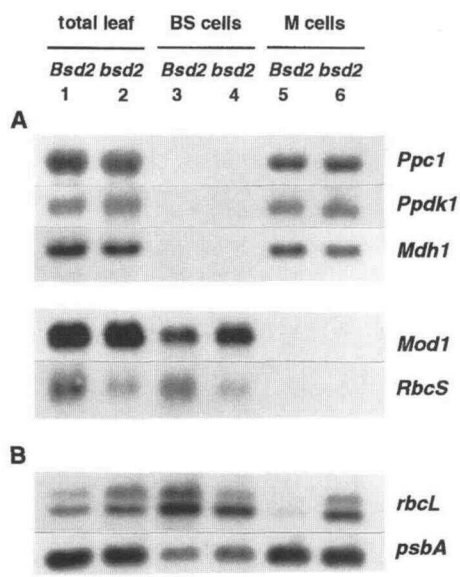

Figure 3. Gel Blot Analysis of Total RNA Isolated from Wild-Type (*Bsd2*) and *bsd2-m1* (*bsd2*) Whole Leaves, Purified Bundle Sheath Cells, and Purified Mesophyll Cells.

(A) Shown is hybridization of nuclear-encoded transcripts with *Ppc1* (3.5 kb), *Ppdk1* (3.5 kb), *Mdh1* (1.6 kb), *Mod1* (2.2 kb), and *RbcS* (1.0 kb).
(B) Shown is hybridization of chloroplast-encoded transcripts with *rbcL* (1.6 and 1.8 kb) and *psbA* (1.4 kb).
Equal amounts of total RNA were loaded in each lane (as determined by ethidium bromide staining of the gel). Lanes 1 and 2 contain whole leaves; lanes 3 and 4, purified bundle sheath (BS) cells; lanes 5 and 6, purified mesophyll (M) cells.

observed in wild-type mesophyll cells (Figure 3B, lane 5) most likely represents cross-contamination of chloroplasts during purification.

To test whether the non–cell-specific accumulation of *rbcL* mRNAs resulted from constitutive expression of the plastid genome in bundle sheath and mesophyll cells, we compared *psbA* (chloroplast gene encoding the D1 protein of photosystem II) mRNA levels in separated bundle sheath and mesophyll cells of *Bsd2* and *bsd2-m1* leaves (Figure 3B, lanes 3 to 6). In wild-type and *bsd2-m1* leaves, *psbA* mRNAs accumulated preferentially in mesophyll cells, with low levels of accumulation in bundle sheath cells. This suggests that the *bsd2-m1* mutation does not cause a general defect in chloroplast gene expression but that it specifically disrupts the spatial accumulation patterns of *rbcL* mRNAs.

To determine whether the aberrant regulation of *rbcL* transcripts is limited to photosynthetic tissues, we examined *rbcL* mRNA levels in wild-type and *bsd2-m1* roots. RNA gel blot analysis revealed that *rbcL* transcripts did not accumulate in wild-type or mutant roots (data not shown). Therefore, the ectopic accumulation of *rbcL* mRNAs in *bsd2-m1* plants appears to be restricted to photosynthetic cell types.

rbcL Transcripts Accumulate Non-Cell Specifically in *bsd2-m1* Leaves throughout Development

In wild-type leaves, *rbcL* mRNA accumulation serves as the earliest marker for bundle sheath cell differentiation (Langdale et al., 1988a). It first appears in bundle sheath progenitor cells

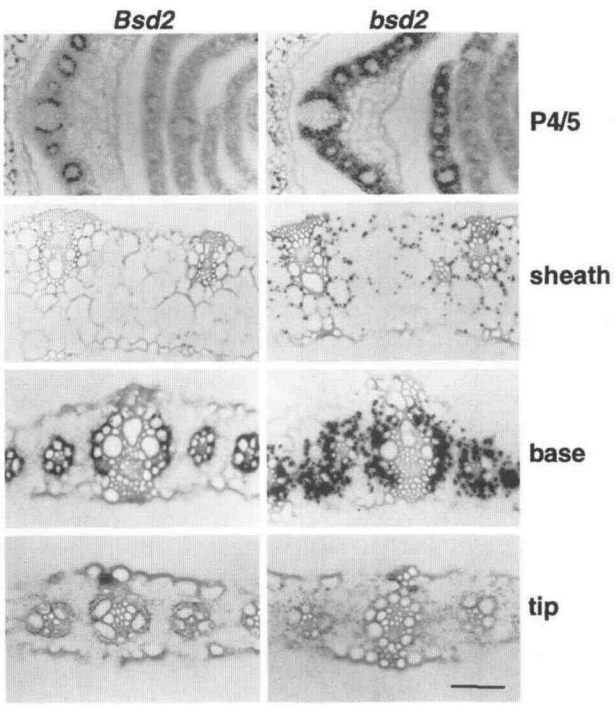

Figure 4. In Situ Detection of *rbcL* Transcripts in Plastochron 4/5 (P4/5) Leaf Primordia, Second Leaf Sheaths, and Third Leaf Blades (Base and Tip) of Wild-Type (*Bsd2*) and *bsd2-m1* (*bsd2*) Siblings.

Bar = 50 μm.

between plastochron 4 (surrounding major vein sites) and plastochron 5 (surrounding intermediate vein sites). To establish how early in leaf development the *bsd2-m1* defect is manifest, *rbcL* gene expression patterns were examined in situ in wild-type and mutant P1–5 leaf primordia. Figure 4 shows that the non–cell-specific accumulation of *rbcL* mRNAs in mutant leaf primordia was observed as early as plastochron 4. This suggests that during normal development in the light, the *Bsd2* gene acts at or before plastochron 4 to suppress *rbcL* transcript accumulation in mesophyll cells.

To establish a role for *Bsd2* gene action during later stages of normal leaf development, we examined C_4 gene expression patterns in the sheath and blade (base and tip) of seedling leaves (Figure 4). In wild-type leaf sheath, low levels of *rbcL* transcripts accumulated in both bundle sheath and mesophyll cells (i.e., a C_3 pattern of differentiation was observed). In *bsd2-m1* leaf sheath, a similar distribution of *rbcL* mRNAs was observed, but transcripts were present at much higher levels (Figure 4). This result provides a possible role for the *Bsd2* gene in regulating overall levels of *rbcL* in C_3-like differentiating tissue. In C_4 blade tissue, *rbcL* transcripts accumulated ectopically in mesophyll cells throughout mutant leaf development. No transcripts were detected in epidermal or vascular cell types (Figure 4). This confirms the hypothesis that the *bsd2-m1* mutation acts to disrupt *rbcL* mRNA levels exclusively in photosynthetic tissues and further suggests that the *Bsd2* gene acts to repress *rbcL* transcript levels in mesophyll cells throughout normal development. Interestingly, accumulation profiles in *bsd2-m1* leaf blades followed normal developmental patterns, with transcript levels higher at the base of the blade than at the tip (Figure 4; Langdale et al., 1988a). There are two possible explanations for this observation. The first is that *rbcL* transcript levels decrease at the tip of mutant leaves as an indirect result of deteriorating chloroplast structure late in development. The second is that in C_4 differentiated tissue, the *Bsd2* gene plays no role in the temporal regulation of *rbcL* transcripts levels.

The *bsd2-m1* Mutation Disrupts RuBPCase Accumulation Patterns in a Light-Independent Manner

During normal development, RuBPCase accumulates non–cell specifically in the dark but bundle sheath cell specifically in the light (Sheen and Bogorad, 1985, 1986; Langdale et al., 1988b). To determine whether the *Bsd2* gene plays a role in this switch, we compared C_4 protein levels in etiolated and greening tissue. Figure 5 shows that the mesophyll cell–specific C_4 enzymes PEPCase, PPDK, and NADP-MDH and the bundle sheath cell–specific NADP-ME accumulated to normal levels in both etiolated and greening *bsd2-m1* leaves. In contrast, both the RuBPCase large and small subunits were absent (Figure 5). These data suggest that the wild-type *Bsd2* gene

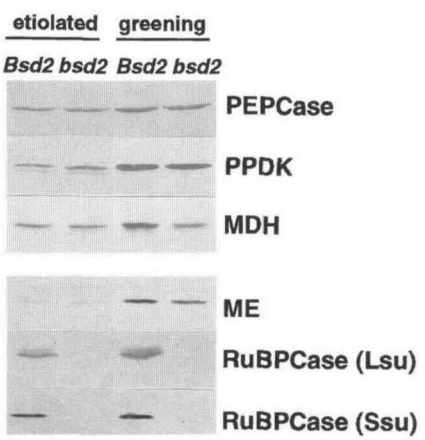

Figure 5. Immunoblot Analysis of Total Protein Extracted from Etiolated and Greening Wild-Type (*Bsd2*) and *bsd2-m1* (*bsd2*) Leaves.

(Top) Mesophyll cell–specific enzymes PEPCase (109 kD), PPDK (95 kD), and NADP-MDH (MDH; 41 kD) are shown.

(Bottom) Bundle sheath cell–specific enzymes NADP-ME (ME; 61 kD), RuBPCase large subunit (Lsu; 53 kD), and RuBPCase small subunit (Ssu; 14 kD) are shown.

Protein was isolated using a standard weight of tissue per volume of extraction buffer. Equal sample volumes were loaded in each lane.

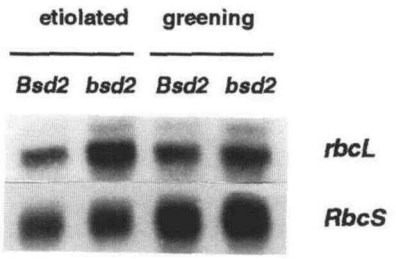

Figure 6. RNA Gel Blot Analysis of *RbcS* and *rbcL* Transcripts in Etiolated and Greening Wild-Type (*Bsd2*) and *bsd2-m1* (*bsd2*) Leaves.

Equal amounts of total RNA were loaded in each lane (as determined by ethidium bromide staining of the gel).

product is required for RuBPCase accumulation in both the light and the dark but is not required for the light-induced accumulation of other C_4 enzymes.

To determine whether the absence of RuBPCase protein in etiolated and greening *bsd2-m1* leaf tissue results from post-transcriptional defects (as seen in light-grown tissue), *RbcS* and *rbcL* levels were analyzed by RNA gel blot analysis. Figure 6 shows that *RbcS* transcript levels were essentially equal in wild-type and mutant etiolated and greening tissue. However, *rbcL* transcript levels were notably higher in *bsd2-m1* leaves, presumably due to the ectopic accumulation of *rbcL* transcripts in mesophyll cells of both etiolated and greening tissue.

To test this hypothesis, the spatial distribution of *rbcL* transcripts in *bsd2-m1* was examined by in situ hybridization of ^{35}S-labeled riboprobes to etiolated and greening leaf sections. Figure 7 shows that in wild-type etiolated leaves, *rbcL* mRNAs accumulated primarily in bundle sheath cells, but they were also present in mesophyll cells. When exposed to light, steady state levels of *rbcL* transcripts decreased in mesophyll cells and increased in bundle sheath cells. In etiolated *bsd2-m1* leaves, *rbcL* transcripts were abundant in both bundle sheath and mesophyll cells; however, exposure to light failed to suppress *rbcL* mRNA accumulation in mesophyll cells. Furthermore, overall levels of transcript were increased relative to the wild type. This was particularly obvious when sections from the tip of *Bsd2* and *bsd2-m1* greening leaves were compared. In wild-type leaves, *rbcL* transcript levels were down-regulated in cells at the tip (Figure 7). Similar patterns of repression were observed with nuclear-encoded transcripts in *bsd2-m1* leaves (data not shown); however, *rbcL* mRNA levels remained high (Figure 7). These findings suggest that during normal development, the *Bsd2* gene acts in a light-independent manner to spatially and temporally regulate *rbcL* transcript levels.

The *bsd2-m1* Mutation Specifically Disrupts Bundle Sheath Cell Chloroplast Structure

Immunolocalization of the NADP-ME protein (Figure 2) and in situ localization of *rbcL* transcripts (Figure 4) both suggested that chloroplast structure was perturbed at the tip of *bsd2-m1* leaves. To investigate the possibility that chloroplast structure deteriorates as development proceeds, we examined the chloroplast ultrastructure in *bsd2-m1* leaves. The spatial accumulation patterns of *rbcL* mRNAs (Figure 4) suggested that the *bsd2-m1* mutation disrupts leaf development as early as plastochron 4 or 5. At this stage, bundle sheath and mesophyll progenitor cells are cytoplasmically dense and can be identified only by their position relative to vein sites; proplastids in both cell types are identical. Figure 8 shows that *Bsd2* and *bsd2-m1* proplastids were indistinguishable in plastochron leaf primordia. This observation suggests that the *Bsd2* gene plays no role in early plastid differentiation.

To determine whether the *bsd2-m1* mutation affects subsequent stages of chloroplast development, the seedling leaf sheath and blade were examined. The leaf sheath represents an earlier developmental stage than the blade and has been shown to be more C_3 than C_4 in character, both with respect to photosynthetic activity and chloroplast differentiation (Sharman, 1942; Kirchanski, 1975; reviewed in Nelson and Langdale, 1992). In the *bsd2-m1* sheath, chloroplast differentiation in both bundle sheath and mesophyll cells was normal (Figure 8). This suggests either that the *bsd2-m1* mutation does not perturb early developmental pathways or that the mutation does not affect chloroplast structure in regions of the leaf that lack C_4 photosynthetic capability. To test this further, chloroplast structure was examined at the base and tip of wild-type and mutant third leaf blades (Figure 8). During all stages of blade development, mesophyll cell chloroplast structure was unaffected by the *bsd2-m1* mutation. Any differences observed between mutant and wild-type mesophyll cell chloroplasts (e.g.,

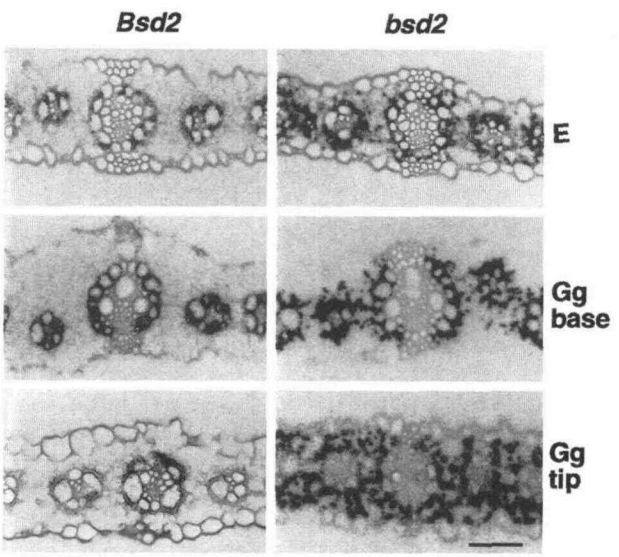

Figure 7. In Situ Detection of *rbcL* Transcripts in Etiolated (E) and Greening (Gg) Leaves of Wild-Type (*Bsd2*) and *bsd2-m1* (*bsd2*) Siblings.

Bar = 50 μm.

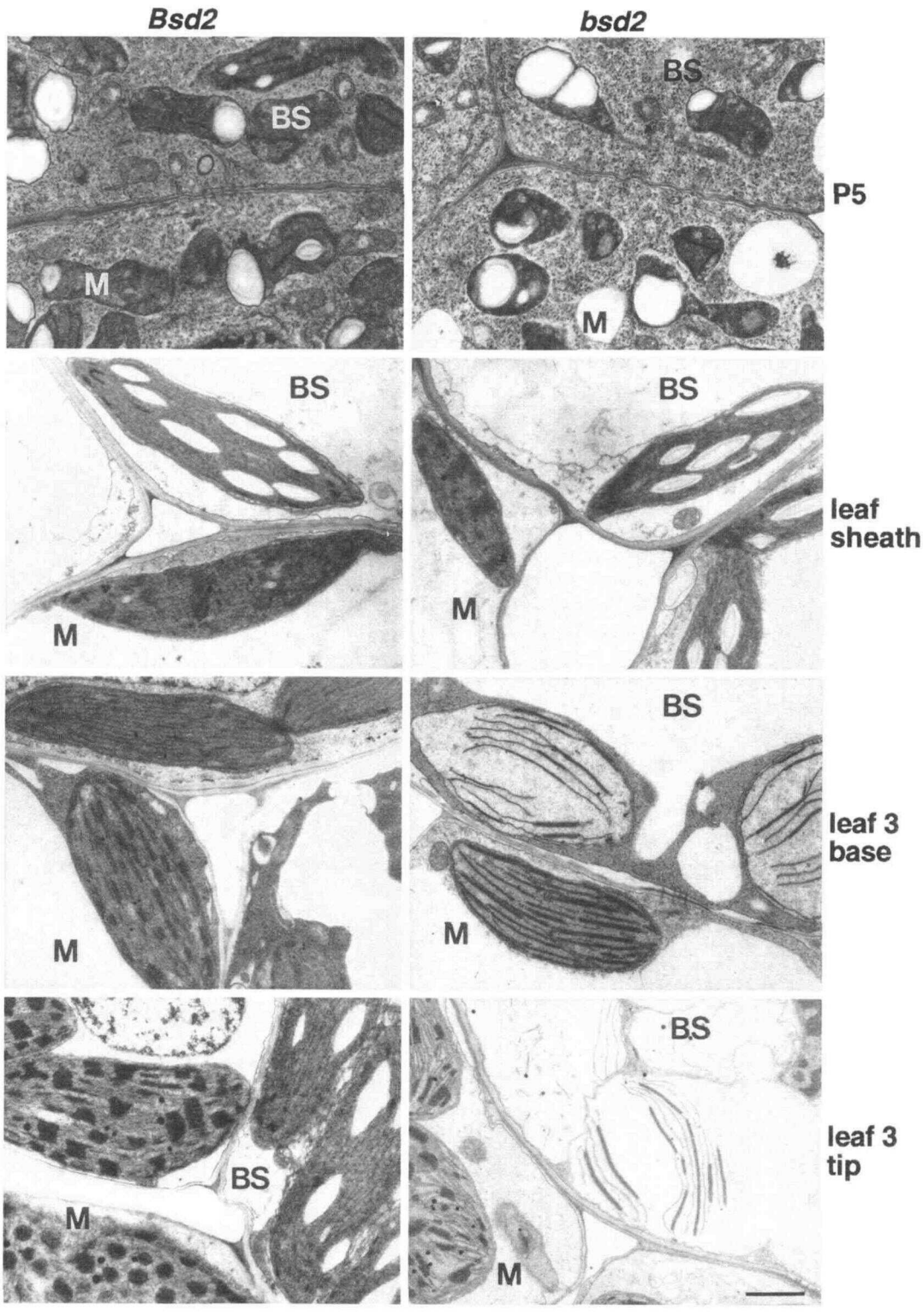

Figure 8. Transmission Electron Microscopy of Plastochron 5 (P5) Leaf Primordia, Second Leaf Sheaths, and Third Leaf Blades (Base and Tip) of Wild-Type (*Bsd2*) and *bsd2-m1* (*bsd2*) Siblings.

BS, bundle sheath; M, mesophyll. Bar = 1.1 μm.

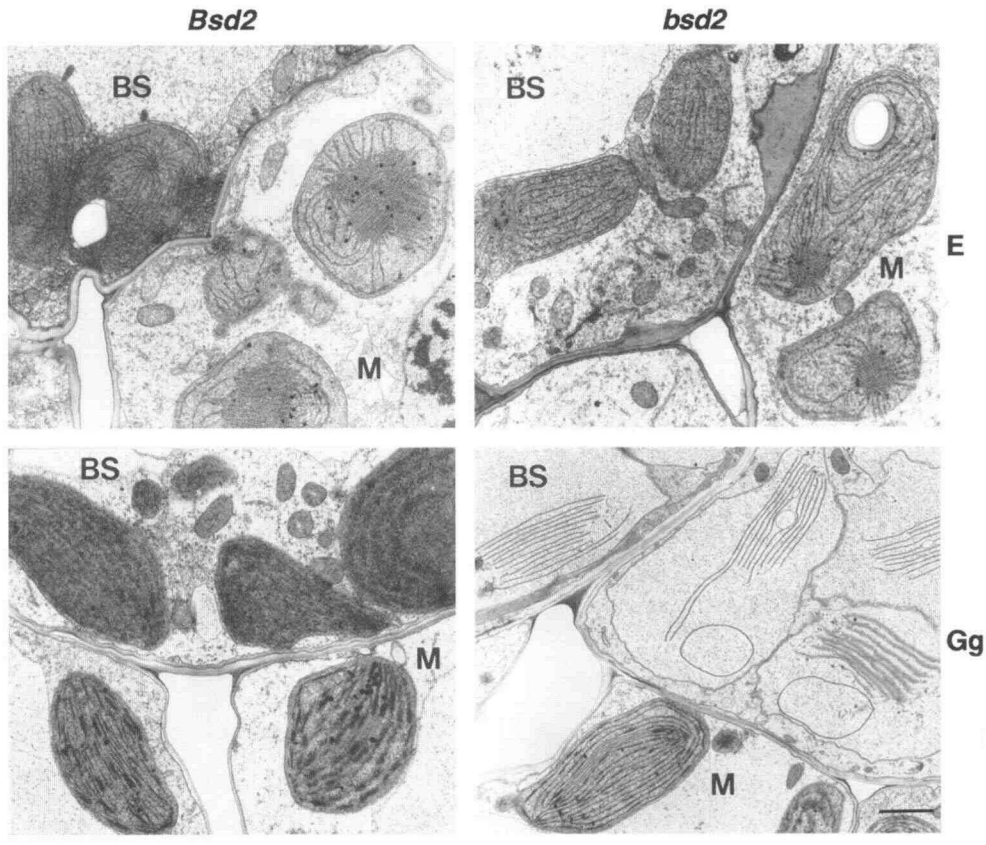

Figure 9. Transmission Electron Microscopy of Etiolated (E) and Greening (Gg) Leaves of Wild-Type (*Bsd2*) and *bsd2-m1* (*bsd2*) Seedlings. BS, bundle sheath; M, mesophyll. Bar = 1.1 μm.

base of leaf three) were inconsistent and could always be explained on the basis of differences in developmental age (Kirchanski, 1975). In contrast, bundle sheath cell chloroplasts at the base of *bsd2-m1* third leaves appeared "swollen" when compared with the wild type. This swelling increased in severity toward the tip of *bsd2-m1* leaves, until the chloroplasts filled the entire bundle sheath cell space (Figure 8). Furthermore, bundle sheath chloroplasts contained only rudimentary lamellae with long regions of appression. These data suggest that the *bsd2-m1* mutation specifically disrupts bundle sheath cell chloroplasts in the C_4 leaf blade and that the degree of disruption is correlated with developmental age.

To distinguish between developmental and light-induced effects of the *bsd2-m1* mutation on bundle sheath cell chloroplast structure, we examined ultrathin sections of etiolated and greening leaves. Figure 9 shows that wild-type and *bsd2-m1* etioplasts looked identical. This suggests that *Bsd2* is not required for normal plastid development in dark-grown leaves. After exposure to light for 24 hr, however, bundle sheath cell chloroplasts in *bsd2-m1* leaves appeared swollen (Figure 9). Interestingly, appressed lamellae were absent from chloroplasts of greening leaves. This suggests that the order of events leading to the phenotype observed in light-grown leaves (Figure 8) is swelling of the outer membrane, followed by disintegration and stacking of the internal thylakoid membranes. These results show that the disruption of bundle sheath cell chloroplast structure is a light-dependent event. Therefore, we propose that the *Bsd2* gene product does not directly regulate bundle sheath or mesophyll chloroplast differentiation but that the observed defects in bundle sheath chloroplast morphology are an indirect consequence of aberrant photosynthetic activity. Furthermore, we propose that in the most severely affected cells, chloroplast deterioration leads to turnover of bundle sheath cell–specific transcripts and proteins.

DISCUSSION

Leaf development involves the coordinated control of cell division patterns and the determination of cell fate. Once cells have become determined, differentiation processes elaborate the final form. In *bsd2-m1* maize leaves, neither overall leaf morphology nor the anatomical arrangement of bundle sheath and

mesophyll cells are disrupted. This implies that cellular division and determination patterns are not regulated by the *Bsd2* gene product. However, the biochemical and morphological differentiation of bundle sheath cells is perturbed in *bsd2-m1* leaves, whereas mesophyll cells appear to differentiate normally. This suggests that *Bsd2* gene action is primarily required in the maturation and maintenance of bundle sheath cell function.

Aberrant regulation of RuBPCase is the only feature of the *bsd2-m1* mutant phenotype that is observed at all developmental stages in both light-grown and dark-grown plants. The absence of large and small subunit proteins throughout *bsd2-m1* leaf development, despite *rbcL* and *RbcS* transcript accumulation, suggests that there is a failure to translate one or both mRNAs, or that both proteins are unstable in *bsd2-m1* leaves. Current evidence suggests that perturbation of either *rbcL* or *RbcS* gene regulation results in the absence of both subunits. For example, a point mutation in the tobacco *rbcL* gene that prevents holoenzyme assembly results in a 98% reduction in steady state levels of both large and small subunit proteins (Avni et al., 1989). Similarly, antisense inhibition of *RbcS* gene expression in transgenic tobacco prevents large subunit protein accumulation, although *rbcL* transcripts accumulate to normal levels and are translated (Rodermel et al., 1988; Jiang and Rodermel, 1995). In *bsd2-m1* leaves, *RbcS* transcript accumulation profiles were essentially normal; however, the spatial and temporal regulation of *rbcL* transcripts was disrupted. On this basis, we suggest that the *Bsd2* gene regulates *rbcL* gene expression patterns but does not directly affect *RbcS* gene expression.

The accumulation of *rbcL* transcripts in both bundle sheath and mesophyll cells of *bsd2-m1* light-grown leaves suggests that during normal development, the *Bsd2* gene acts to destabilize *rbcL* transcripts in mesophyll cells. In greening leaf tips of *bsd2-m1* leaves, the abnormally high levels of *rbcL* transcripts observed further suggest that *Bsd2* acts to control temporally the absolute transcript levels in both bundle sheath and mesophyll cells. Thus, during normal development, *Bsd2* acts post-transcriptionally to regulate both the spatial and temporal profiles of *rbcL* transcripts. Similar post-transcriptional mechanisms may be involved in the cell-type–specific (Boinski et al., 1993) and temporal (Berry et al., 1985) expression of the *rbcL* gene in *Amaranthus hypochondriacus*.

At the tip of light-grown *bsd2-m1* leaves, where *rbcL* transcripts are turned over, other (perhaps more general) degradation mechanisms may act. In particular, senescence-related events may be involved because mutant plants discolor and start to wilt ~12 days after germination. It has been proposed that reduced chloroplast gene expression during leaf senescence results from decreased chloroplast function followed by increased chloroplast breakdown (Camp et al., 1982; Mae et al., 1984; Wardley et al., 1984; Ford and Shibles, 1988; Kura-Hotta et al., 1990). In *RbcS* antisense-inhibited transgenic tobacco, the developmental reduction of *rbcL* transcripts observed in older leaves has been correlated with a decrease in plastid DNA template availability (Jiang and Rodermel, 1995). In a similar fashion, the reduction in *rbcL* mRNA levels at the tips of *bsd2-m1* light-grown leaves could be a consequence of chloroplast breakdown.

Nuclear genes that regulate different aspects of chloroplast photosynthetic gene expression have been identified; however, the specific mechanisms by which such genes act remain elusive (Mayfield et al., 1995). In Chlamydomonas, nuclear genes have been reported to regulate chloroplast transcription (*rbcL*) (Hong and Spreitzer, 1994), RNA processing (cytochrome b_6f complex *petD* mRNA) (Barkan et al., 1994), *trans*-splicing (photosystem I subunit *psaA* transcripts) (Goldschmidt-Clermont et al., 1990), stability (photosystem II subunit transcripts *psbC* and *psbB*) (Sieburth et al., 1991), and translation (*psbA*) (Danon and Mayfield, 1991; Girard-Bascou et al., 1992). In spinach, a nuclear-encoded 28-kD RNA binding protein (28RNP) appears to be responsible for 3′ processing and/or stability of four plastid mRNAs (including *psbA* and *rbcL*) (Schuster and Gruissem, 1991). The 28RNP protein, which is differentially expressed during plant development in concert with plastid RNAs, exhibits two conserved RNA binding domains. RNA binding occurs upon dephosphorylation of an acidic domain at the N terminus, but the exact nature of the protein–RNA interaction is not known (Lisitsky and Schuster, 1995). It is conceivable that *Bsd2* may play a similar role in the regulation of *rbcL* mRNA stability and/or translation.

In maize, a genetic approach has been used to identify a number of genes that regulate chloroplast RNA metabolism. For example, *hcf38* mutants in maize show alterations in the abundance and size of many chloroplast transcripts, suggesting that the *Hcf38* gene regulates the synthesis or maturation of a group of chloroplast mRNAs (Barkan et al., 1986). In contrast, *chloroplast protein synthesis1-1* (*cps1-1*), *cps1-2*, *cps2*, and *hcf7* mutants contain reduced levels of many chloroplast-encoded proteins without corresponding deficiencies in chloroplast transcripts (Barkan, 1993). In *cps* mutants, chloroplast mRNAs are associated with abnormally few ribosomes, suggesting that *Cps1-1*, *Cps1-2*, and *Cps2* regulate general chloroplast translation processes. However, *Hcf7* appears to regulate 16S rRNA processing. An additional maize mutant, *chloroplast RNA processing1 (crp1)*, exhibits defects in both mRNA processing and translation in that unprocessed polycistronic mRNAs are not translated (Barkan et al., 1994). Although the *bsd2-m1* mutant also displays an *hcf* phenotype, preliminary mapping data suggest that the mutation is genetically distinct from those described above (L.N. Hall and J.A. Langdale, unpublished data). Complementation analysis is currently under way to determine whether *bsd2-m1* is allelic to other *hcf* mutants that map to the same chromosome arm (T.P. Brutnell and J.A. Langdale, unpublished data).

A number of features of the *bsd2-m1* mutant phenotype can only be observed at the tip of light-grown leaves. We propose that these bundle sheath cell–specific disruptions (i.e., reduction in NADP-ME levels, reduction in *RbcS* mRNA levels, and aberrant chloroplast structure) are secondary effects that result from an absence of RuBPCase protein. Interestingly, the observed swelling of chloroplasts is similar to the chloroplast phenotype observed in tobacco mutants that fail to assemble

the RuBPCase holoenzyme (Foyer et al., 1993). There are a number of possible explanations for the bundle sheath cell–specific nature of the *bsd2-m1* phenotype. First, because the mesophyll cell–specific enzymes accumulate to normal levels in *bsd2-m1* leaves, it is conceivable that they are functional. As such, malate would be generated in the mesophyll and then transferred to the bundle sheath. The final outcome of this scenario would depend on whether NADP-ME present in the bundle sheath is active. Functional NADP-ME would result in elevated levels of CO_2, which would increase the proton load in the cytoplasm. Whether this would cause bundle sheath cell–specific pH changes depends on the buffering capacity of the cytoplasm and the activity of various pH-regulating transport processes. Interestingly, studies with tomato have shown that elevated cellular CO_2 results in the repression of *RbcS* but not *rbcL* transcript levels; this result is consistent with our observations of *bsd2-m1* bundle sheath cells (Van Oosten and Besford, 1994). If NADP-ME is inactive in the bundle sheath, levels of malate tend to increase. It is not clear what the physiological consequence of this increase would be both in terms of pH regulation and organic acid cycling, because there will be no corresponding movement of pyruvate back to the mesophyll.

Another, more likely explanation for the bundle sheath cell–specific phenotype is that the absence of RuBPCase, and hence lack of Calvin cycle activity, leads to excess reduction of photosystem I due to a decrease in the metabolic demand for ATP. Under these conditions, the futile cycling of electrons through photosystem I would generate an increase in levels of reactive-oxygen intermediates. Such reactive-oxygen intermediate species could trigger photooxidative damage in the bundle sheath cell chloroplasts (Allen, 1995). Photooxidative damage has previously been correlated with chloroplast breakdown and repression of photosynthetic gene expression in a number of carotenoid-deficient mutants (reviewed in Taylor, 1989). For example, carotenoid-deficient maize plants that exhibit photooxidative damage under high-light conditions have reduced levels of *RbcS* mRNAs (Mayfield and Taylor, 1987). This evidence supports our hypothesis that the reduction of transcript levels at the tip of light-grown *bsd2-m1* leaves could be due to photooxidative damage in bundle sheath cells. Furthermore, recent experiments have shown that if *bsd2-m1* plants are grown at low light intensity, bundle sheath cell chloroplasts are not disrupted (R. Roth and J.A. Langdale, unpublished observations).

We have previously characterized the *bsd1-m1* mutant of maize and shown that the *Bsd1* gene acts to regulate both the morphological and functional differentiation of bundle sheath cells in the light (Langdale and Kidner, 1994). In dark-grown plants, *Bsd1* regulates the accumulation of RuBPCase in both cell types but directs chloroplast differentiation specifically in bundle sheath cells. Together, these data suggest that the *Bsd1* gene product plays a direct role in regulating bundle sheath cell chloroplast differentiation. Furthermore, they suggest that *Bsd1* acts downstream of genes that act to restrict RuBPCase accumulation to bundle sheath cells in the light. As such, the *bsd1-m1* mutant differs in two significant ways from the *bsd2-m1* mutant. First, the disruption of chloroplast structure in *bsd2-m1* mutants appears to be a secondary effect of the mutation, suggesting that the *Bsd2* gene is not inherently involved in chloroplast differentiation. Second, *bsd2-m1* mutants fail to respond to the signal that normally restricts *rbcL* gene expression to bundle sheath cells. Double-mutant analysis should provide further insights into the respective roles of *Bsd1* and *Bsd2* during bundle sheath cell differentiation and maintenance.

Interestingly, our mutagenesis program has yielded six mutants that disrupt C_4 differentiation in bundle sheath cells but none that disrupts mesophyll cell development (Langdale et al., 1995). The most obvious explanation for this bias is that our screen precludes the identification of mesophyll cell–specific mutants. We have no reason to believe that this is the case, however, because our primary screen was very general (i.e., we examined pale green plants). Therefore, our data suggest either that bundle sheath cell differentiation is dependent on mesophyll cell differentiation, such that perturbation of mesophyll cell development necessarily disrupts bundle sheath cell development, or that bundle sheath cell maturation is more easily perturbed by general defects in photosynthesis. Characterization of the *bsd2-m1* mutant has provided support for the latter explanation because the primary effect of the mutation appears to be non–cell specific, yet the majority of phenotypic perturbations are manifest specifically in bundle sheath cells.

METHODS

Plant Material and Growth Conditions

The *bundle sheath defective2–mutable1* (*bsd2-m1*) allele was isolated from a line containing active *Mutator* transposable elements by W.F. Sheridan (University of North Dakota, Grand Forks). *bsd2-m1* plants are pale green and die after the endosperm reserves are depleted (usually ~13 days after planting). Occasionally, however, sectors of dark green tissue permit growth beyond the seedling stage. Such sectored plants were used to cross and backcross *bsd2-m1* into the inbred line W22. Heterozygous progeny of the second cross into W22 were self-pollinated, and the resultant progeny were used for the experiments described in this report. In all cases, *bsd2-m1* mutants segregated 1:4, thus indicating the recessive nature of the mutation.

Seedlings were grown in soil in a growth chamber maintained at 28°C with a 16-hr-light (131 μmol m^{-2} sec^{-1})/8-hr-dark cycle. Plastochron 1 to 5 (P1–5) leaf primordia were harvested 6 days after planting, when the seedlings were 1.5 to 2 cm tall and all leaves were still enclosed in the coleoptile. For all experiments, the young shoots were excised 2 to 3 mm above the shoot apical meristem, after which the seedlings were transferred to the growth chamber and grown until mutant phenotypes could be scored. For RNA and immunoblot analyses, the coleoptile was removed before harvesting the leaf primordia. Two 2- to 3-mm transverse sections were taken from the base of the immature shoot for in situ experiments, thus comprising plastochron

3 to 5 leaf primordia enclosed within an outer coleoptile. For transmission electron microscopy (TEM), the coleoptile and all internal leaf primordia were removed before harvesting 2-mm^2 tissue samples across the base of leaf 1 (plastochron 5). Third leaves from light-grown seedlings were harvested 11 days after sowing, as the fourth leaf was emerging. For in situ and TEM analyses, tissue samples were taken from the first centimeter of the base and tip of the third leaf. Sheath tissue for light microscopy was harvested by taking transverse sections across the shoot (2 cm below the ligule of leaf 1), thus comprising immature leaves 1 to 3 and the sheath of leaf 2 within the sheath of leaf 1. For TEM, the second leaf sheath was dissected, and 2-mm^2 tissue samples were harvested across the midregion.

Etiolated seedlings were grown in vermiculite in complete darkness at 28°C for 6 days until the first centimeter of the unexpanded leaf 1 was visible above the coleoptile. Tissue was harvested by excising the seedling 3 to 4 mm above the shoot apical meristem (under green safe light), after which plants were transferred to the growth chamber and grown until mutant phenotypes could be scored. For RNA and immunoblot analyses, the first leaf was harvested by removing the coleoptile and internal leaves. Tissue samples for TEM and in situ analyses were taken within 1 cm of the tip of leaf 1. Light-shifted (greening) seedlings were grown in complete darkness, as described above, and then shifted to the growth chamber for 24 hr. Seedlings were shifted at the beginning of the 16-hr-light/8-hr-dark cycle. Leaf 2 was harvested by cutting across the shoot (1 cm below the ligule of leaf 1).

Purification of Separated Bundle Sheath and Mesophyll Cells

Bundle sheath and mesophyll cells were isolated from wild-type and mutant leaves, according to Sheen and Bogorad (1985).

cDNA and Antibody Probes

The ribulose bisphosphate carboxylase (RuBPCase) small subunit (*RbcS*), RuBPCase large subunit (*rbcL*), phosphoenolpyruvate carboxylase (PEPCase) (*Ppc1**Zm1), NADP-dependent malic enzyme (NADP-ME) (*Mod1*Zm1*), NADP-dependent malate dehydrogenase (NADP-MDH) (*Mdh1**Zm1), and pyruvate phosphodikinase (PPDK) (Ppdk1*Zm1) cDNA clones (pJL10, pJL12, pTN1, pTN5, C30, and pH 2, respectively) have been described previously (Langdale et al., 1988a; Glackin and Grula, 1990; Langdale and Kidner, 1994). A *psbA* clone was kindly provided by A. Barkan (University of Oregon, Eugene).

Antisera raised against RuBPCase holoenzyme (wheat), RuBPCase large subunit (*Flaveria*), RuBPCase small subunit (maize), PEPCase (*Amaranthus hypochondriacus*), PPDK (maize), NADP-ME (maize), and NADP-MDH (maize) have been described previously (Langdale et al., 1987; Langdale and Kidner, 1994). With the exception of the large subunit antiserum, all antibodies used were polyclonal.

Preparation of Total Leaf Protein and Immunoblot Analysis

The isolation of total leaf protein has been described previously (Langdale et al., 1987). Samples were electrophoresed on 10% SDS–polyacrylamide gels and blotted onto 0.2-mm nitrocellulose membranes by using a Bio-Rad Mini Protean II apparatus according to the manufacturer's instructions. Immunoblot analysis was performed using horseradish peroxidase–conjugated secondary antibody and 4-chloro-1-naphthol substrate, as described by Langdale et al. (1987).

Preparation of RNA and RNA Gel Blot Analysis

RNA was purified, electrophoresed on 1% formaldehyde–agarose gels, blotted onto Nytran membranes (Schleicher & Schuell, Dassel, Germany), and hybridized as reported previously by Langdale et al. (1988a).

In Situ Localization of C_4 Gene Products

Plant tissues were fixed in 3:1 ethanol–acetic acid for 30 min at room temperature and stored at 4°C in 70% ethanol. Samples were embedded in Paraplast Plus (Sherwood Medical Co., St. Louis, MO) and sectioned to 8 mm as reported previously (Langdale et al., 1987). Immunolocalization assays were also performed as described previously (Langdale et al., 1987). Briefly, after rehydration, sections were incubated with NADP-ME primary antibody diluted 1:500 in PBS (10 mM sodium phosphate buffer, pH 7.5, 0.15 M NaCl) containing 1 mg mL^{-1} BSA. Bound antibodies were detected using a biotinylated secondary antibody and a streptavidin–horseradish peroxidase detection system. RNA in situ hybridization assays were performed using ^{35}S-labeled riboprobes, as described by Langdale et al. (1988a).

Electron Microscopy

All tissues were cut under and fixed in Karnovsky's fixative (3% paraformaldehyde, 3% gluteraldehyde, 0.025 M phosphate buffer, pH 7.2) for 2 hr at room temperature. All plant tissues that needed to be scored for a mutant phenotype (e.g., etiolated and P1–5 leaves) were stored in 0.025 M phosphate buffer, pH 7.2, after prefixing (for a maximum of 7 days). Tissue samples were postfixed for 1 hr in 2% OsO_4, followed by two consecutive 20-min washes in 0.025 M phosphate buffer, pH 7.2. Following dehydration through an acetone series, samples were gradually infiltrated with 25% TAAB resin/acetone (TAAB Laboratory Equipment, Reading, UK) overnight, followed by 50% (for 5 hr), 75% (overnight), and finally 100% (overnight) TAAB. The resin was subsequently polymerized at 60°C for 24 hr. Ultrathin sections (60 to 70 nm) were cut using a diamond knife on a Sorvall MT5000 microtome. Sections were mounted on Butvar B98 (Agar Aids, Essex, UK) slots, stained with 0.5% uranyl acetate for 1 hr, and then counterstained with 2.7% lead citrate for 30 min. All rinses were performed with CO_2-free distilled water. Sections were examined on a Jeol JEM-2000EX transmission electron microscope and photographed using AGFA Scientia EM film 23 D 56 (Leverkusen, Germany).

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