bundle sheath defective, a mutation that disrupts cellular differentiation in maize leaves

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SUMMARY

Post-primordial differentiation events in developing maize leaves produce two photosynthetic cell types (bundle sheath and mesophyll) that are morphologically and biochemically distinct. We have isolated a mutation that disrupts the differentiation of one of these cell types in light-grown leaves. *bundle sheath defective 1-mutable 1* (*bsd1-m1*) is an unstable allele that was induced by transposon mutagenesis. In the bundle sheath cells of *bsd1-m1* leaves, chloroplasts differentiate aberrantly and C4 photosynthetic enzymes are absent. The development of mesophyll cells is unaffected. In dark-grown *bsd1-m1* seedlings, morphological differentiation of etioplasts is only disrupted in bundle sheath cells but photosynthetic enzyme accumulation patterns are altered in both cell types. These data suggest that, during normal development, the *Bsd1* gene directs the morphological differentiation of chloroplasts in a light-independent and bundle sheath cell-specific fashion. In contrast, *Bsd1* gene action on photosynthetic gene expression patterns is cell-type independent in the dark (C3 state) but bundle sheath cell-specific in the light (C4 state). Current models hypothesize that C4 photosynthetic differentiation is achieved through a light-induced interaction between bundle sheath and mesophyll cells (J. A. Langdale and T. Nelson (1991) *Trends in Genetics* 7, 191-196). Based on the data shown in this paper, we propose that induction of the C4 state restricts *Bsd1* gene action to bundle sheath cells.

Key words: differentiation, maize, bundle-sheath, chloroplast development, C4 photosynthesis

INTRODUCTION

The development of vegetative leaves is an indispensable feature of the life cycle of non-succulent vascular plants. In spite of this fact, our understanding of events that control differentiation in the leaf is limited. The maize leaf is an excellent system for the study of cellular differentiation events since the final differentiated state is well defined both morphologically and functionally. The mature leaf is composed of sheath and blade regions which are delimited by an epidermal fringe known as the ligule. As a consequence of cell division patterns, a developmental gradient exists with the oldest cells at the tip of the blade and the youngest at the base of the sheath (Sharman, 1942; Sylvester et al., 1990). Both sheath and blade contain a series of parallel veins running the length of the leaf (Esau, 1942; Russell and Evert, 1985). Surrounding these veins are concentric circles of two distinct photosynthetic cell types. Bundle sheath (BS) cells, which are situated immediately adjacent to the vein, develop co-ordinately with neighbouring mesophyll (M) cells to interact in the fixation of CO_2 in the C4 photosynthetic cycle [reviewed in Langdale and Nelson (1991) and Nelson and Langdale (1992)].

At maturity, BS and M cells are structurally and biochemically distinct. BS cells have agranal chloroplasts that are arranged centrifugally within the cell whereas M cells have granal chloroplasts that are randomly arranged (Brown, 1975). Each of the two cell types accumulates a distinct complement of C4 photosynthetic enzymes. Ribulose bisphosphate carboxylase (RuBPCase) and NADP-malic enzyme (NADP-ME) function in the BS cells whereas phosphoenolpyruvate carboxylase (PEPCase), pyruvate phosphate dikinase (PPdK) and NADP-malate dehydrogenease (NADP-MDH) function in the M cells (Edwards and Huber, 1979). Current evidence suggests that this cell-specific accumulation of photosynthetic enzymes is regulated by cell position and light (Langdale et al., 1988b). BS and M cells differentiate in concert with the vascular system. Cells around the midvein mature first, followed by cells surrounding lateral veins and finally by those surrounding intermediate veins (Langdale et al., 1987). In light-grown plants, M cells positioned adjacent to a vascular bundle accumulate the appropriate complement of C4 enzymes. M cells located further than two cells away from the vein, however, accumulate RuBPCase instead of M cell-specific enzymes and photosynthesize using the C3 pathway (Langdale et al., 1988b). In dark-grown tissues, both BS and M cells accumulate RuBPCase although the leaves are non-photosynthetic (Sheen and Bogorad, 1986; Langdale et al., 1988b). These results suggest that maize develops a C3 pattern of cell-type differentiation (RuBPCase in all photosynthetic cells) by default and that C4 specialization is achieved through the inter-

pretation of a light-induced signal that emanates from the leaf vasculature (Langdale et al., 1988b). M cells at a distance from a vein either do not receive the signal or cannot interpret it. The concept of a positional control of photosynthetic cell-type differentiation has been supported by cell lineage analysis. M cells in the central layer of the leaf blade are more closely related to BS cells than to other M cells (Langdale et al., 1989). Therefore, as all M cells differentiate in the same way, cell position must play a greater role than cell lineage in directing cell-type differentiation.

Current models to explain mechanisms of C4 development imply that photosynthetic differentiation in BS and M cells results from a light-induced interaction between the two cell types (Langdale and Nelson, 1991; Nelson and Dengler, 1992; Nelson and Langdale, 1992). Since the differentiation of BS and M cells is temporally co-ordinate, however, it has not been possible to dissect this signalling process. To address this problem, we have isolated and characterized a mutation that disrupts the differentiation of a single cell type in light-grown leaves. The *bundle sheath defective 1-mutable 1* (*bsd1-m1*) allele was induced by transposon mutagenesis. In this paper, we show that the *bsd1-m1* mutation uncouples the tightly co-ordinated development of BS and M cells that is essential for C4 photosynthetic function in maize leaves. Based on the data shown, we propose that after induction of the C4 state, *Bsd1* gene action directs chloroplast differentiation and photosynthetic gene expression in BS cells. C4 photosynthetic differentiation in M cells is not dependent on *Bsd1* gene function.

MATERIALS AND METHODS

Plant material and growth conditions

The *bsd1-m1* allele was identified in a random mutagenesis programme, which utilized the maize transposable element *Spm* (McClintock, 1954) as an insertional mutagen. *opaque2-mutable20* (*o2-m20*), an allele containing an autonomous *Spm* at the *O2* locus (Schmidt et al., 1987), was backcrossed to a stable *o2* line and germinal revertants were selected. Plants from 346 *O2* revertant kernels were self-pollinated, and the progeny screened for segregating mutant phenotypes. *bsd1-m1* was initially identified in a family that had pale green lethal seedlings segregating in a 3:1 ratio.

Field crosses were carried out in Connecticut, USA in the summers of 1989-1992. Seedlings were screened in sand benches in a greenhouse maintained at 25°C with a light cycle of 16 hours light (400 μE/m^2/s) and 8 hours dark. Segregating populations of plants used to characterize the mutant phenotype were grown in soil in the greenhouse as above. Etiolated seedlings were grown in vermiculite in total darkness at 28°C. After harvesting tissue samples, etiolated plants were transferred to the greenhouse and grown until mutant phenotypes could be scored. All tissue samples were harvested early in the morning so that starch content in the leaves was minimal.

Antibody and cDNA probes

RuBPCase holoenzyme (wheat), large subunit (Lsu) (*Flaveria*) and small subunit (Ssu) (maize) antisera were gifts from Drs Rachel Leech (University of York), Carole Bassett (USDA/ARS, Athens, GA) and Jen Sheen (Massachussetts General Hospital, Boston), respectively. PEPCase (*Amaranthus*) antibody was a gift from Dr Jim Berry (State University of New York, Buffalo). PPdK, NADP-ME and NADP-MDH antisera have been described previously (Langdale et al., 1987). With the exception of the Lsu antiserum, all antibodies used were polyclonal.

RuBPCase Ssu (*RbcS*), RuBPCase Lsu (*RbcL*), PEPCase (*Ppc1*Zm1*) and NADP-ME (*Mod1*Zm1*) cDNA clones have been described previously (pJl10, pJL12, pTN1 and pTN5 respectively) (Langdale et al., 1988a). An NADP-MDH (*Mdh1*Zm1*) cDNA subclone was obtained from Drs M. Metzler and T. Nelson (Yale University). This subclone (C30) has a 1.1kb *Eco*R1 fragment derived from the full-length cDNA clone described in Metzler et al. (1989), inserted into the *Eco*R1 site of pBS(+) (Stratagene, San Diego, California). A PPdK (*Ppdk1*Zm1*) cDNA clone (pH2) was obtained from Dr J. Grula (Phytogen) (Hudspeth et al., 1986). A 200bp *Pst*I fragment of this cDNA was subcloned into pBS(+) using standard procedures (Sambrook et al., 1989).

Preparation of total leaf protein and western analysis

Total leaf protein was isolated as previously described (Langdale et al., 1987). Samples were electrophoresed on 10% SDS-polyacrylamide gels and blotted onto 0.2 μm nitrocellulose using a Bio-Rad Mini Protean II apparatus according to the manufacturer's instructions. Western analysis was carried out using horseradish peroxidase (HRP)-conjugated secondary antibody and 4-chloro-1-naphthol substrate, also as previously described (Langdale et al., 1987).

Preparation of RNA and northern analysis

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

In situ localization of photosynthetic gene products

Tissue samples were fixed in ethanol:acetic acid (3:1), embedded in Paraplast plus and sectioned as previously described (Langdale et al., 1987). Immunocytochemistry was carried out using monospecific primary antibodies, a biotinylated secondary antibody and a streptavidin-HRP detection system, also as in Langdale et al. (1987). Sections were stained with Safranin/Fast Green as in Langdale et al. (1988b) and hybridized in situ with ^{35}S-labelled riboprobes as in Langdale et al. (1988a).

Electron microscopy

Leaf sections (<1 mm) were cut under, and prefixed, in Karnovsky's fixative (Karnovsky, 1965) for 3 hours. Samples were washed in 0.1 M phosphate buffer pH7.2, postfixed for an hour in 2% OsO$_4$ and transferred back into phosphate buffer overnight. After dehydration through an acetone series, sections were put in 25% TAAB resin (TAAB Laboratory Equipment, Reading, UK) (in acetone) overnight. Samples were subsequently transferred into 50%, 75% and 100% resin (2 hours each) before polymerization at 60°C for 24 hours. Ultrathin sections were cut using a glass knife on a Reichart OMO2 microtome and with a diamond knife on a Sorvall MT5000 microtome. Sections were mounted on pioloform slots, stained with 0.5% uranyl acetate for 10 minutes, rinsed with CO$_2$-free distilled water and then counterstained with 2.7% lead citrate for 10 minutes. Sections were examined on a JEOL JEM-2000EX transmission electron microscope. Micrographs were taken using Ilford EM film.

RESULTS

bsd1-m1 is a recessive pale green-mutable allele

bsd1-m1 seedlings were identified in the M2 generation of a transposon mutagenesis experiment (see Materials and Methods). Mutant leaf blades display a variegated phenotype with pale green and dark green sectors (Fig. 1A). Preliminary molecular and genetic evidence suggests but does not prove that *bsd1-m1* is *Spm*-induced (J. Langdale, unpublished observations). However, we have not yet determined whether the

Fig. 1. Mutant phenotype of *bsd1-m1* second (A) and eighth (B) leaves. The seedling leaf shown in A was 15 cm from the ligule to the tip, whereas the mature leaf in B was 88 cm from ligule to tip.

Fig. 2. Western blot analysis of total leaf proteins isolated from (1) wild-type sibling leaf 3; (2) *bsd1-m1* leaf 3 - mutant tissue; (3) *bsd1-m1* leaf 7 - revertant sector in basal half of leaf; (4) *bsd1-m1* leaf 7 - mutant tissue in basal half of leaf; (5) *bsd1-m1* leaf 7 - revertant sector in upper half of leaf; (6) *bsd1-m1* leaf 7 - mutant tissue in upper half of leaf. Third leaves were harvested as the fourth leaves emerged (26 days after planting) whereas the seventh leaf was harvested as the ninth leaf emerged (43 days after planting). Third leaves were 28 cm (wild-type) and 23 cm (*bsd1-m1*) from ligule to tip whereas the seventh leaf was 64 cm from ligule to tip. Protein extracts were prepared using a standard weight of tissue per volume of extraction buffer. Equal sample volumes were loaded for western analysis. M cell-specific enzymes are shown in the top panel whereas BS cell-specific enzymes are shown in the bottom panel. RuBPCase Lsu ($M_r = 53 \times 10^3$) and Ssu ($M_r = 14 \times 10^3$) proteins are shown.

observed variation represents somatic excision events (i.e. dark green excision sectors on a pale green mutant background), the activity of an *Spm*-suppressible allele (i.e. pale green 'suppressed' sectors on a dark green non-suppressed background) or the activity of an *Spm*-dependent allele (i.e. dark green 'induced' sectors on a pale green 'non-induced' background) (McClintock, 1954). Since both large (early) and small (late) sectors of dark green tissue are seen throughout the blade and sheath, the *bsd1-m1* phenotype may result from the combined action of excision and induction or suppression events. Regardless of the genetic mechanism by which it is produced, dark green tissue in *bsd1-m1* leaves is phenotypically indistinguishable from wild-type leaf tissue. We therefore assume that *Bsd1* gene function is normal in the dark green sectors and consequently refer to this tissue as 'revertant'. Plants with several revertant sectors occasionally develop beyond depletion of endosperm food reserves. Mutant tissue in such plants greens towards the tip of the leaf but can still be distinguished from revertant sectors (Fig. 1B). Mutant plants are always stunted when compared to wild-type siblings.

bsd1-m1 sectored plants occasionally produce pollen, but rarely produce ears that set seed. Pollen from such *bsd1-m1* plants was used to fertilize ears of homozygous *Bsd1/Bsd1* plants. The resulting *bsd1-m1/+* heterozygotes exhibited no phenotype themselves, but when self-pollinated approximately a quarter of their progeny (46/216=0.212) were pale green-mutable. Deviation from the expected 25% frequency of pale green seedlings most likely results from germinal excision events at the *bsd1-m1* locus. The *bsd1-m1* defect therefore results from a recessive mutation in a single nuclear gene locus.

C4 gene expression patterns are disrupted in *bsd1-m1* leaves

Mutant *bsd1-m1* leaf tissue is phenotypically pale green and photosynthesizes at 10% the rate of wild-type sibling leaf tissue, as determined by measurements of oxygen evolved per unit leaf area (C. Grof and J. Langdale, unpublished observation). To assay the extent to which individual C4 photosynthetic enzymes are affected by the *bsd1-m1* mutation, we examined protein levels on western blots (Fig. 2). Protein
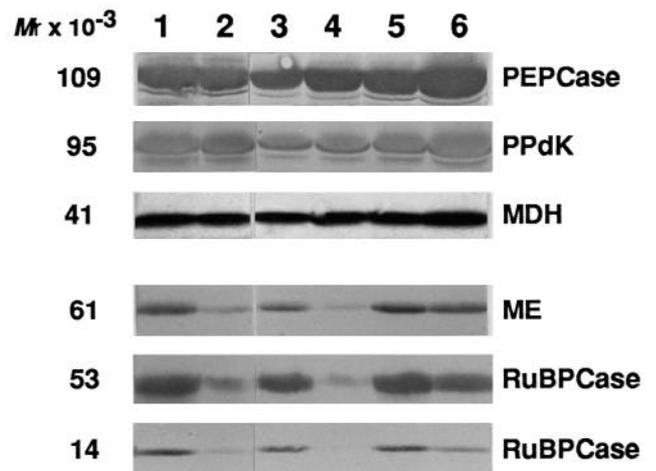
samples isolated from wild-type and *bsd1-m1* (mutant tissue only) seedling leaves were compared with samples from mutant and revertant tissue at both the base and the tip of a mature *bsd1-m1* leaf. Blots were reacted with antibodies against both M cell-specific enzymes (PEPCase, PPdK, MDH) and BS cell-specific enzymes (RuBPCase Lsu, RuBPCase SSu, ME). The data clearly demonstrate that while M cell-specific proteins accumulate to the same extent in both wild-type and mutant seedlings, BS cell-specific protein levels are dramatically reduced in the mutant as compared to wild-type (Fig. 2, lanes 1 and 2). At the base of mature leaves, an identical situation to that seen in seedling leaves is observed (Fig. 2, lanes 3 and 4). However, in mutant tissue at the tip of mature leaves, BS cell-specific proteins appear to accumulate to almost the level seen in revertant sectors (Fig. 2, lanes 5 and 6). Levels of photosynthetic enzymes in revertant sectors also increase towards the tip of the leaf but the increase is not as dramatic (Fig. 2, lanes 3 and 5).

As the western blot data suggested that mutant cells could recover to some extent at the tip of mature leaves, we examined the accumulation pattern of BS cell-specific proteins in these leaves in situ. Fig. 3 compares RuBPCase (A,C) and ME (B,D) accumulation patterns in mutant tissue at the base (A,B) and at the tip (C,D) of a mature *bsd1-m1* leaf. In cells at the base of the leaf, both RuBPCase and ME are virtually absent. However, both proteins are detected in most cells at the tip of the leaf. Since no particular group of cells (e.g. those on the adaxial side of the leaf or those around major veins) fail to
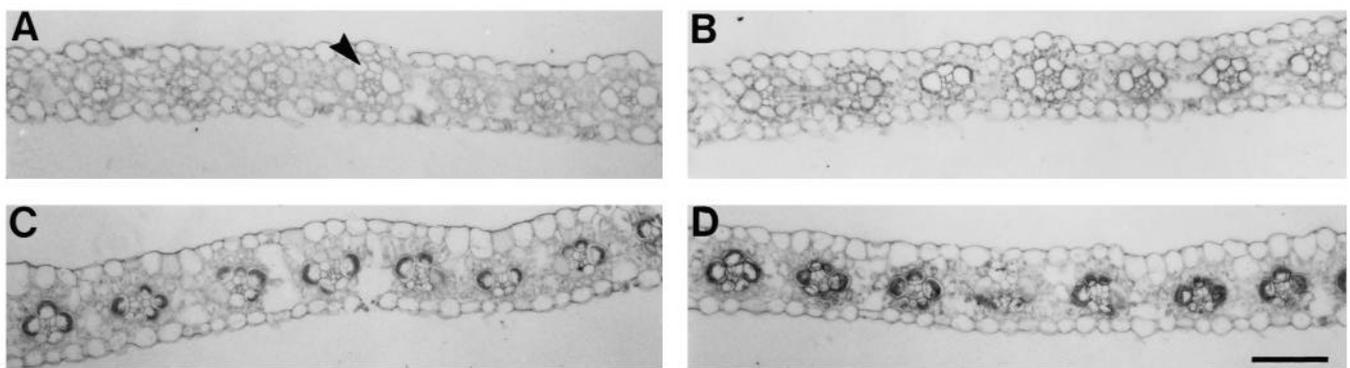
Fig. 3. Immunolocalization of RuBPCase (A,C) and ME (B,D) proteins in mutant tissue at the base (A,B) and at the tip (C,D) of a *bsd1-m1* sixth leaf. For each antibody reaction, base and tip sections were analyzed on the same slide. Leaf 6 was harvested as leaf 9 was emerging (43 days after planting). Leaf length was 63 cm from ligule to tip. Arrowhead highlights BS cells which are arranged in circles around each vein. M cells constitute the remaining non-epidermal tissue. Bar, 100 µm.

recover, we assume that all mutant BS cells are equally competent to overcome the *bsd1-m1* defect.

To determine whether the observed reduction of BS cell-specific enzyme levels in mutant leaves is manifest at the level of transcript accumulation or at the level of translation, C4 gene products were analysed on northern blots. We compared equal amounts of total RNA isolated from the base of mature wild-type and *bsd1-m1* (mutant tissue only) leaves. As in the case of protein accumulation patterns, equivalent amounts of M cell-specific transcripts are seen in both wild-type and mutant leaves whereas BS cell-specific transcripts are greatly reduced in mutant leaves as compared to wild-type (Fig. 4). PEPCase transcript and protein levels were slightly higher in mutant leaves than in wild-type leaves (Fig. 2, lanes 5 and 6; Fig. 4). In the absence of PEPCase activity measurements, however, it would be unwise to speculate on the biochemical significance of this observation. The observed levels of BS cell-specific transcripts suggest that the *bsd1-m1* defect acts on C4 photosynthetic gene expression at the level of transcript accumulation.

BS cell chloroplast differentiation is aberrant in *bsd1-m1* leaves

When viewed under the light microscope, the anatomical arrangement of BS and M cells in *bsd1-m1* leaves is identical to that seen in leaves of wild-type siblings except that BS cell chloroplasts are not visible. This suggested that, in addition to disrupting BS cell-specific photosynthetic gene expression, the *bsd1-m1* mutation affects chloroplast differentiation. To test this possibility, we examined ultrathin sections of mutant seedling and mature leaves using transmission electron microscopy. Fig. 5 shows chloroplast ultrastructure in BS and M cells of mutant tissue in a seedling leaf (A), a revertant sector in a mature leaf (B) and mutant tissue at the base (C) and at the tip (D) of a mature sectored leaf. Dimorphic chloroplasts are clearly distinguishable in all sections. BS cell chloroplasts contain agranal thylakoid membranes arranged in parallel, whereas M cell chloroplasts exhibit clear granal stacks. Neither chloroplast type contains starch since leaves were harvested after overnight depletion of stores. In all cases examined, chloroplast structure in revertant sectors was identical to that seen in wild-type sibling leaves (data not shown). M cell chloroplast structure in mutant tissue does not differ significantly from that seen in revertant sectors or wild-type siblings at any given
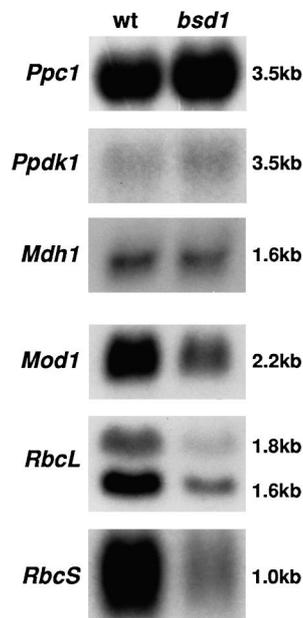

Fig. 4. Northern blot analysis of total RNA isolated from wild-type sibling and *bsd1-m1* leaves. RNA was prepared from leaf 8 of plants in which leaf 9 was just emerging (40 days after planting). Wild-type leaf length was 63 cm whereas *bsd1-m1* leaf length was 53 cm. RNA was prepared from the basal half of the leaf in each case and from mutant tissue in the *bsd1-m1* leaf. Equal amounts of total RNA were loaded in each lane, as judged by ethidium bromide staining of ribosomal RNA bands. M cell-specific transcripts are shown in the upper panel whereas BS cell-specific transcripts are shown in the lower panel. The two *RbcL* transcripts observed represent the unprocessed (1.8 kb) and processed (1.6 kb) forms.

developmental stage (Fig. 5). In contrast, BS cell chloroplasts in mutant seedling leaves (Fig. 5A) or in mutant tissue at the base of mature leaves (Fig. 5C) exhibit only rudimentary lamellae that do not extend the length of the plastid. BS cell chloroplasts in mutant tissue at the tip of mature leaves, however, are virtually indistinguishable from those seen in adjacent revertant sectors (compare Fig. 5B and D). Mitochondrial structure appears identical in both BS and M cells of mutant and revertant tissue (Fig. 5B, 5C and data not shown); however, mitochondrial function may be affected by the *bsd1-m1* mutation. The aberrant differentiation of BS cell chloroplast development in mutant tissue, and the apparent recovery at the tip of mature leaves reflects the observed disruption of C4 gene expression patterns. Consequently, we conclude that in light-grown leaves, the *bsd1-m1* mutation disrupts both the morphological and biochemical differentiation of BS cells without affecting M cell development.

Revertant sectors in *bsd1-m1* leaves have discrete boundaries

Throughout this work, we use the term 'revertant sector' to
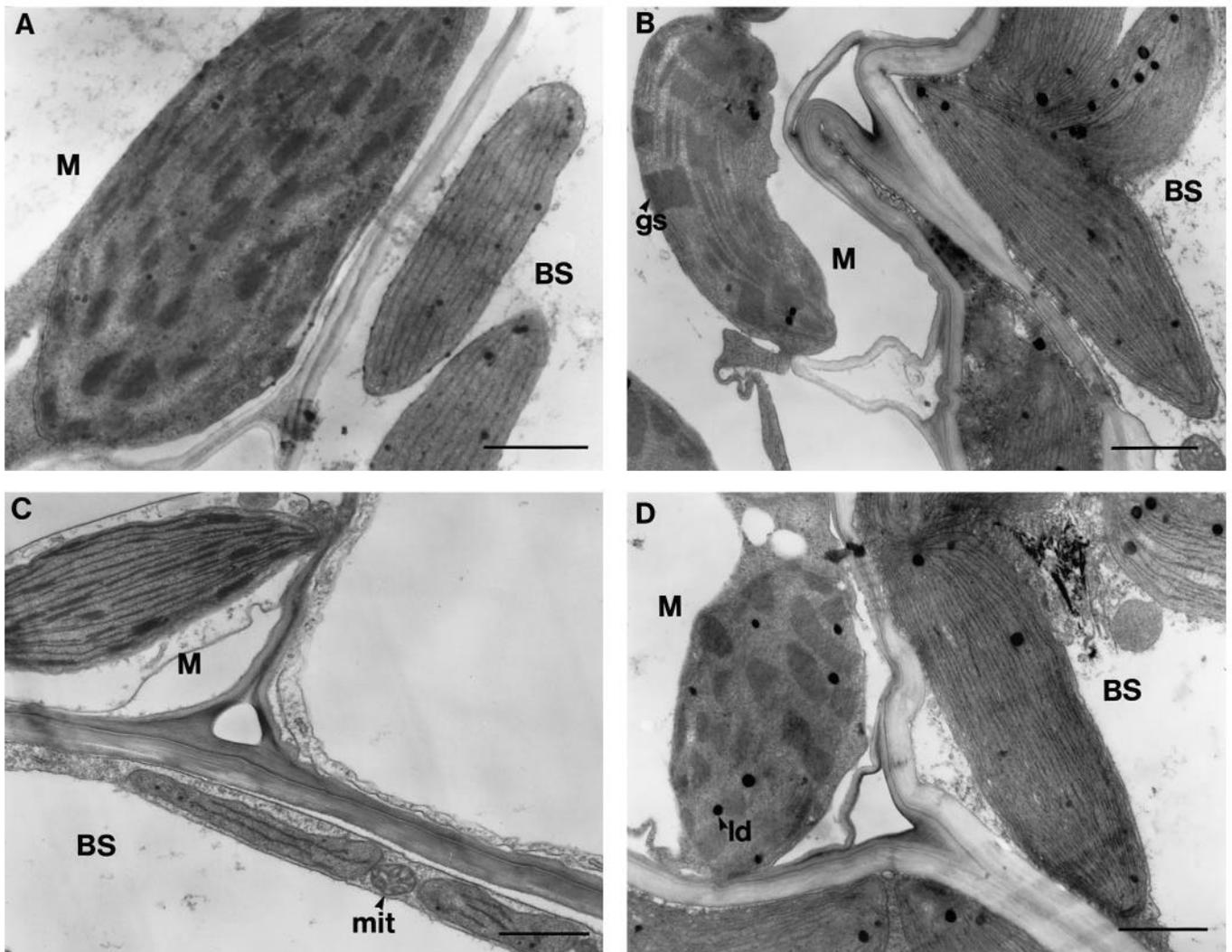
Fig. 5. Transmission electron micrographs of (A) *bsd1-m1* leaf 3 - mutant tissue 6 cm above the ligule, (B) *bsd1-m1* leaf 9 - revertant sector 32 cm above the ligule, (C) *bsd1-m1* leaf 9 - mutant tissue 18 cm above the ligule, (D) *bsd1-m1* leaf 9 - mutant tissue 62 cm above the ligule. Leaf 3 was harvested as the fourth leaf emerged (26 days after planting) whereas leaf 9 was harvested as leaf 13 emerged (51 days after planting). Leaf 3 was 10.3 cm from the ligule to the tip whereas leaf 9 was 83 cm from the ligule to the tip. BS, bundle sheath cell; M, mesophyll cell; gs, granal stack; ld, lipid droplet; mit, mitochondria. BS chloroplasts in C flank the mitochondrion. Bar, 1 μm.

indicate phenotypic reversion to a wild-type phenotype (i.e. pale green to dark green). Furthermore, we have correlated reversion with the presence of BS cell-specific markers. All cells within phenotypically revertant sectors, however, need not carry a revertant *Bsd1* allele. If *Bsd1* gene action was non-cell autonomous, revertant sectors would have indistinct boundaries with mutant tissue or would not be visible. Discrete sector boundaries would indicate that gene action was either cell autonomous or effective at a limited cell range. To determine the extent to which the *Bsd1* gene acts cell-autonomously, we used probes against both M and BS cell-specific gene products to analyze sector boundaries in *bsd1-m1* leaves. Fig. 6 shows serial sections of a sector boundary in a mature leaf reacted with ^{35}S-labelled antisense transcripts. The M cell-specific transcript *Ppc1* is present in both the mutant and revertant halves of the leaf (Fig. 6A) whereas BS cell-specific transcripts are detected only in revertant tissue (Fig. 6B-D). Sections decorated with BS cell-specific probes illus-

trate very abrupt sector boundaries. Since a single cell in the vascular bundle at the boundary can be phenotypically revertant (e.g. Fig. 6C), we propose that *Bsd1* gene action is either cell-autonomous or is non-autonomous over a limited cell range. These two possibilities will not be distinguished until we can determine whether *Bsd1* gene expression at sector boundaries is coincident with observed photosynthetic gene expression patterns.

Mutant BS cells in *bsd1-m1* leaves are distinct at plastochron 4

The earliest marker of BS cell differentiation in wild-type leaves is the accumulation of *RbcL* transcripts between plastochron 4 (in cells around major vein sites) and plastochron 5 (in cells around intermediate vein sites) (Langdale et al., 1988b). At this time, BS and M cells are densely cytoplasmic and cannot be distinguished morphologically. To establish how early in development the *bsd1-m1* defect is manifest, we
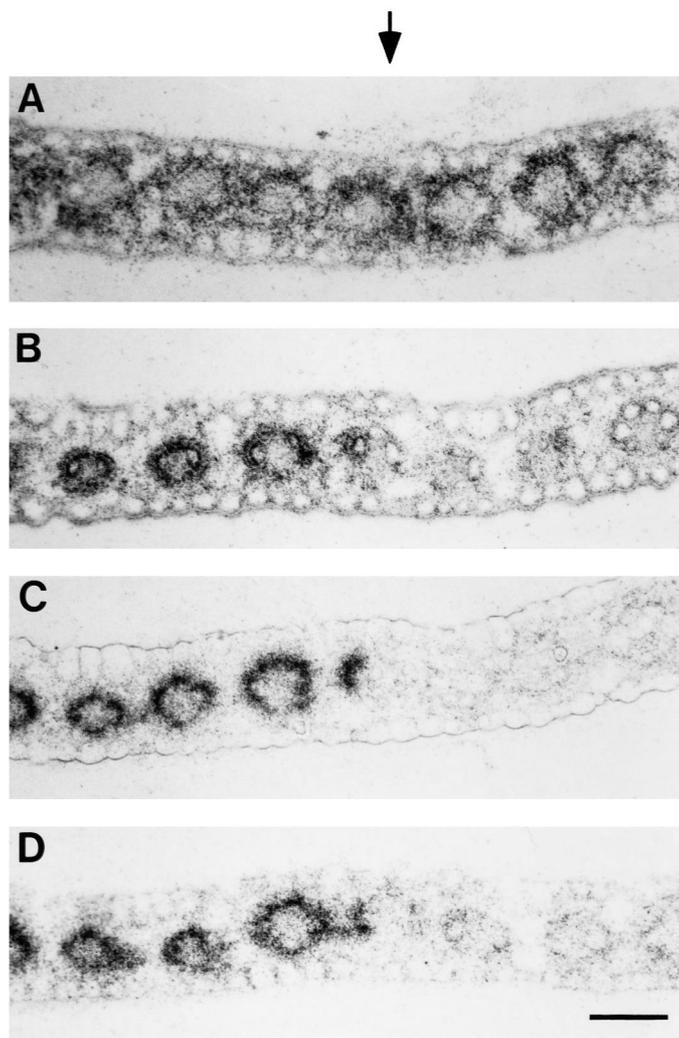
Fig. 6. In situ hybridization to *Ppc1* (A), *Mod1* (B), *RbcL* (C) and *RbcS* (D) antisense transcripts across a sector boundary in the seventh leaf of a *bsd1-m1* plant. The arrow highlights a sector boundary with revertant tissue to the left and mutant tissue to the right. The leaf used was the same as that used for the western analysis shown in Fig. 2. Sections were taken from half way between the ligule and the tip of the leaf. Bar, 100 μm.

hybridized ^{35}S labelled *RbcL* antisense transcripts to sections of young mutant leaf primordia. *bsd1-m1* seedlings were harvested when five leaves had been initiated at the shoot apical meristem. As such, leaf 1 was at plastochron (P) 5, leaf 2 at P4 and leaf 3 at P3 (Fig. 7A). In wild-type siblings harvested at this stage, *RbcL* transcript can be detected in all BS progenitor cells of leaf 1 (data not shown). Mutant first leaf primordia, however, are composed of some cells that accumulate *RbcL* transcripts and some cells that do not (Fig. 7B). We assume that this mosaicism reflects the presence of revertant and mutant tissue. As we occasionally observe this mosaic expression pattern in the second leaf of *bsd1-m1* seedlings, we propose that the normal *Bsd1* gene functions at or before P4.

The *bsd1-m1* mutation disrupts differentiation in etiolated leaves

In wild-type etiolated maize leaves, RuBPCase accumulates in
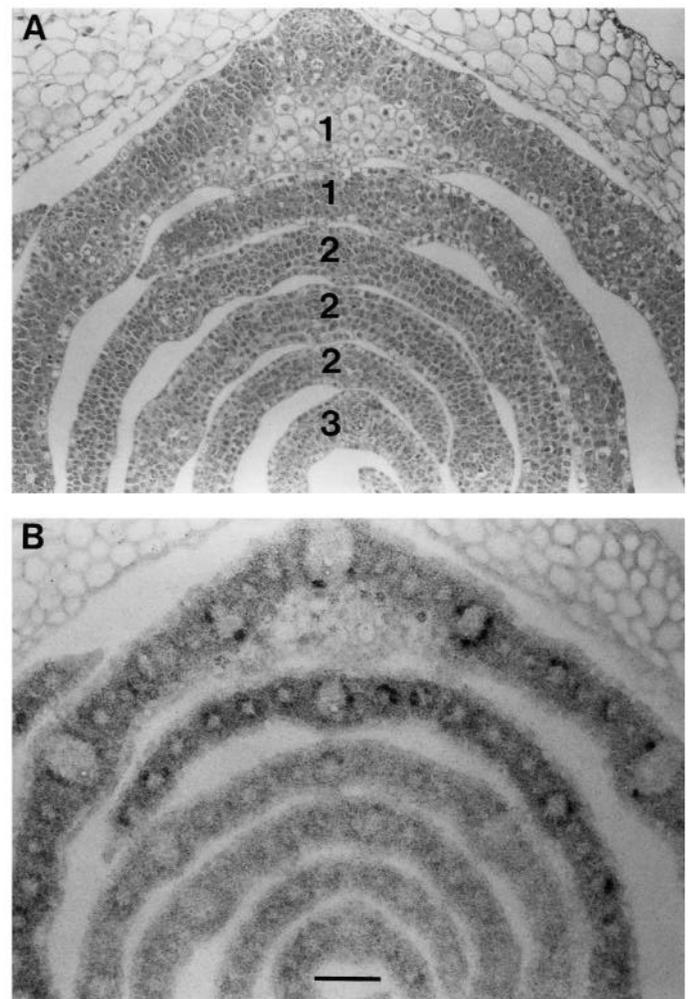

Fig. 7. In situ hybridization to young leaf primordia in a *bsd1-m1* seedling. Sections are shown stained with the histological stain Fast Green (A) and hybridized to *RbcL* antisense transcripts (B). Leaf primordia 1, 2 and 3 are as indicated. With both sense and antisense probes, high levels of non-specific background hybridization are consistently observed in young leaf sections (Langdale et al., 1988b). Cell-specific signals can still be distinguished clearly, however, as seen in some of the BS cells of leaf 1. Seedlings were harvested 5 days after planting, just as the first leaf emerged from the coleoptile. Seedling length was 3 cm from meristem to tip. Sections were sampled approximately 0.75 cm above the meristem. Bar, 100 μm.

both BS and M cells suggesting that a C3 pattern of photosynthetic gene expression is implemented in the dark (Langdale et al., 1988b). To determine whether the *Bsd1* gene influences this developmental programme, we examined RuBPCase protein accumulation patterns in etiolated *bsd1-m1* leaves. In wild-type siblings used as controls, RuBPCase was detected in BS cells of light-shifted leaves (Fig. 8A) and in both BS and M cells of etiolated leaves (Fig. 8B). In etiolated *bsd1-m1* leaves, however, RuBPCase could only be detected in BS and M cells of revertant sectors (Fig. 8C). Similarly, *RbcL* and *RbcS* transcripts were undetectable in mutant etiolated tissue (data not shown). These data suggest that *Bsd1* gene action in etiolated plants is not cell-type specific since the *bsd1-m1* mutation prevents (or reduces) RuBPCase accumulation in both BS and M cells. However, an examination of
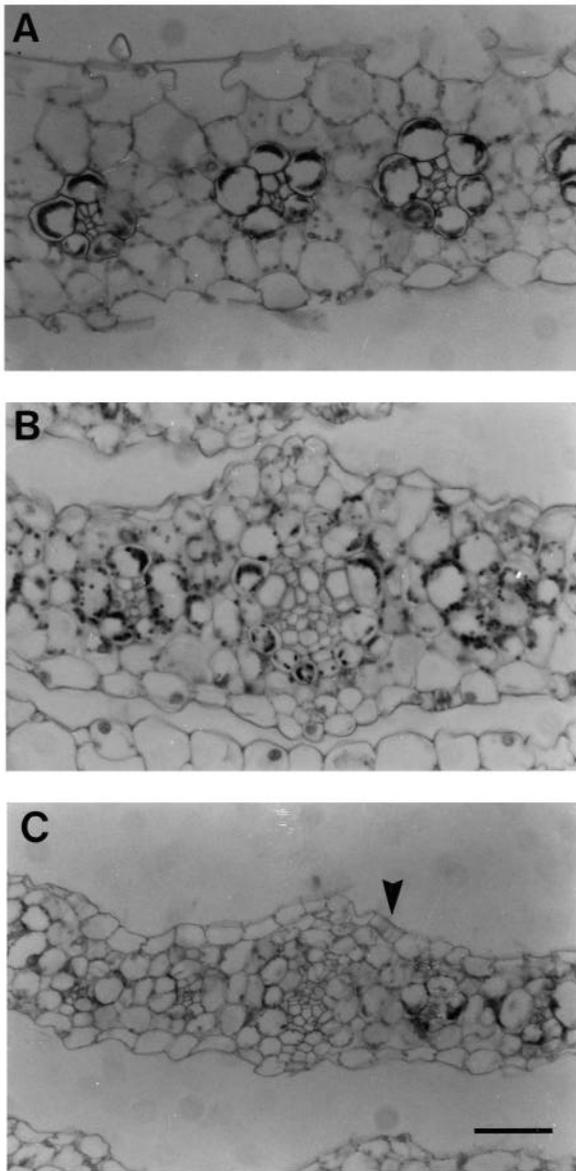
Fig. 8. Immunolocalization of RuBPCase protein in wild-type sibling light-shifted (A) and etiolated (B) leaves and in an etiolated *bsd1-m1* leaf (C). The arrow indicates a sector boundary with mutant tissue to the left and revertant tissue to the right. Plants were grown for 7 days in the dark and then shifted to the light for 7 days. The tips of first and second leaves were harvested from etiolated plants. Third leaves of light-shifted plants were harvested half way between the ligule and leaf tip, as the fourth leaf emerged. Bar, 35 μm.
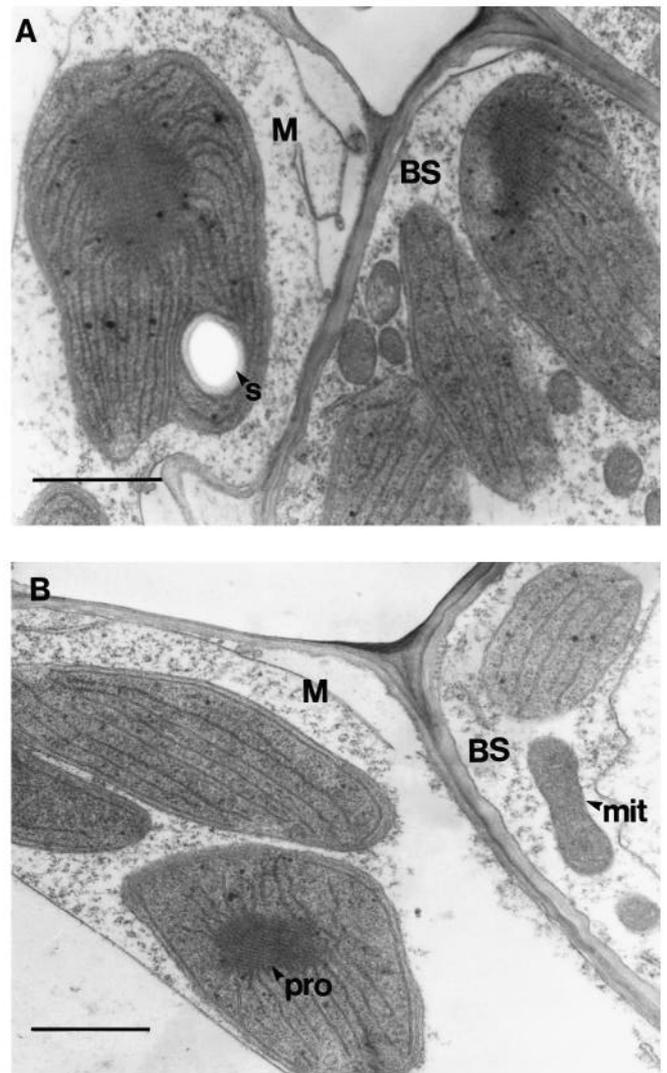

Fig. 9. Transmission electron micrographs of revertant (A) and mutant (B) tissue in an etiolated *bsd1-m1* leaf. Approximate measurements of chloroplast surface area in cells around intermediate veins ($n=30$ in each case) revealed sizes of 1.3 ± 0.7 μm^2 (mutant BS), 2.9 ± 1.1 μm^2 (revertant BS), 4.7 ± 1.5 μm^2 (mutant M) and 4 ± 1.5 μm^2 (revertant M). Plants were grown for 7 days in the dark after which time samples were taken from the tip of the first leaf. BS, bundle sheath cell; M, mesophyll cell; mit, mitochondrion; pro, prolamellar body; s, starch granule. Bar, 1 μm.

chloroplast structure in etiolated mutant seedlings revealed that *Bsd1* gene action is cell-type specific with respect to chloroplast differentiation. Fig. 9 shows electron micrographs of mutant and revertant tissue in an etiolated *bsd1-m1* leaf. In revertant sectors, etioplast structure is identical to wild type (data not shown), where both BS and M cell plastids have thylakoid membranes radiating from distinct prolamellar bodies (Fig. 9A). Etioplasts in mutant tissue also exhibit these characteristics; however, BS cell plastids are reduced in size as compared to those seen in revertant sectors (Fig. 9B). Since M cell etioplasts are identical in mutant and revertant tissue,

the BS cell-specific effect of the *bsd1-m1* mutation on chloroplast differentiation is light-independent. However, as the differences between BS cell chloroplasts in mutant and revertant tissue are more dramatic in light-grown plants than in etiolated plants (compare Figs 5B,C, 9A,B), the effect of the mutation is enhanced in the presence of light. The more severely disrupted chloroplasts observed in light-grown leaves may be a consequence of photooxidative damage (Taylor, 1989).

DISCUSSION

Maize leaf primordia are initiated in the shoot apical meristem and emerge in a distichous phyllotaxy. Subsequent division

and differentiation produces an organ containing multiple distinct cell types. We have isolated and characterized a mutation that disrupts the differentiation of a single cell type in developing leaves grown in the light. The *bsd1-m1* allele is recessive indicating that phenotypic abnormalities most likely occur through loss of function of the normal *Bsd1* gene. Instability at the *bsd1-m1* locus leads to restoration of *Bsd1* function such that revertant sectors are phenotypically indistinguishable from wild type. Mutant leaves have normal cellular anatomy with BS and M cells arranged in concentric circles around vascular centres. However, loss of *Bsd1* function leads to both morphological and biochemical disruptions in BS cells such that BS cell chloroplasts develop aberrantly and BS cell-specific C4 photosynthetic gene products fail to accumulate. M cell differentiation proceeds as normal in mutant leaves, although it is unknown whether M cell-specific enzymes are active in mutant tissue.

We have shown that, in light-grown plants, the *Bsd1* gene functions within a limited cell range, at or before P4. Since BS cells are not fully differentiated until P8 (Nelson and Dengler, 1992), *Bsd1* must influence early differentiation events. However, as revertant sectors (dark green) in *bsd1-m1* plants can be large (early) or small (late), cells must be able to respond to *Bsd1* at any stage in development. One of the characteristics of *bsd1-m1* plants is the ability of mutant cells at the tip of mature leaves to overcome the defect imposed by the mutation. In wild-type mature leaves, BS cell chloroplasts are fully differentiated 30 cm above the ligule (Kirchanski, 1975). An identical situation is seen in revertant sectors of *bsd1-m1* plants. In mutant tissue, however, BS cell chloroplasts undergo most differentiation between 30 cm and 60 cm above the ligule. A similar retardation is observed with respect to the accumulation of photosynthetic gene products. BS cell-specific photosynthetic enzyme levels increase towards the tip of both wild-type and *bsd1-m1* leaves, but peak levels in mutant leaves are not reached until further up the leaf. Although differences between cells at the tip and the base of a maize leaf presumably reflect the combined action of spatial and temporal mechanisms, it is generally accepted that the maize leaf presents a developmental gradient with the oldest cells at the tip. As such, the observed differences between wild-type and mutant leaves invoke three possible explanations. First, the *bsd1-m1* mutation may delay normal *Bsd1* action such that cells mature at a later stage than seen in wild-type siblings. Alternatively, the *Bsd1* gene may function in conjunction with a second gene product that is unaltered by the *bsd1-m1* mutation. For example, *Bsd1* could act early in development while the second gene acts at later stages. The activity of the second gene must be somewhat impaired in *bsd1-m1* leaves, however, as mutant tissue can be distinguished from revertant sectors at the leaf tip. Both of the above explanations predict that BS cells at the base of the leaf eventually recover to the same extent as those at the tip. Consistent with this prediction, the phenotypic gradient in mutant tissue diminishes as the leaves age. Since the gradient does not disappear completely, however, spatial mechanisms must also play a role. A final consideration is that *Bsd1* function may be partially restored through the developmentally programmed action of *Spm* (Fedoroff and Banks, 1988). For example, if *bsd1-m1* is caused by an *Spm* insertion and *Bsd1* function is suppressed by *Spm* activity, revertant sectors would represent excision events or the nonsuppressed (inactive *Spm*) state and pale green tissue would represent the suppressed (active *Spm*) state. The phenotypic gradient observed within the pale green tissue would therefore reflect a gradient of incomplete (tip) to complete (base) suppression. This hypothesis awaits genetic confirmation; however, similar effects have been observed with other *Spm*-induced (Masson et al., 1987) and *Mutator*-induced (Martienssen et al., 1990) alleles.

Based on the *bsd1-m1* mutant phenotype, we propose that, during normal development in both the light and the dark, the *Bsd1* gene influences the morphological differentiation of BS cell chloroplasts. The *Bsd1* gene product plays no role in the differentiation of M cell chloroplasts. In addition to directing morphological differentiation in BS cells, the *Bsd1* gene also affects photosynthetic enzyme accumulation patterns. However, *Bsd1* gene action on photosynthetic gene expression is only BS cell-specific in the presence of light. This apparent requirement for light may correspond to a requirement for the induction of C4 photosynthesis. Wild-type etiolated leaves develop a C3 pattern of photosynthetic differentiation in that both BS and M cells accumulate RuBPCase (Sheen and Bogorad, 1986; Langdale et al., 1988b). A similar C3 pattern is observed in light-grown leaf-like organs such as husk leaves, where M cells are positioned at a distance from a vein (Langdale et al., 1988b). In *bsd1-m1* etiolated leaves neither cell type accumulates RuBPCase. Furthermore, preliminary evidence suggests that *Rbc* transcripts cannot be detected in mutant husk leaf tissue of *bsd1-m1* plants (J. Langdale, unpublished observation). An obvious extrapolation from these data is that *bsd1-m1* is a mutation in the *RbcS* structural gene that contributes 80% of *RbcS* transcripts (Sheen and Bogorad, 1986). We discount this possibility, however, on the basis that *RbcS* gene structure is unaffected by the *bsd1-m1* mutation (L. Hall and J. Langdale, unpublished observation). We suggest that, during normal development, one function of the *Bsd1* gene product is to activate *RbcS* gene expression. In tissues that normally display a C3 pattern of photosynthetic gene expression, *Bsd1* gene action is cell-type independent such that RuBPCase accumulates in both BS and M cells. In contrast, *Bsd1* gene action in C4 photosynthetic tissues is BS cell specific.

Current models hypothesize that in maize leaves, the switch from a C3 to a C4 photosynthetic state occurs through a light-induced interaction between BS and M cells, whereby RuBPCase becomes repressed in M cells close to a vein (Langdale and Nelson, 1991; Nelson and Langdale, 1992). Cell-specific C4 differentiation proceeds coincident with this repression. Since M cells differentiate appropriately in light-grown *bsd1-m1* plants, the switch to C4 development is unaffected by the *bsd1-m1* mutation. We therefore propose that during normal development in the light, the *Bsd1* gene functions downstream of genes that induce the C4 state. After C4 induction, the *Bsd1* gene product becomes restricted to BS cells where it acts to direct both morphological and biochemical differentiation events. This hypothesis ramifies and extends existing models of C4 development.

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