

Arabidopsis Membrane Steroid Binding Protein 1 Is Involved in Inhibition of Cell Elongation^W

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A putative Membrane Steroid Binding Protein (designated MSBP1) was identified and functionally characterized as a negative regulator of cell elongation in *Arabidopsis thaliana*. The MSBP1 gene encodes a 220–amino acid protein that can bind to progesterone, 5-dihydrotestosterone, 24-epi-brassinolide (24-eBL), and stigmasterol with different affinities in vitro. Transgenic plants overexpressing MSBP1 showed short hypocotyl phenotype and increased steroid binding capacity in membrane fractions, whereas antisense MSBP1 transgenic plants showed long hypocotyl phenotypes and reduced steroid binding capacity, indicating that MSBP1 negatively regulates hypocotyl elongation. The reduced cell elongation of MSBP1-overexpressing plants was correlated with altered expression of genes involved in cell elongation, such as expansins and extensins, indicating that enhanced MSBP1 affected a regulatory pathway for cell elongation. Suppression or overexpression of MSBP1 resulted in enhanced or reduced sensitivities, respectively, to exogenous progesterone and 24-eBL, suggesting a negative role of MSBP1 in steroid signaling. Expression of MSBP1 in hypocotyls is suppressed by darkness and activated by light, suggesting that MSBP1, as a negative regulator of cell elongation, plays a role in plant photomorphogenesis. This study demonstrates the functional roles of a steroid binding protein in growth regulation in higher plants.

INTRODUCTION

Steroids, derived from isoprenoid, are lipophilic, low molecular weight compounds that act as signaling molecules during complex developmental processes in higher eukaryotes (Kliwer et al., 1998). Based on the lipophilic properties, steroid molecules are relatively insoluble in water and biological fluids and are thus usually detected either in a conjugated form (linked to a hydrophilic moiety, such as sulfate or glucuronide derivatives) or bound to binding proteins in a noncovalent reversible manner (Johnson and Everitt, 1980). Steroid binding proteins can function either as steroid receptors that initiate cellular responses or steroid carrier proteins that control the transport or metabolism of steroids. In animals, steroid binding proteins (SBPs) are classified as plasma or membrane SBPs, according to their subcellular localizations. The plasma SBPs often serve as receptors or their coactivators for steroid hormones and thereby function as ligand-activated transcription factors (Evans, 1988; Glass, 1994; Beato et al., 1995; Mangelsdorf et al., 1995). In addition, plasma SBPs seem to act as a buffer or reservoir for

active hormones to likely protect them from peripheral metabolism and may increase the half-life of biologically active forms (Rosner, 1990). Because of their highly conserved binding domains, many SBPs possess high affinities for more than one kind of steroid (Wingfield et al., 1984; Cenedella et al., 1999; Deviche et al., 2001). In recent years, many membrane SBPs (MSBPs), characterized by a highly conserved single membrane-spanning domain in the N-terminal region, have been isolated and studied in animals (Falkenstein et al., 1996; Meyer et al., 1996; Cenedella et al., 1999). MSBPs were proposed to mediate high affinity binding of steroid hormones and rapid nongenomic actions (Meyer et al., 1996; Falkenstein et al., 1998; Zhu et al., 2001), such as stimulation of the Na⁺/H⁺ exchanger (Wehling et al., 1991) and rapid cellular ion fluxes (Blackmore, 1993; Turner and Meizel, 1995; Wehling, 1997).

Many steroid molecules have been identified as essential growth regulators in plants as well as in animals (Geuns, 1978; Jones and Roddick, 1988). In plants, the best studied steroid molecules are brassinosteroids (BRs), which are detected being involved in the regulation of multiple developmental processes, including cell elongation, fertility, flowering, senescence, and photomorphogenesis (Clouse and Sasse, 1998). Mutant plants deficient in BR biosynthesis or sensitivity showed marked developmental defects (Clouse et al., 1996; Li et al., 1996; Szekeres et al., 1996), indicating that steroids play important roles as plant hormones. However, no previous work has identified plant homologs of the well-characterized animal nuclear steroid receptors (McCarty and Chory, 2000), nor were such homologs identified during the complete annotation of the *Arabidopsis*

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thaliana genome sequence (Arabidopsis Genome Initiative, 2000). The observation that steroid signaling in plants leads to altered transcription and/or physiological responses in the absence of such homologs indicates that these processes are likely mediated by alternative, plant-specific mechanisms.

Accumulating genetic and biochemical evidence indicates that BRs are perceived by the membrane-localized receptor kinase brassinosteroid insensitive 1 (BRI1; Li and Chory, 1997; Yamamuro et al., 2000; Bishop and Koncz, 2002; Wang and He, 2004). BRI1 is a Leu-rich repeat receptor-like kinase required for BR response and structurally resembles animal receptor kinases. Wang et al. (2001) demonstrated the membrane-bound BRI1-complex functions as the receptor of brassinolide (the most active BR). However, there has been no evidence for direct binding between BRs and BRI1. In fact, it has been suggested that an SBP might be part of the BRI1 receptor complex (Li et al., 2001; Nam and Li, 2002). Genome sequence annotation has identified four putative SBPs in Arabidopsis; however, their physiological functions have yet to be determined.

Here, we present our studies on a putative MSBP (MSBP1) in Arabidopsis. We have demonstrated that (1) *MSBP1* encodes as SBP in higher plants, (2) MSBP1 functions as a negative regulator of cell elongation in Arabidopsis, (3) overexpression of *MSBP1* affects the expression of specific genes involved in cell elongation and sterol metabolism, and (4) expression of *MSBP1* in Arabidopsis hypocotyl is suppressed under dark conditions but activated by light, suggesting a role of MSBP1 in the light inhibition of hypocotyl elongation.

RESULTS

Isolation and Structural Analysis of MSBP1, a Putative SBP

For identification of SBPs in Arabidopsis, the porcine membrane progesterone binding protein (PGC1_PIG, the first discovered MSBP; Falkenstein et al., 1996; Meyer et al., 1996; Bairoch and Apweiler, 2000) coding sequence was used as bait to perform a tBlastN search (protein query versus translated database; Altschul et al., 1997) against the National Center for Biotechnology Information Arabidopsis genome database. We identified a highly homologous cDNA named *MSBP1* (E-value 2×10^{-18} , accession number NM_124603, Arabidopsis Genome Initiative locus At5g52240), which encodes a putative membrane-associated progesterone receptor or membrane progesterone binding protein (Mifsud and Bateman, 2002). Specific primers were then designed and used for PCR-based screening (Alfandari and Darribère, 1994) of a cDNA library generated from Arabidopsis hypocotyl tissues, resulting in the isolation of the full-length *MSBP1* cDNA. Comparison between the cDNA and Arabidopsis genomic sequences indicated that the *MSBP1* gene is located on chromosome V and consists of two exons (381 and 525 bp, respectively) and one intron (774 bp) (Figure 1A). It was noted that the sequence of *MSBP1*, termed *AtMP1* (accession number AF153284), was originally released to the National Center for Biotechnology Information database.

The *MSBP1* cDNA was deduced to encode a polypeptide of 220 amino acids with a calculated molecular mass of 24.4 kD and a theoretical isoelectric point of 4.37. Structural analysis of

MSBP1 revealed the presence of an N-terminal transmembrane region (13 to 35 amino acids) and a conserved steroid binding domain (74 to 171 amino acids), indicating that the structure of this protein is similar to that of the other known membrane-associated progesterone receptors (Figure 1B). Homologous comparison showed that MSBP1 shared an overall identity of 44 and 45% with the porcine and human membrane progesterone binding proteins, respectively. Secondary and stereostructural analyses revealed that the steroid binding domain, which is ~70% identical to that of PGC1_PIG and PGC2_HUMAN, consists a mixed α + β structure with two pairs of α -helices forming a sandwich-pocket structure to one side of β -sheets (Mifsud and Bateman, 2002), which is likely responsible for holding steroid molecules (Figures 1C and 1D).

MSBP1 Binds Progesterone and Plant Steroid Molecules in Vitro

Previously, lino et al. (2003) indicated that progesterone is a ubiquitous constituent in plants, and the level of which is similar to that of other plant hormones; hence, the progesterone binding activity of recombinant MSBP1 was analyzed to test whether *MSBP1* indeed encoded a SBP. *Escherichia coli*-expressed recombinant MSBP1 was induced at 28°C and proteins were harvested 2.5 h after supplementation with isopropylthio- β -galactoside (IPTG) (1 mM). Purified recombinant proteins were found to show the expected molecular mass (Figure 2A) and were subsequently used for binding assays using [3 H]-progesterone. MSBP1 bound [3 H]-progesterone in vitro in a dose-dependent and specific binding manner, whereas the control protein (AtIPK2 α -His, with similar molecular mass, ~48 kD) couldn't bind [3 H]-progesterone (Figure 2B). Further analysis revealed that the K_d of MSBP1 to progesterone is 31.2 ± 2.8 nM, which is rather higher than that of porcine MSBP to progesterone (11 nM; Meyer et al., 1996) and Arabidopsis BRI1 to brassinolide (7.4 ± 0.9 nM; Wang et al., 2001). The saturation concentration (B_{max}) of MSBP1 to progesterone is 4.13 ± 0.26 pmol/ μ g proteins.

To further clarify the binding specificity of MSBP1 to other steroid molecules, unlabeled progesterone, 5 α -dihydrotestosterone (5 α -DHT), plant steroids (24-epi-brassinolide [24-eBL], the hormonal steroid; and stigmasterol, the membrane constituent steroid), and abscisic acid (ABA) (as negative control) were used as competitors for [3 H]-progesterone in the competition assay. The results showed that supplemented ABA had no effects on the binding of [3 H]-progesterone to MSBP1, whereas unlabeled progesterone drastically inhibited the binding. In addition, supplementation of 5 α -DHT, 24-eBL, or stigmasterol reduced the binding of MSBP1 to progesterone to a different degree. Analysis of the binding specificity of MSBP1 for steroidal molecules through comparing the IC_{50} (concentration for 50% inhibition) and K_i [inhibition constants, $K_i = IC_{50}/(1 + [^3H]\text{-progesterone}/K_d)$] indicates that MSBP1 has highest affinity to progesterone (IC_{50} 88 nM; K_i 33.8 nM), followed by 5 α -DHT (IC_{50} , 126 nM; K_i , 48.5 nM), 24-eBL (IC_{50} , 277 nM; K_i , 106.4 nM), and stigmasterol (IC_{50} , ~10 μ M) (Figure 2C). These results indicate that MSBP1 can bind to multiple steroids with different affinities. Such behavior of MSBP1 is similar to the SBPs in animals that can bind several steroid compounds with different

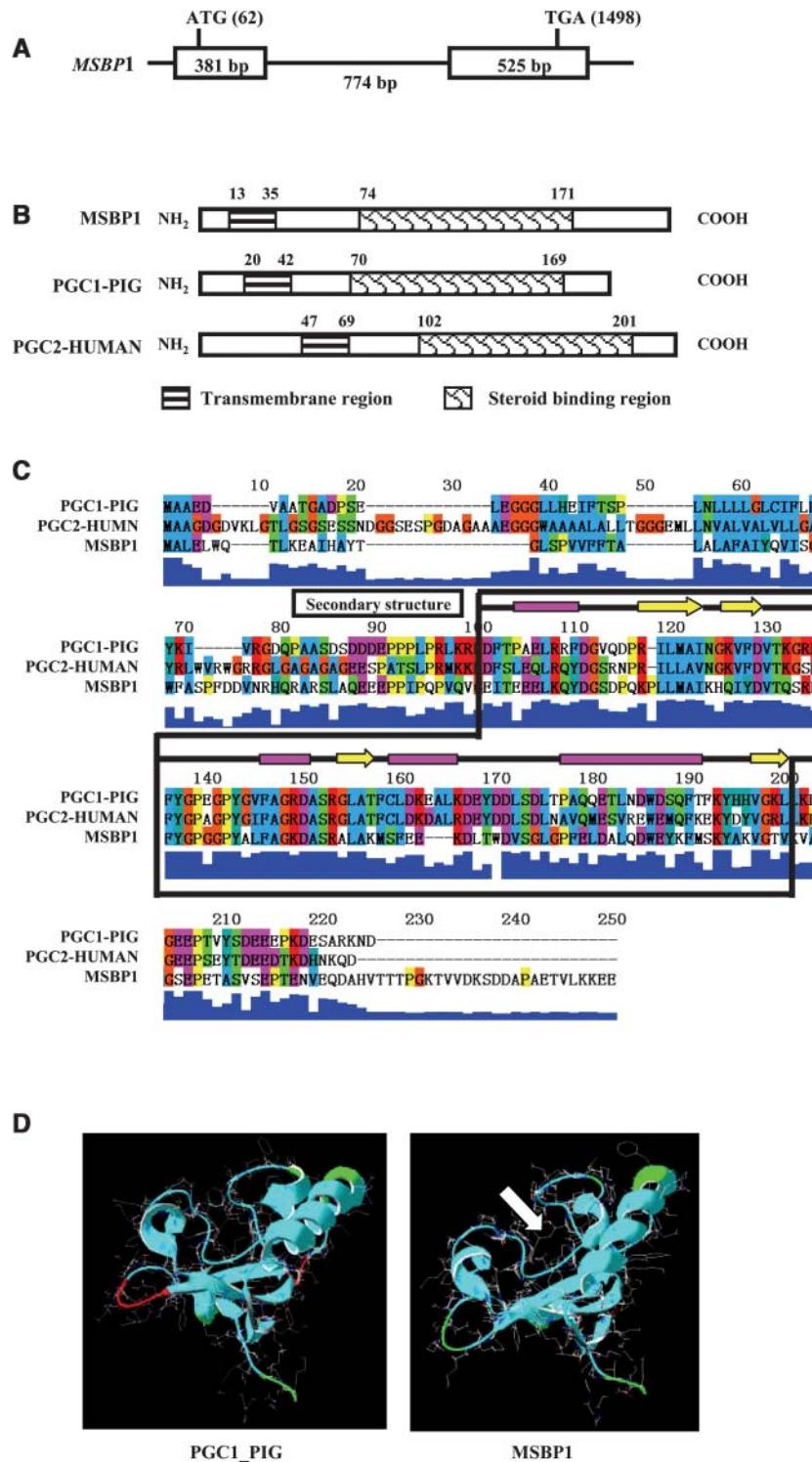


Figure 1. Structure of MSBP1.

(A) Exon/intron structure of the *MSBP1* gene. Two exons (381 and 525 bp; boxed) were identified via comparison of the *MSBP1* cDNA and genomic sequences. Numbers indicate sizes (in nucleotides).

(B) Schematic representation of the SBPs, indicating conserved transmembrane and steroid binding regions. Accession numbers are Q95250 (PGC1-PIG) and O15173 (PGC2-HUMAN). Numbers indicate positions of amino acid residues.

affinities (Wingfield et al., 1984; Cenedella et al., 1999; Deviche et al., 2001).

MSBP1 Is Expressed in Various Tissues but Suppressed in Darkness and Activated under Light Conditions

Both RT-PCR and promoter-reporter gene fusion studies were employed to study the expression patterns of *MSBP1*. RT-PCR showed that *MSBP1* was expressed almost in all tissues (cotyledon, stem, root, leaf, and floral tissues; Figure 3A). The expression patterns were then analyzed in detail through promoter-reporter gene fusion studies. The promoter region of *MSBP1* (1.5 kb) was isolated from Arabidopsis genomic DNA and fused to the β -glucuronidase (*GUS*) gene. Histochemical analysis of *GUS* activities in transgenic plants harboring this construct showed that *MSBP1* was expressed at a high level in the aboveground parts of seedlings and expressed only weakly in root tissues. *GUS* activity was also observed in the rosettes, bolts, flower stalks, and silique stalks but barely detectable in the mature flowers and siliques (Figure 3B). The expression of *MSBP1* in the pistil and stigma but not in the anther suggests that it may play a role in the reproductive development.

Further analysis via RT-PCR indicated that the expression level of *MSBP1* was not obviously affected by treatment with different phytohormones (auxin, brassinolide, gibberellin, cytokinin, and ABA) and osmotic stresses (Ca^{2+} and NaCl). By contrast, the expression was dramatically suppressed in darkness (Figure 3C). Histochemical analysis of *MSBP1*-*GUS* showed that *MSBP1* was constitutively expressed in cotyledons under either dark or light conditions but showed light-dependent changes in the hypocotyl. As shown in Figure 3D, expression of *MSBP1*-*GUS* under darkness or light was similar during the initial stages of germination (days 0 to 2) but differed thereafter as hypocotyls began to elongate. *MSBP1*-*GUS* was highly expressed in hypocotyls during days 3 to 7 after germination under light but was almost undetectable in darkness. When the seedlings were transferred from dark to light conditions, *MSBP1*-*GUS* expression increased rapidly. Conversely, *MSBP1*-*GUS* expression was quickly suppressed when seedlings were transferred from light to darkness (Figure 3E), which is consistent with our RT-PCR observations that expression of *MSBP1* was suppressed under darkness and restored by light (Figure 3C, bottom panel), although this phenomenon was only observed in the biological apex of the hypocotyls.

To further explore the effects of different wavelengths of light on the expression of *MSBP1*, *GUS* activities were analyzed in the

seedlings grown under the conditions of darkness, blue light, red light, and far-red light. The results revealed that *MSBP1* was constitutively expressed in cotyledons, dramatically activated by blue and far-red light, and slightly stimulated by red light in hypocotyls (Figure 3F), suggesting that *MSBP1* is regulated by both phytochromes and cryptochromes. Interestingly, the *MSBP1* gene was highly expressed under the far-red light conditions, under which Arabidopsis seedlings show very short hypocotyls, and was less expressed under the red light conditions, which results in a longer hypocotyl, suggesting that *MSBP1* is involved in hypocotyl inhibition by various photoreceptors.

MSBP1 Negatively Regulates Cell Elongation

Transgenic approaches were employed to study the biological function of *MSBP1*. The complete coding region of *MSBP1* was subcloned into the pCAMBIA1302 binary vector under the control of the 35S promoter of *Cauliflower mosaic virus* in both sense and antisense orientations, and the constructs were transformed into Arabidopsis. Twenty-two independent T1 plants were obtained, and T-DNA integration was confirmed by PCR analysis of genomic DNA (data not shown). Homozygous transgenic lines containing a single copy of the T-DNA insert (showing 3:1 segregation of antibiotic resistance) were analyzed by RT-PCR for *MSBP1* expression. We found that *MSBP1* expression was enhanced in the *MSBP1*-overexpression plants (O-*MSBP1*) (Figure 4A) and reduced in *MSBP1* antisense plants (A-*MSBP1*) (Figure 4B) in comparison with that of control plants. To further confirm the transgenic plants, the progesterone binding activity of membrane fractions from the wild-type and transgenic plants with altered *MSBP1* expression was measured. An increase of progesterone binding activity in the microsomal proteins of the transgenic plants overexpressing *MSBP1* and a decrease in that of *MSBP1*-deficient plants were observed (Figure 4C). The number of specific binding sites in *MSBP1*-overexpressing plants (39.2 versus 11.9 fmol/ μg -membrane protein) was approximately twofold higher than that of wild-type plants ($B_{\text{max}} = 23.9 \pm 2.5$ fmol/ μg -membrane protein), with similar binding affinities ($K_d = 36.7 \pm 2.5$ nM compared with 31.5 ± 4.6 nM). The increased binding sites were consistent with the increased expression of *MSBP1* in transgenic plants (approximately threefold of that in the wild type; see supplemental data online).

According to the expression pattern analysis, we focused our analysis on hypocotyls elongation. Transgenic plants

Figure 1. (continued).

(C) Multiple amino acid sequence alignment of *MSBP1* and selected PGC1_PIG and PGC2_HUMAN. Alignments are colored using the ClustalX scheme in Jalview. Orange, Gly (G); light green, Pro (P); blue, small and hydrophobic amino acids (A, V, L, I, M, F, and W); dark green, hydroxyl and amine amino acids (S, T, N, and Q); magenta, negatively charged amino acids (D and E); red, positively charged amino acids (R and K); dark blue, His (H) and Tyr (Y). The α -helix and β -sheet are indicated as magenta rectangles and yellow arrows, respectively. Note that the secondary structure of the steroid binding region is highly conserved.

(D) Predicted stereostructure of *MSBP1* (right panel) and PGC1_PIG (left panel). The steroid binding domain consists of a mixed α + β structure with two pairs of α -helices forming a binding pocket to one side of β -sheets. The prediction was performed at <http://swissmodel.expasy.org> and viewed with the program of Deep View Swiss-Pdb Viewer written by Nicolas Guex, Alexandre Diemand, Manuel C. Peitsch, and Torsten Schwede. Arrow indicates the putative binding pocket.

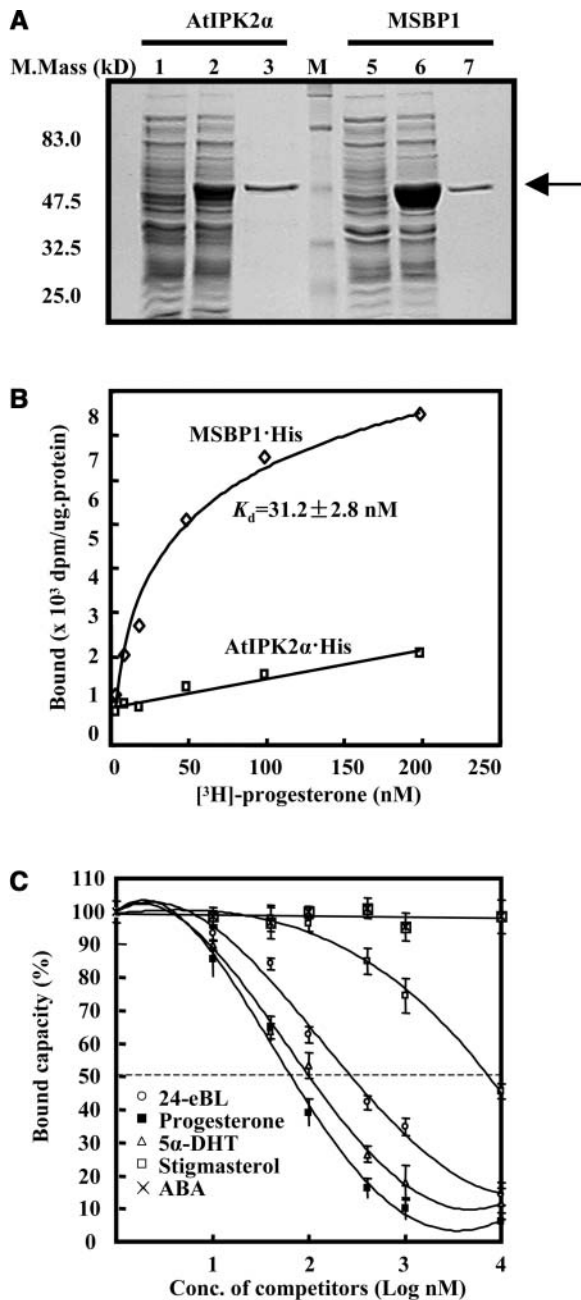


Figure 2. Steoid Binding Assays for MSBP1.

(A) Expression and purification of recombinant MSBP1. M. mass, molecular mass marker; lanes 1, 2, and 3, uninduced or induced crude extract and purified recombinant control proteins (AtIPK2 α); lanes 5, 6, and 7, uninduced or induced crude extract and purified MSBP1. Induction was performed with the supplementation of IPTG (1 mM in final concentration) for 2.5 h. Arrow shows the position of recombinant MSBP1.

(B) Recombinant MSBP1 binds progesterone in vitro. The K_d value and B_{max} were calculated from data of three independent experiments. AtIPK2 α , which was used as control protein, showed no binding to labeled progesterone.

(C) Binding of MSBP1 to [³H]-progesterone was competitively inhibited

overexpressing *MSBP1* showed inhibited hypocotyl growth compared with control plants. After 4 or 6 d of growth under dark conditions, seedlings (hypocotyls) overexpressing *MSBP1* were shorter, with only ~75% the length of control plants (Figure 4D). By contrast, the antisense transgenics showed the opposite seedling growth phenotypes under the same conditions (i.e., more elongated hypocotyls than the wild type). Similar phenotypes were observed when plants were either grown in light for 6 d or transferred from darkness (4 d) to light (4 d) (Figure 4E). The phenotypes of the *MSBP1* antisense plants were less dramatic when grown in the dark than in light (Figures 4D and 4E), and this is probably because of the fact that, under dark conditions, the transcripts of *MSBP1* in hypocotyls of control plants are very low, as well as those in the antisense transgenic ones. Longitudinal sections of hypocotyls revealed that the cell length of dark-grown *MSBP1* overexpressing plants was ~27% shorter than that of control plants (Figure 4F) and that of light-grown *MSBP1* deficiency plants was ~18% longer (Figure 4G). Thus, the differences in cell length are similar to the differences in hypocotyl length of these plants, suggesting that the altered hypocotyl lengths of *MSBP1* transgenic plants were because of altered cell elongation rather than cell division. These results indicated that overexpression of *MSBP1* led to reduced growth, and suppression of *MSBP1* resulted in enhanced growth.

To further study the functional mechanism of *MSBP1* suppression on hypocotyl growth, we detected expression of marker genes involved in cell expansion or division, including *AtExp1*, *KORRIGAN (KOR)*, *TCH4*, *Meri-5*, and *β -tubulin2* (Cosgrove, 2000; Yin et al., 2002; He et al., 2003) in transgenic plants with altered transcripts of *MSBP1*. As shown in Figure 4H, transgenic plants deficient for *MSBP1* showed no obvious changes in expression of the tested genes, whereas cell elongation-related genes were obviously suppressed in transgenic plants overexpressing *MSBP1*. *AtExp1*, a cell wall extension factor belonging to the expansin gene family, was suppressed in *MSBP1* overexpressing plants, but almost unaltered (with a slight increase in line 3) in *MSBP1*-deficient plants. A similar, though less obvious, pattern was observed in the case of *KOR*, which encodes a membrane-bound endo- β -1,4-glucanase critical for cell wall assembly, cell expansion, and cytokinesis. By contrast, expression of *β -tubulin2* (a microtubule subunit important for the cytoskeleton, cell growth, and mitosis), *TCH-4*, and *Meri-5* (a member of the xyloglucan endotransglycosylase gene family involved in the cell wall loosening required for plant cell expansion) was unchanged in the transgenic plants. The expressions of the above tested genes were further confirmed by Affymetrix oligonucleotide chip hybridizations (*AtExp1* in *MSBP1*-enhanced plants was downregulated to ~66% of that in wild-type ones; see supplemental data online). Taken together, these results indicate that *MSBP1* regulates some cell elongation-related genes, such as *AtExp1*, resulting in shortened cells.

by unlabeled progesterone or steroid molecules 5 α -DHT, 24-eBL, and stigmasterol but not by ABA. IC₅₀ showed the different binding affinity of MSBP1 to various steroids.

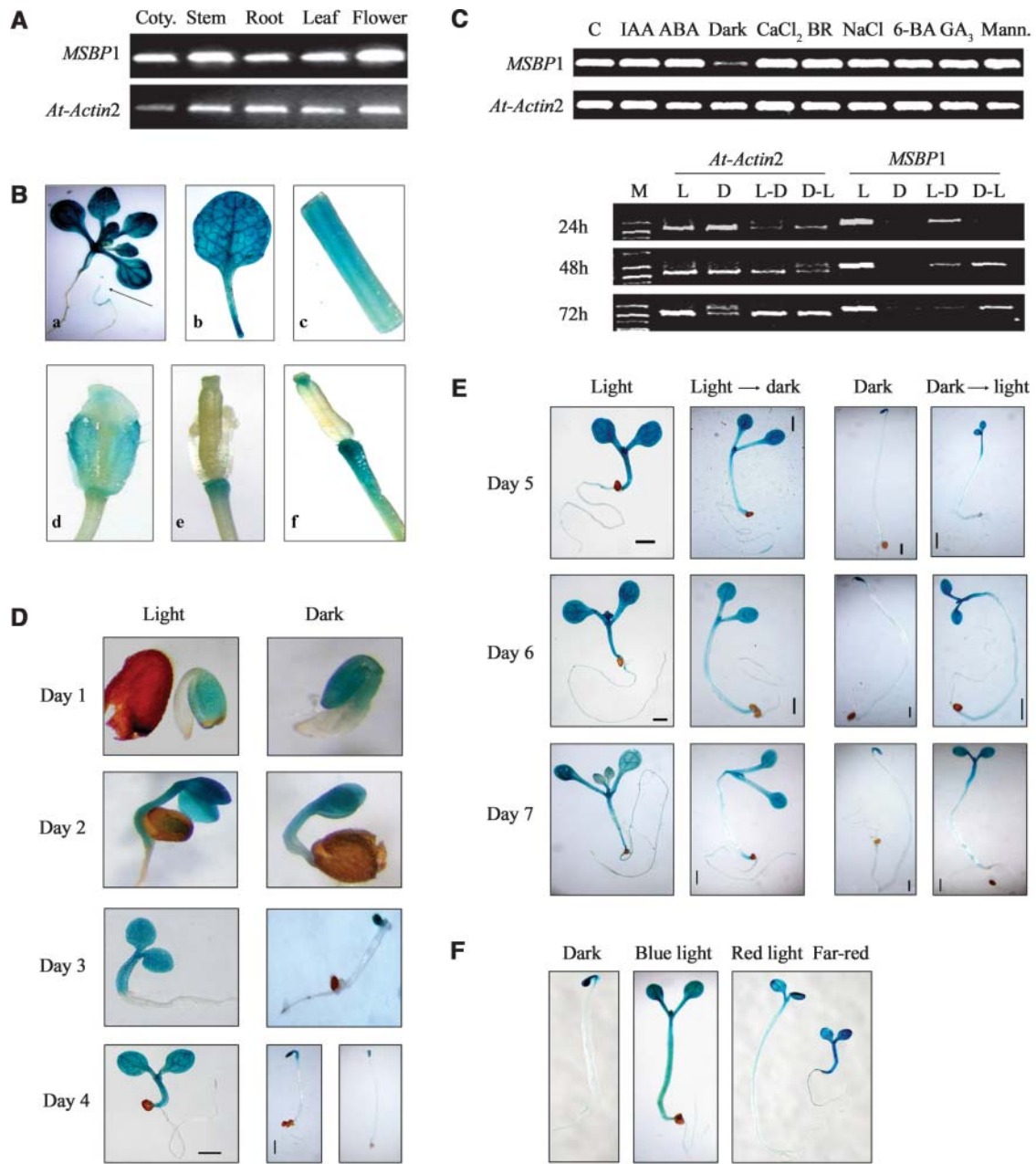


Figure 3. Expression Pattern Analysis of MSBP1 in Various Tissues and Differential Expression of which under Light and Darkness.

(A) Semiquantitative RT-PCR analysis revealed constitutive expression of *MSBP1* in various tissues, including cotyledon, stem, root, leaf, and flower. *Actin2* was used as a positive internal control.

(B) Promoter-reporter gene fusion studies indicated that *MSBP1* was expressed in Arabidopsis seedlings and root tip (a), leaf (b), stem (c), petal (d), base of flower (e), and stigma (f).

(C) Semiquantitative RT-PCR analysis revealed that expression of *MSBP1* was not altered after treatment of seedlings with osmotic stresses and plant hormones, including auxin, BR, gibberellin, ABA, and cytokinin, but was severely suppressed in darkness (top panel). Detailed studies of *MSBP1* expression indicated normal transcript levels under light and very low levels in darkness and induction when seedlings were transferred to light. By contrast, *MSBP1* expression was suppressed when seedlings were transferred from light to darkness (bottom panel). M, DNA marker; C, untreated control; L, light; D, darkness; L-D, the seedlings were transferred from light to darkness for 24, 48, and 72 h; D-L, the seedlings were transferred from darkness to light for 24, 48, and 72 h. The *Actin2* gene was used as a positive internal control.

(D) Differential expression of *MSBP1*-GUS in hypocotyls under dark and light conditions. After 2 d of germination, *MSBP1*-GUS was strongly expressed in light and obviously suppressed by dark. Bar = 1 mm.

(E) *MSBP1* expression was not detected in hypocotyls after germination for 5, 6, and 7 d under darkness but was induced by transferring 4-d-old

Additionally, the downstream genes regulated by *MSBP1* were studied through the whole-genome microarray chip (Affymetrix ATH1) using 7-d-old etiolated seedlings of the wild type and two independent lines of *MSBP1*-enhanced plants (Table 1; see supplemental data online). Differential expression of some genes related to cell growth and cytoskeleton were detected in the *MSBP1* enhanced transgenic plants, which is consistent with the observation of the involvement of *MSBP1* in cell elongation. Of the 17 differential genes involved in cell elongation and growth, 13 genes were downregulated. These include many genes encoding expansins and extensins, which promote cell elongation. For instance, the expression of *AtExp18*, *AtExp14*, and *AtExp7* was almost reduced by 50 to 60% compared with the control plants. These results provide strong evidence that enhanced *MSBP1* resulted in reduced cell elongation and shortened hypocotyls because of the suppression of the cell elongation-related genes. In addition, expression of several genes involved in steroid/sterol metabolism (including cytochrome P450 involved isoprenoid metabolism) was altered (15 were upregulated and three suppressed), whereas those related to BR synthesis were not obviously changed.

Modified Expression of *MSBP1* Results in Altered Sensitivities to Progesterone and Brassinolide

Because *MSBP1* can bind progesterone and 24-eBL *in vitro*, we tested the sensitivities to exogenous progesterone and 24-eBL in the transgenic plants with altered expression of *MSBP1*. In *Arabidopsis* wild-type plants, exogenous BRs can have stimulating or inhibitory effects on hypocotyl growth, depending on its concentration and light conditions (Li et al., 1996). In the dark, low concentrations of BRs ($\sim 10^{-10}$ M) slightly promote the seedling's growth (Ephritikhine et al., 1999), and high concentrations of which ($>10^{-8}$ M) inhibit hypocotyl elongation. In light, BRs promote hypocotyls elongation at a wide range of concentrations. The promotion effects of BRs under light conditions were observed at lower concentration (<0.01 μ M, compared with >0.05 μ M in wild-type seedlings) in the *MSBP1*-deficient plants and higher concentrations (>0.5 μ M) in the *MSBP1*-overexpressing ones (Figure 5A), indicating the modulated sensitivities of plants to exogenous BRs by the altered expression of *MSBP1*. Similar tendencies were observed by the seedling growth under darkness (Figure 5B), in which the *MSBP1*-overexpressing seedlings showed reduced sensitivities compared to those of wild-type and *MSBP1*-deficient plants.

Although Iino et al. (2003) have found that progesterone is a ubiquitous constituent in plants, studies for its physiological functions and effects on cell responses are still lacking. To

determine whether exogenous progesterone affects plant growth, various concentrations of progesterone (0 to 1 μ M) were used to treat *Arabidopsis* seedlings (under light or darkness for 6 d), and lengths of hypocotyls were measured. As shown in Figure 5C, under light conditions, treatment with low concentrations of progesterone (0 to 0.1 μ M) resulted in elongated hypocotyls, and high concentration of progesterone (>0.5 μ M) inhibited hypocotyl elongation, indicating its complicated roles in plant growth. However, the response curve was shifted to lower concentrations in the *MSBP1*-deficient plants, suggesting that suppressed expression of *MSBP1* resulted in hypersensitivity to progesterone. By contrast, enhanced expression of *MSBP1* reduced the response to exogenous progesterone (i.e., the promotion effects of progesterone were restricted at higher concentrations). Under darkness, the wild-type and *MSBP1*-overexpressing plants showed no obvious response to progesterone (Figure 5D). The *MSBP1*-deficient plants responded to progesterone with reduced hypocotyl lengths.

The responses of *MSBP1*-modified transgenic plants to exogenous phytohormones involved in hypocotyl elongation, such as gibberellin and auxin, were also examined. The results showed that, in the darkness, increasing concentrations of gibberellin and auxin could not rescue the short hypocotyl phenotype of *MSBP1*-overexpressing plants, and the *MSBP1*-overexpressing plants showed similar responses to exogenous auxin and gibberellic acid (GA) as the wild-type plants. Taken together, these results indicate that *MSBP1* functions to regulate hypocotyl elongation via a pathway that does not involve auxin or gibberellin.

MSBP1 Localizes to the Plasma Membrane

The presence of an N-terminal transmembrane region indicates that *MSBP1* may localize to the membrane. To confirm this, we subcloned the coding region of *MSBP1* into pCambia1302, resulting in the expression of green fluorescent protein (GFP) fused to the C terminus of *MSBP1*. The construct was transformed into onion epidermal cells through bombardment and examined by confocal microscopy. The results confirmed that *MSBP1* localized to the plasma membrane, a finding that was further confirmed in transgenic plants harboring CaMV35S:*MSBP1:GFP* (Figure 6).

Enhanced *MSBP1* Results in the Differential Expression of a Variety of Genes

To further reveal the molecular mechanisms underlying the shortened hypocotyl resulting from the enhanced expression of

Figure 3. (continued).

dark-grown seedlings into the light condition for 1 (Day 5, Dark \rightarrow light), 2 (Day 6, Dark \rightarrow light), and 3 d (Day 7, Dark \rightarrow light). By contrast, expression of *MSBP1* (L) was suppressed when 4-d-old light-grown seedlings were transferred to darkness for 1 (Day 5, Light \rightarrow dark), 2 (Day 6, Light \rightarrow dark), and 3 d (Day 7, Light \rightarrow dark). Bar = 1 mm.

(F) Expression of *MSBP1*-GUS under various light conditions (continuous blue light, red light, and far-red light). Seedlings were grown in each light condition for 6 d.

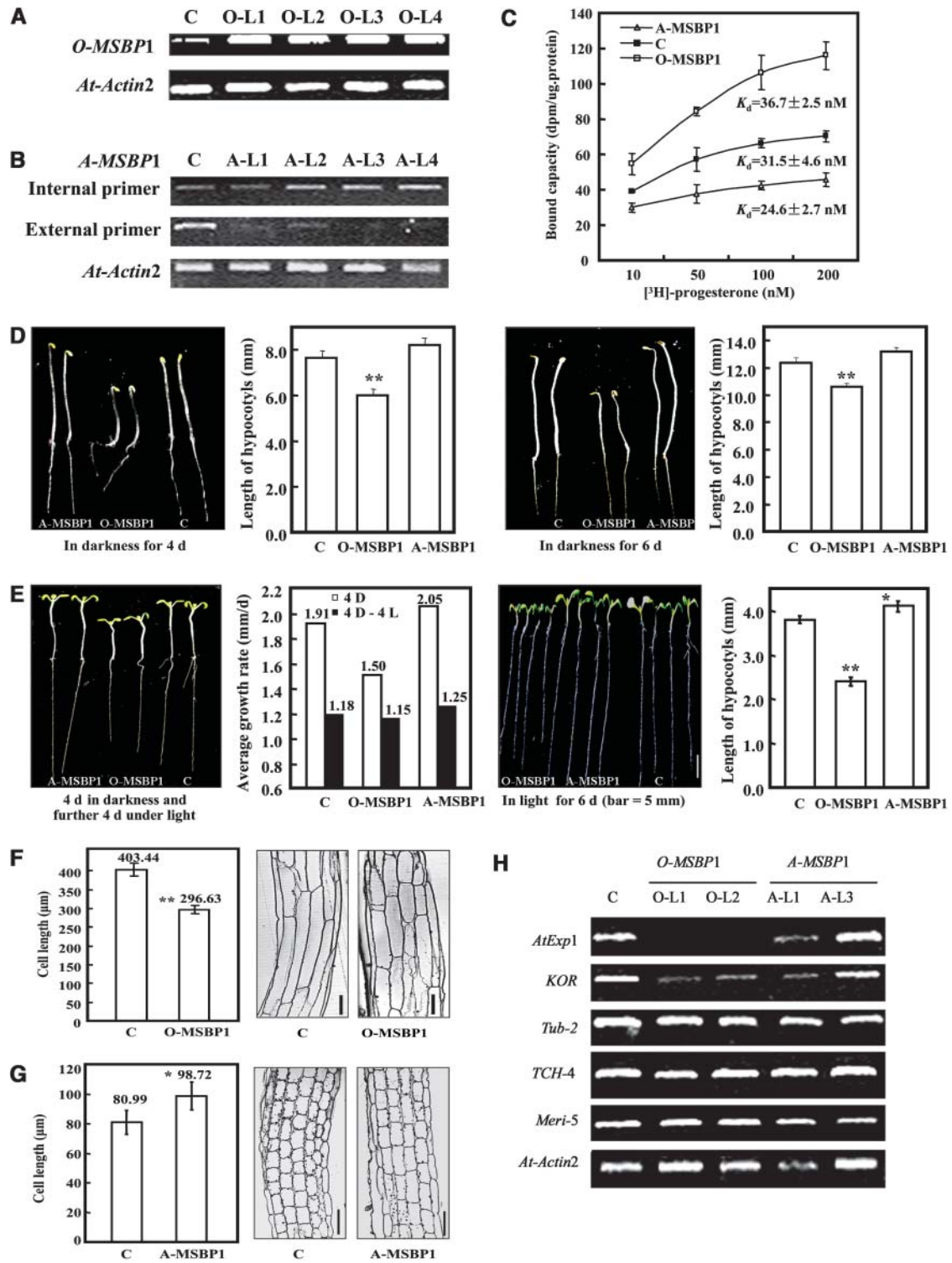


Figure 4. Enhanced Expression of MSBP1 Results in Shortened Hypocotyls Because of Reduced Cell Elongation, whereas Its Suppressed Expression Results in Elongated Hypocotyls with Increased Cell Length.

(A) Overexpression of *MSBP1* in transgenic plants harboring CaMV35S:*O-MSBP1*. Expression of *MSBP1* RNA was analyzed by RT-PCR in wild-type plants (labeled as C in [A] to [H]) and independent transgenic lines (O-L1, O-L2, O-L3, and O-L4). The *Actin2* gene was used as an internal control.

(B) Deficiency of *MSBP1* in transgenic plants harboring CaMV35S:*A-MSBP1*. Expression of *MSBP1* RNA was analyzed by RT-PCR in wild-type (C) and

Table 1. Numbers and Percentage of the Differentially Expressed Genes Involved in the Processes of Cell Elongation and Growth, Cytoskeleton, Photomorphogenesis, or Steroid Metabolism and Signaling in *MSBP1*-Overexpressing Plants Grown under Darkness

Functional Characterization	Upregulated (115 Genes)	Downregulated (64 Genes)
Cell elongation and growth	5 (4.4%)	12 (18.8%)
Cytoskeleton	4 (3.5%)	1 (1.6%)
Photomorphogenesis	2 (1.8%)	1 (1.6%)
Steroid/sterol metabolism and signaling (including cytochrome P450-involved isoprenoid metabolism)	15 (13.0%)	3 (4.7%)

Detailed information is available at the Web site <http://www.plant-signal.cn>.

MSBP1, expression profiling of the downstream genes was performed using the commercially available ATH1 Arabidopsis whole-genome microarray chip. Seven-day-old etiolated seedlings of the control and two independent lines of *MSBP1*-overexpressing plants were used. When combined, the data of two independent lines, 178 genes, including 114 upregulated genes and 64 downregulated ones, showed significant differential expressions (in two dependent lines, at least one with fold variation ≥ 2 and the other with ≥ 1.5) among $\sim 22,000$ ones tested. Functional characterizations indicated the involvement of the encoded proteins in plant photomorphogenesis, sterol metabolism, signal transduction, cell growth, and cytoskeleton (Table 1; see supplemental data online). Noticeably, in the upregulated genes, $\sim 13\%$ were involved in isoprenoid/sterol/sterol metabolism and signaling, whereas $\sim 19\%$ of those downregulated ones were involved in the processes of cell elongation and cell growth. In the case of the genes involved in photomorphogenesis, besides those in light-harvesting and photosynthetic systems, some other genes encoding photo-

receptors and/or coactivators were modified, such as *COP1 interactive protein 1* and homeobox gene *ATH1*, indicating that *MSBP1* is involved in photomorphogenesis of Arabidopsis.

Proteins related to steroid/sterol metabolism and signaling were also detected with differential expressions, including pentacyclic triterpene synthase, terpene synthase, squalene monooxygenase, hydroxysteroid dehydrogenase, and FK506 binding protein. Additionally, differential expression of the cell growth- and cytoskeleton-related genes, especially downregulation of many expansins and extensins, leads to the reduced cell elongation and shortened hypocotyl under enhanced *MSBP1*.

DISCUSSION

SBPs are essential for growth and development of animals. Most known animal SBPs not only regulate the concentrations of general available steroids and control their metabolic clearance rates, but also function in steroid signal transduction through interacting with specific receptors on the plasma membrane of target cells, thus directly involve in regulation of cell growth and differentiation (Rosner et al., 1999; Breuner and Orchinik, 2002). SBP coding genes have been annotated in higher plants, including 4 in Arabidopsis and 2 in rice. Although these genes are expected to function in plant steroid signaling pathways (Li et al., 2001; Nam and Li, 2002), there has been no previous reports on the functional characterization of plant SBPs. This study for the first time demonstrates that at least one of these proteins, *MSBP1*, functions as a SBP in regulating plant growth and development.

MSBP1, an SBP of Higher Plants

MSBP1 resembles the animal membrane progesterone binding proteins in size and stereostructure. Structural analysis of *MSBP1* identified an N-terminal transmembrane domain and a C-terminal steroid binding region, both of which are present in similar positions within the animal MSBPs, specifically the

Figure 4. (continued).

independent transgenic lines (A-L1, A-L2, A-L3, and A-L4). External primers at the 5' and 3' untranslated regions were used for detecting sense *MSBP1* transcript, and internal primers were used to detect both sense and antisense transcripts. The *Actin2* gene was used as an internal control.

(C) The membrane fractions from *MSBP1*-overexpressing plants presented a higher binding capacity to progesterone than that from wild-type plants (B_{max} , 63.0 ± 5.4 compared with 35.8 ± 3.8 fmol/ μ g-membrane protein), with the similar K_d constant ($K_d = 36.7 \pm 2.5$ nM compared with 31.5 ± 4.6 nM), whereas the binding capacity of the membrane fractions from *MSBP1*-deficient plants indicated the presence of other unknown binding proteins (B_{max} , 23.9 ± 2.5 fmol/ μ g-membrane protein). For information of transgenic plants, please refer to Figures 4A and 4B.

(D) Growth of control and transgenic plants in darkness for 4 (left) and 6 (right) d. Heteroscedastic *t* test analysis showed the changes of P-value < 0.01 (**). Error bar represents SE.

(E) Growth and relative growth rates of control and transgenic plants in darkness for 4 d (open bars) then transferred to light for 4 d (closed bars, left panels) and under light for 6 d (right). Heteroscedastic *t* test analysis showed the changes of P-value < 0.05 (*) and P-value < 0.01 (**). Error bar represents SE.

(F) Calculations of cell length (left) and longitudinal section (right) indicate decreased cell elongation in transgenic plants overexpressing *MSBP1*. Six-day-old seedlings under dark conditions were used. Heteroscedastic *t* test analysis showed the changes of P-value < 0.01 (**). Bar = 100 μ m. Error bar represents SE.

(G) Calculations of cell length (left) and longitudinal section (right) indicate enhanced cell elongation in *MSBP1*-deficient transgenic plants. Seedlings were grown for 6 d under light conditions. Heteroscedastic *t* test analysis showed the changes of P-value < 0.05 (*). Bar = 100 μ m. Error bar represents SE.

(H) RT-PCR analyses of the expression of cell division- and cell elongation-related genes in *MSBP1*-sense (O-*MSBP1*) or -antisense (A-*MSBP1*) transgenic plants. O-L1, O-L2, A-L1, and A-L3, independent transgenic lines.

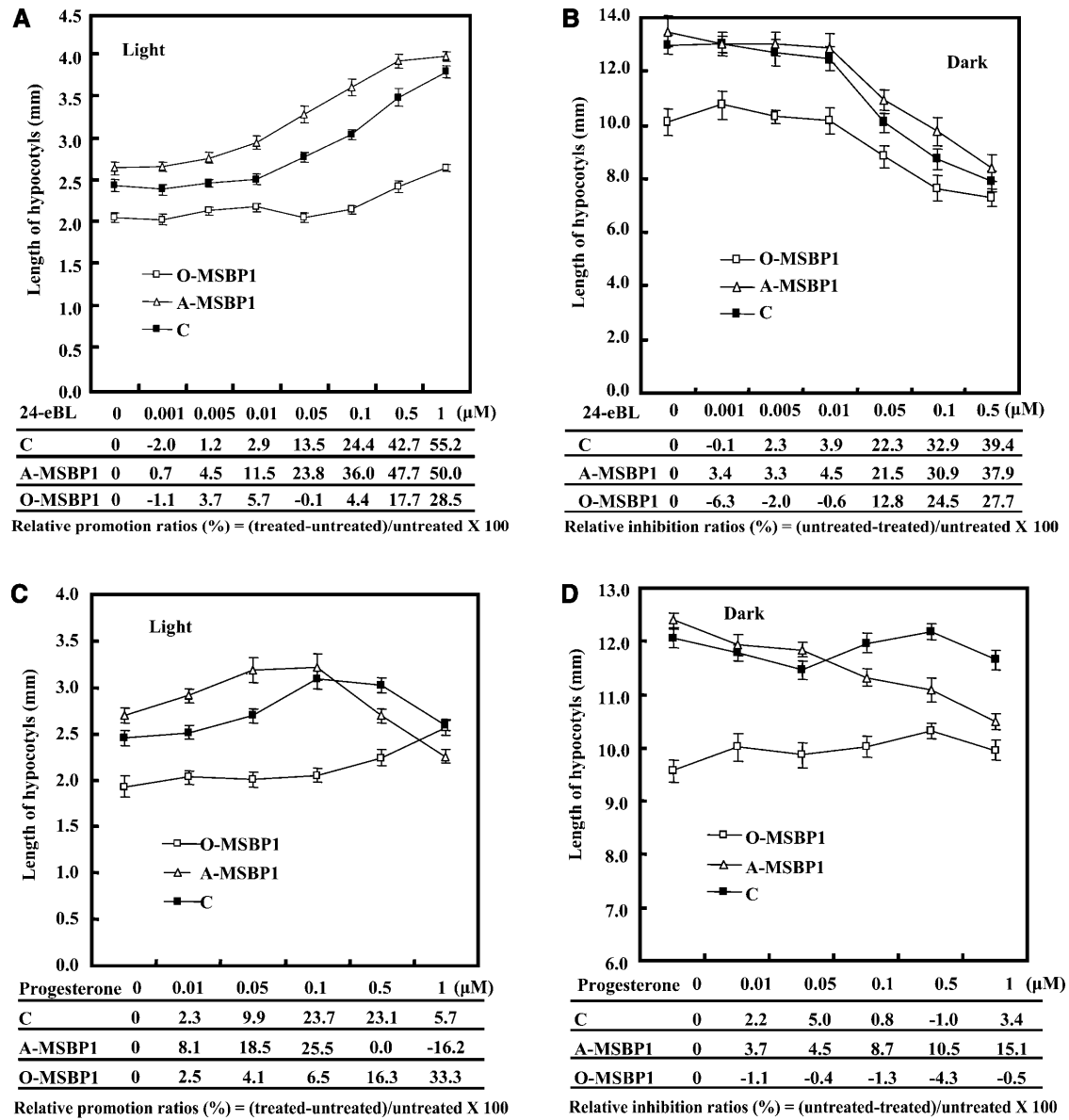


Figure 5. Transgenic Plants Overexpressing or Deficient for MSBP1 Showed Altered Sensitivities to Exogenous Progesterone and 24-eBL.

Seven-day-old seedlings grown under light or darkness conditions were measured. Error bar represents SE.

(A) *MSBP1*-overexpressing (*O-MSBP1*) plants showed reduced sensitivities to 24-eBL under light conditions, and *MSBP1*-deficient plants (*A-MSBP1*) showed increased sensitivity. Seedlings were grown on media containing various concentration of 24-eBL for 7 d. The relative promotion ratios (%) are shown at bottom

(B) Effect of 24-eBL on hypocotyl lengths of wild-type (C), *A-MSBP1*, and *O-MSBP1* plants in the dark. Seedlings were grown on media containing various concentration of 24-eBL for 7 d. The relative inhibition ratios (%) are shown at bottom.

(C) Effect of 24-eBL on hypocotyl lengths of wild-type (C), *A-MSBP1*, and *O-MSBP1* plants grown under light conditions. Seedlings were grown on media containing various concentration of progesterone for 7 d. The relative promotion ratios (%) are shown at bottom.

(D) Increasing progesterone inhibited the hypocotyls elongation of the wild-type (C) and *MSBP1*-deficient plants but not the *O-MSBP1* plants under darkness. Seedlings were grown in the dark on media containing various concentration of progesterone for 7 d. The relative inhibition ratios (%) are shown at bottom.

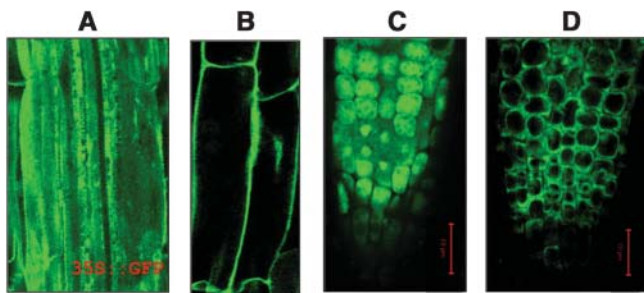


Figure 6. MSBP1 Localizes to the Plasma Membrane.

Green fluorescence in transgenic plants harboring mock vector (CaMV35S:mGFP5) shows that mGFP5 is ubiquitously distributed in the cell cytoplasm (**A**), hypocotyl cells; **C**], root-tip cells), whereas that in transgenic plants expressing CaMV35S:MSBP1:mGFP5 is localized to the plasma membrane (**B**], hypocotyl cells; **D**], root-tip cells). Bars = 20 μ m.

membrane progesterone binding proteins (Figure 1B). The presence of these regions is consistent with our subcellular localization studies showing plasma membrane localization (Figures 6B and 6D) as well as stereostructure predictions indicating a conserved sandwich-pocket structure for binding of steroid molecules (Figure 1D). The plasma membrane localization of MSBP1 suggests that it might act, either directly or by forming membrane-associated complexes, to afford cells greater flexibility in using membranous plant steroids (Lindsey et al., 2003). Consistent with the suggestion by Mifsud and Bateman (2002) that the proximity of cell membranes to a steroid binding template pocket could give rise to new ligand binding pockets with specificity for membrane-soluble molecules such as steroids, the localization of SBPs to the cell membrane probably represents a cellular adaptation facilitating the use of steroids as triggers for rapid response mechanisms.

The *MSBP1* gene indeed encodes an SBP in plants. Recombinant MSBP1 and membrane fractions from Arabidopsis plants can bind progesterone in vitro (Figures 2B and 4C). Similar to animal SBPs, MSBP1 also has the binding capacities to several other steroids with different affinities (Figure 2C). In birds, the corticosteroid binding protein binds not only glucocorticoids and progesterone with high affinity but testosterone and estradiol with lower affinity (Wingfield et al., 1984; Deviche et al., 2001). The porcine MSBP also binds several steroidal molecules, including progesterone, corticosteroid, and testosterone (Meyer et al., 1996). Arabidopsis MSBP1 could bind several steroidal molecules, including animal steroid (5 α -DHT), progesterone, and 24-eBL, with relatively high affinity and stigmasterol with very low affinity. The ability to bind to diverse steroid molecules is likely because of interaction with the common tetracyclic skeleton, and the different affinities to different steroids suggest that binding is also affected by different substitutions in the side chain and number and position of double bonds in the steroidal nucleus. Analysis of IC₅₀ (concentration for 50% inhibition) and K_i (inhibition constants) indicates that the potency of 5 α -DHT bound to MSBP1 is \sim 68% and that of 24-eBL is only \sim 30% of progesterone to MSBP1. Interestingly, similar to Arabidopsis MSBP1, porcine MSBP also presents relatively higher specificity

to progesterone than testosterone (binding affinity to testosterone is only 16% of that to progesterone; Meyer et al., 1996). Such similarity might reflect evolutionary conservation of binding specificity between the Arabidopsis and porcine SBPs, which share around 70% amino acid sequence similarity in their steroid binding domain.

Although both progesterone and BRs have been detected in plants, the binding constants (K_d) of MSBP1 to progesterone (\sim 30 nM) and 24-eBL (up to 100 nM) are higher than the in vivo concentrations of them in Arabidopsis plants. Progesterone has been found as a ubiquitous constituent in plants, and the level of which is similar to that of other plant hormones (lino et al., 2003), raising the possibility that MSBP1 might play a role in the signal transduction of progesterone. Although the binding activity of MSBP1 to BRs is lower than that to progesterone, its function in BR action can't be excluded, like the role of corticosteroid binding protein in testosterone signaling in birds (Deviche et al., 2001). The altered sensitivities of the *MSBP1*-modified plants to BRs suggest that *MSBP1* can affect BR response. However, compared with the K_d constant of 7.9 nM of the BRI1 complex, the relatively low affinity (K_d constant $>$ 35 nM) of MSBP1 to BRs suggested that it may not be one component of the BRI1 complex. MSBP1 might be involved in a novel BR signaling pathway. It is possible that MSBP1 might serve as a low affinity SBP and be involved in the developmental processes under high concentrations of BR.

It is also possible that MSBP1 is involved in action of a yet uncharacterized sterol or steroid. Although MSBP1 could bind both progesterone and 24-eBL, the short hypocotyl phenotype of *MSBP1*-overexpressing plants could not be completely rescued or mimicked by treatment with these compounds, suggesting that the suppressed cell elongation might not result from modulated action of these steroids. This notion is strongly supported by the microarray analysis employing Affymetrix chips, which showed that the genes affected in BR-deficient plants or by BR treatments, including *CPD* and *DWF4* (Mussig et al., 2002), were not changed in the *MSBP*-enhanced plants (see supplemental data online). Furthermore, the altered expressions of the genes involved in steroid/sterol metabolism (including cytochrome P450 involved isoprenoid metabolism) suggests that MSBP1 may be involved in signaling of a novel steroid or sterol because most signaling feedback pathways regulate the biosynthesis of the signal molecules. The physiological ligands of MSBP1 are yet to be established by further studies.

MSBP1 Functions as a Negative Regulator in Hypocotyl Elongation through Downregulating Cell Elongation-Related Genes

Our studies of transgenic plants overexpressing or underexpressing *MSBP1* demonstrate that *MSBP1* negatively regulates hypocotyl elongation (Figures 4D and 4E) through inhibition of cell elongation. The correlation between the inhibitory effects of *MSBP1* on cell elongation and reduced expression of genes known to promote cell elongation, such as expansins, in *MSBP1*-overexpressing plants strongly suggests that *MSBP1* is involved in a regulatory pathway for cell elongation. Cell elongation requires loosening of the cell wall with enzymes like hydrolases

and xyloglucan-endo-transglycosylases, synthesizing new wall components and expanding the cytoplasm to fill the new space. MSBP1 regulates the expression of the related genes to suppress hypocotyl elongation. The underlying mechanism by which enhanced *MSBP1* affects a regulatory pathway for cell elongation awaits further elucidation.

MSBP1 Is Involved in Photomorphogenesis

The expression of *MSBP1* is almost constitutive in various tissues and hardly regulated by application of different phytohormones and osmotic stresses. However, *MSBP1* in Arabidopsis hypocotyl is expressed rather weakly in the dark and is activated by light, especially by blue light and continuous far-red light (Figures 3D to 3F). The expression levels of *MSBP1* are positively correlated with the hypocotyl inhibition under various light conditions and in the wild-type and transgenic plants with altered expression of *MSBP1* (Figures 4D and 4E). The *MSBP1*-overexpressing transgenic plants showed shortened hypocotyls under both light and darkness, and the increased hypocotyl length of *MSBP1*-deficient plants in the light but not in the dark was consistent with the differential expression of *MSBP1* in hypocotyls of light- and dark-grown wild-type plants. These results demonstrate that the light-induced expression of *MSBP1* contributes to the inhibition of hypocotyls by light.

The different growth of hypocotyls under darkness and light conditions is very significant for plants, which ensures seedlings to emerge from the soil and to transit the growth stage from heterotrophic to photoautotrophic physiology. Seedlings that germinate in darkness are etiolated, with extended hypocotyls and folded cotyledons. Exposure to light leads to de-etiolation, during which hypocotyl elongation is inhibited, cotyledons unfold, and chloroplast development begins, allowing the formation of an optimal body plan for the transition from heterotrophic to photoautotrophic physiology (Maloof et al., 2001). Our studies provide strong evidence that *MSBP1* is involved in the process of de-etiolation as a negative regulator to inhibit hypocotyl elongation.

Based on these observations, we propose the roles of *MSBP1* during seedling de-etiolation. Upon germination in the dark, the absence of *MSBP1* allows hypocotyl elongation. Once the seedling emerges from the soil, *MSBP1* is activated by light, and it activates a regulatory pathway, perhaps through binding with steroids, leading to decreased expression of cell elongation-related genes and subsequent reduced cell elongation (Figure 7A). Another possibility is that *MSBP1*, through binding, sequester/transport a cell elongation-stimulating steroid from its action site, resulting in the suppressed cell elongation, supported by the responses of O-*MSBP1* and A-*MSBP1* plants to progesterone—O-*MSBP1* reduced responses to the steroid it binds to and A-*MSBP1* plants are hypersensitive.

MSBP1 Is Involved in Multiple Plant Developmental Processes

Expression profiling analysis of the differentially expressed genes under enhanced *MSBP1*, using Affymetrix oligonucleotide

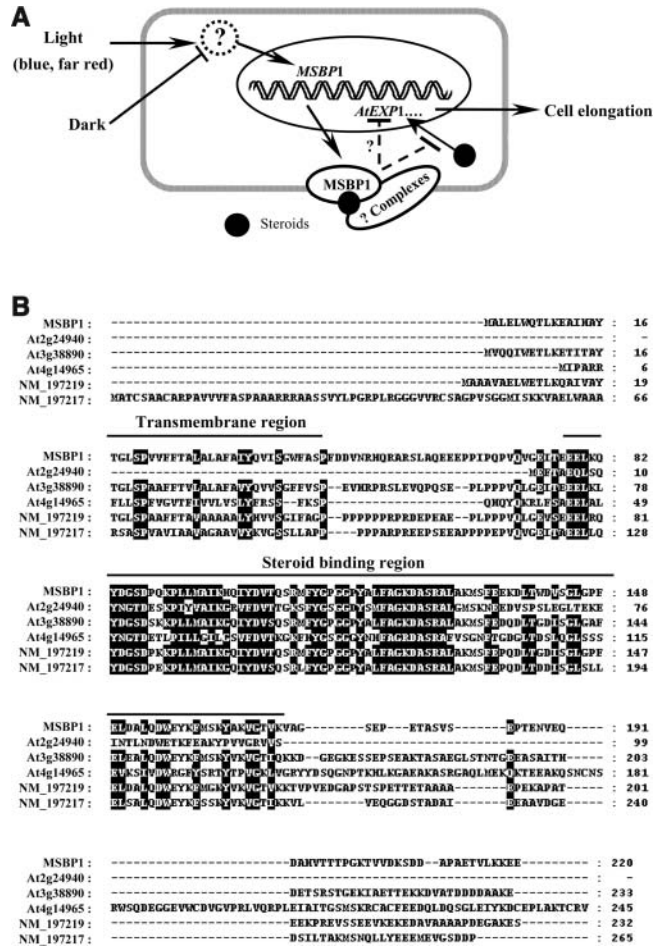


Figure 7. A Possibly Conserved Function of MSBP1 in Higher Plants.

(A) A functional model for MSBP1. Transcription of *MSBP1* is activated by light signaling. The MSBP1 protein is localized to the plasma membrane. MSBP1 may perceive a growth-inhibiting steroid signal. Alternatively, MSBP1 may sequester or transport a growth-promoting steroid away from its site of action (such as inside versus outside of the cell).

(B) Presence of multiple SBPs in higher plants. Alignment of MSBP1 with other predicted SBPs found in the genomes of Arabidopsis and *O. sativa*. Accession numbers are as follows: At2g24940, At3g38890, At4g14965, NM_197219, and NM_197217. The transmembrane and steroid binding regions are indicated.

chips, results in the identification of a variety of multigene families involved in plant signal pathway and transcriptional regulation (Table 1). Very interestingly, *MSBP1* is involved in photomorphogenesis probably through regulating the expression of photoreceptor-related proteins, in the steroid signaling pathway through those potentially involved in sterol or steroid metabolism and perception, and in cell growth and the cytoskeleton through the relative proteins. The exact mechanism by which *MSBP1* regulates the expression of these genes awaits further studies.

Multiple SBP Isoforms Are Present in Arabidopsis and *Oryza sativa*

The steroid binding capacities of the membrane fraction of *MSBP1*-deficient plants revealed the presence of other unknown SBPs in Arabidopsis (Figure 2D). Searches of various plant genomes revealed the presence of four and at least two SBP isoforms in Arabidopsis and rice, respectively, indicating that there is likely a multiple-isoform SBP gene family in both monocot and dicot plants. Furthermore, comparative analyses using the available sequences of other plants, such as rapeseed (*Brassica napus*), pea (*Pisum sativum*), and soybean (*Glycine max*), showed that *MSBP1* shared high homology to other predicted SBPs (Figure 7B), including identities of 71% to putative Arabidopsis At3g38890, 52% to At2g24940, 30% to At4g14965, 73% to putative rice NM_197219, and 71% to NM_197217. The identities in the predicted steroid binding domain were even higher, reaching 88% in rice NM_197219. Secondary structural analysis indicated that most of the putative SBPs had similar structures, especially in the putative steroid binding regions. Protein spatial structure predictions revealed that all of them possessed similar sandwich-pocket structures consisting of $\alpha+\beta$ mixtures essential for binding to steroids, although some tiny differences in the niche are likely responsible for their specific binding to different steroids.

Presently, most putative SBPs in plants are predicted to be membrane-associated proteins, which is very different from the animal SBPs, most of which are soluble cytoplasmic molecules. Surprisingly, a soluble SBP (At2g24940) was annotated in the Arabidopsis genome and has been confirmed by subcellular localization studies as being present primarily as a nuclear protein (data not shown). Taken together, these results suggest that the SBP activities of plants and animals may occur via distinct mechanisms.

In summary, a membrane-localized SBP, *MSBP1*, was identified in higher plants and shown to play a role in cell elongation and light regulation of seedling growth. The presence of multiple SBPs with differential expression and subcellular locations suggests important roles and diverse mechanisms of action of SBPs in multiple physiological and cellular processes in plants.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Columbia-0 was used as the wild type, and transgenic plants were transformed through *Agrobacterium tumefaciens* GV3101 with the floral dipping methods (Clough and Bent, 1998). Plant seeds were surface sterilized and sown on 0.5× MS medium containing 2% (w/v) sucrose and 0.8% (w/v) bacto-agar. The plates were wrapped with two layers of aluminum foil for dark cultures and kept at 4°C for 2 d to break dormancy. For measurement of hypocotyl length, plates were vertically oriented in a dark growth chamber at 20 ± 2°C, and seedlings were grown under long-day growth conditions (20-h light at ~45 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Ten to fourteen days after germination, seedlings were hand-transferred to soil and grown in a growth room under the same controlled conditions.

Continuous blue light was provided by eight 40-W fluorescent bulbs and filtered through one layer of blue Plexiglas filter (wavelength, 470 ±

5 nm); the fluence rate under the filter was 36.5 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Continuous red light was filtered through one layer of red Plexiglas (670 ± 5 nm) with a fluence rate of 25 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Continuous far-red light was output by filtering through one layer of Plexiglas with a wavelength of 730 ± 5 nm and a fluence rate of 0.8 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

Isolation and Identification of the *MSBP1* cDNA

An Arabidopsis cDNA clone encoding a putative SBP was identified through homologous search using PGC1-PIG (accession number Q95250) as bait. Primers *MSBP1*-1 (5'-ATGGCGTTAGAACTATGGC-3') and -2 (5'-CTACTCCTCCTTCTCAAC-3') were used to isolate positive clones through PCR-based screening (Alfandari and Darribère, 1994) of a cDNA library constructed from Arabidopsis hypocotyl tissues. Positive clones were converted into pBluescript SK- derivatives using ExAssist helper phage according to the supplier's instructions (Stratagene, La Jolla, CA). The clone with the longest insert (designated as *MSBP1*) was used for further analysis.

Sequence alignments were analyzed with the Jalview program written by Michele Clamp (<http://www.ebi.ac.uk/clustalw/index.html>). Protein domain analysis was performed at <http://www.sanger.ac.uk/Software/Pfam/>, and stereostructure analysis was performed at <http://swissmodel.expasy.org>.

Semiquantitative RT-PCR Analysis

Semiquantitative RT-PCR analyses were employed to study *MSBP1* transcription levels in various tissues or 7-d-old seedlings after different treatments with hormones and chemicals. Total RNA was isolated with the TRIzol[®] reagent (Invitrogen, Carlsbad, CA) and then reverse-transcribed according to the manufacturer's instructions (RNA PCR kit (AMV), version 2.1; TaKaRa, Dalian, China). Reverse transcription was performed in a total volume of 40 μL using 4 μg total RNA as template and incubated at 42°C for 60 min. Equal amounts of first strand cDNAs were used as templates for PCR amplification using primers *MSBP1*-1 and *MSBP1*-2 for 28, 33, or 36 cycles. The Arabidopsis *Actin2* gene was amplified using primers 1 (5'-TCTTCTCCGCTCTTCTTTCC-3') and 2 (5'-TCTTACAATTTCCGCTCTGC-3') and served as internal positive control for quantification of the relative amounts of cDNA. Amplified DNA products were analyzed with GelDoc 2000 (Furi, Shanghai, China).

For hormone treatments, 7-d-old seedlings were treated with 100 μM indoleacetic acid, ABA, GA₃, or benzyladenine or 1 μM 24-eBL for 8 h. For osmotic stress tests, 150 mM NaCl (12 h), 250 mM mannitol (8 h), and 5 mM CaCl₂ (8 h) were used and 24-h continuous darkness was used for dark treatments. Treated materials were harvested and used for RNA extraction.

Expression of Recombinant *MSBP1* and in Vitro Binding Assays

To study the biochemical characteristics of *MSBP1* in vitro, recombinant *MSBP1* was expressed in *Escherichia coli* and further used to test for progesterone binding activities. The *MSBP1* coding region, amplified via PCR using primers 5'-CATGCCATGGCTATGGCGTTAGAACTATGGC-3' (added *NcoI* site underlined, ATG italic) and 5'-GACCGCTCGAGCTACTCCTCCTTCTCAAC-3' (added *XhoI* site underlined), was cloned into pET-32a(+) (Novagen, Madison, WI) precut with *NcoI* and *XhoI*. The resultant construct was sequenced to confirm the in-frame reading and sequence validity and then transformed into *E. coli* strain AD494(DE)-pLysS. The *E. coli* cells were grown in 5 mL of LB medium at 37°C overnight, and 1 mL of culture was transferred into 100 mL of LB medium and shaken at 37°C until the OD₆₀₀ reached 0.6. The culture was then transferred to 28°C for an additional 30 min, protein expression was

induced by addition of IPTG (final concentration of 1 mM), and samples were incubated for an additional 2.5 h with shaking. Cultured cells were harvested by centrifugation and washed once with ice-cold phosphate-buffered saline (140 mM NaCl in 10 mM PBS, pH 7.4). Cells were disrupted by sonication, the lysate was centrifuged, and the supernatant was separated on 12% SDS-PAGE gels and stained with Coomassie Brilliant Blue R 250. Alternatively, the MSBP1-His-tag fusion proteins were purified from the supernatant by nickel-nitrilotriacetic acid agarose resin affinity chromatography according to the manufacturer's instructions (high level expression and protein purification system; Qiagen, Valencia, CA). Protein concentrations were quantified by the Bradford method (Bradford, 1976) using BSA as the standard. Arabidopsis inositol polyphosphate kinase (IPK2 α), with similar protein size, was recombinantly expressed in the same system and used as control.

In vitro binding assays were performed as described by Ma et al. (2001) with a few modifications. Briefly, an ELISA plate (Nunc-Immuno plates; Maxisorp, Wiesbaden, Germany) was coated with MSBP1 and AtIPK2 α (10 μ g/well) at 4°C for 18 h and washed three times with binding buffer (0.25 M mannitol, 50 mM Tris-Mes, pH 5.8, 5 mM MgCl₂, 0.1 mM CaCl₂, and protease inhibitor cocktail [Sigma-Aldrich, St. Louis, MO]), blocked with binding buffer containing 2% BSA at room temperature for 1 h, and then washed three more times with cool binding buffer. Binding buffer (200 μ L) containing [³H]-progesterone (Amersham, Piscataway, NJ) in final concentrations of 0, 5, 10, 20, 50, 100, and 200 nM was added, and the plate was incubated on ice for 2 h. After incubation, the plate was washed three times with cold binding buffer, the bound protein was dissolved with 100 μ L of 0.3 M NaOH and 10 g/L of SDS, and the radioactivity was measured by a liquid scintillation counter (Beckman BS6500; Fullerton, CA).

For the assays on the inhibitory effects, the binding buffer (200 μ L) contains [³H]-progesterone (50 nM) and unlabeled progesterone, or 5 α -DHT, 24-eBL, stigmasterol, or ABA (Sigma-Aldrich) in gradually increasing concentrations (0, 10, 50, 100, 500, 10³, or 10⁴ nM). The data of competitive assays, including IC₅₀ and K_i, were analyzed using Kaleida-Graph software (Synergy Software, Vermont, Canada).

Preparation of Microsomal Membranes and in Vitro Binding Assays

Membrane fractions were isolated from Arabidopsis seedlings of wild-type and transgenic plants with enhanced or reduced *MSBP1* according to the protocol of Wang et al. (2001), with subsequent operations conducted at 4°C. Briefly, 8 g of plant materials (4-week-old seedlings) were homogenized in the extraction buffer (5 mL/g) containing 20 mM Tris-HCl, pH 7.5, 250 mM mannitol, 5 mM MgCl₂, 0.1 mM CaCl₂, and protease inhibitor cocktail (Sigma-Aldrich), followed by filtering through four layers of cheesecloth. After centrifugation at 10,000g for 15 min, the supernatant was centrifuged at 150,000g for 1 h to collect membranes, which was resuspended and diluted to 2 mg membrane protein/mL in binding buffer (as above mentioned). Each binding assay contains 50 μ L of membrane suspensions, indicated amount of [³H]-progesterone, 1 mg/mL of BSA, and binding buffer in 100 μ L total volume. The binding reactions were incubated for 1 h at 25°C. The bound and free [³H]-progesterone was separated by filtering the mixture through a glass-fiber filter (Whatman GF/F; Clifton, NJ) and washing with 10 mL of ice-cold binding buffer and was quantified by scintillation counting.

Transgenic Modification of MSBP1 Expression

The *MSBP1* coding region was amplified using primers 5'-GCATGC-CATGGCGTTAGAACTATGGCAA-3' (with added *Nco*I site underlined and ATG in italics) and 5'-GGACTAGTCTCCTCTTCTTCAACACAG-3' (added *Spe*I site underlined) and subcloned into pCAMBIA1302 pre-cut with *Nco*I and *Spe*I. The coding region and reading frame were confirmed by sequencing, and the resulting construct was used for both *MSBP1*

overexpression and GFP fusion studies. The full-length *MSBP1* cDNA was cut from pBSK using *Bam*HI and *Sal*I and then subcloned into pCAMBIA1301 to create antisense *MSBP1* under the control of the CaMV35S promoter.

The fusion constructs were used for transformation of Arabidopsis by the floral dipping procedure. Resistant plants were screened on plates containing 25 μ g/mL of hygromycin and confirmed as transgenics through the following two strategies: (1) genomic DNA was extracted with the cetyl-trimethyl-ammonium bromide method (Sambrook et al., 1989) and PCR amplified with primers from the transformed construct, and (2) total RNA was isolated from leaves of both resistant and untransformed plants, and RT-PCR was employed to determine whether *MSBP1* expression had been altered using internal primers (in the fragment for transformation) 5'-ATGGCGTTAGAACTATGGC-3' and 5'-CTACTCCTCTTCTTCAAC-3' and external primers (beyond the fragment for transformation) 5'-CTTTTCAACACTACTGATC-3' and 5'-CAAGATTATCATTGACACTTC-3'.

The confirmed transformants were transplanted to soil for self-crossing, and T2 seeds from individual T1 plants were germinated in the same medium for generation of homozygous T2 lines, which were used for further analysis.

Phenotypic Observation of Transgenic Plants

The hypocotyl lengths of homozygous transgenic plants were calculated at 4 or 6 d after germination under both darkness and light from an average of at least 30 seedlings. All experiments were performed in triplicate. To test the sensitivity to supplemented progesterone or 24-eBL, seeds of control and transgenic plants were germinated on half-strength MS medium supplemented with or without progesterone (0.01, 0.05, 0.1, or 1 μ M) or 24-eBL (0.001, 0.005, 0.01, 0.05, 0.1, 0.5, or 1 μ M) for 7 d. In addition, different amounts of GA₃ (0, 0.5, 1, 5, and 10 μ M) and indole-acetic acid (0, 0.05, 0.1, 0.5, and 1 μ M) were supplemented to test their effects on plant growth. All measurements were performed in triplicate, and the results given are the average of at least 25 seedlings.

Preparation of Hypocotyl Sections for Microscopic Observation

Hypocotyl sections (~2 mm) from control and transgenic plants germinated and grown on vertically oriented plates for 6 d were sampled, fixed in 2.5% glutaraldehyde (16 to 48 h at 4°C), and dehydrated through a graded ethanol series. The samples were embedded in Epon812 resin (Fluka, Buchs, Switzerland) and polymerized at 60°C. Longitudinal sections (3 μ m) were cut and stained briefly with filtered 1% toluidine blue. Sections were microscopically examined (Leica DMR; Wetzlar, Germany) and photographed (Liu et al., 2003).

Promoter-Reporter Gene Fusion Studies and GUS Activity Analysis

The promoter region of *MSBP1* was PCR amplified using primers 5'-ACGCGTCTCGACAACCATCAGAGATCC-3' (added *Sal*I site underlined) and 5'-CGCGGATCCTGCCATAGTTCTAACGCCAT-3' (added *Bam*HI site underlined) with Arabidopsis genomic DNA as the template. The resultant 1540-bp fragment was subcloned into pCAMBIA1300+pBI101.1 (modified in our lab; Liu et al., 2003), and the resulting construct was transformed into *A. tumefaciens* and Arabidopsis. The obtained transgenic plants were used for detection of GUS activity as described (Jefferson et al., 1987). Germinated seeds or seedlings grown for 1, 2, 3, 4, 5, 6, and 7 d in darkness and light were stained. Seedlings cultured in light or darkness for 4 d were switched to darkness or light, respectively, and stained 1, 2, and 3 d after the switch. Photography was performed using a Nikon microscope with a digital camera (Tokyo, Japan).

Expression Pattern Analysis of Cell Division- or Elongation-Related Genes

Total RNA was extracted from 14-d-old control and transgenic seedlings and used for semiquantitative RT-PCR analysis with equal amounts of first-strand cDNA as templates. The corresponding primers for the tested genes are as follows: *AtExp1* (5'-TCTACGGTGGTGGTATGC-3' and 5'-TTACTCTGCCAGTTCTGTCC-3'), *β-tubulin2* (5'-TTCGACCTGATAACTTCGTC-3' and 5'-TCTGCTCGTCAACTTCTTT-3'), *KOR* (5'-CCTTCAATCGTGTGGGTT-3' and 5'-GTGGTGCTGGATGTATATG-3'), *TCH-4* (5'-CGGTAAGAAGCAGTAAAGG-3' and 5'-TGGTCGTGGACAGATCAAG-3'), and *Meri-5* (5'-CAATCGTCTGCGTTCCATA-3' and 5'-ACGCCAGCTTCTACTCT-3'). *Actin2* was amplified and used as an internal positive control.

Confocal Microscopy

Root apices and hypocotyls from 8-d-old transgenic plants harboring *CaMV35S:MSBP1:GFP* were analyzed by confocal microscopy using a Bio-Rad Lasersharp 2000 (Hercules, CA) with a Kr/Ar laser 488 (FITC488, Zeiss LSM500; Herts, UK).

Expression Profiling of Genes in *MSBP1*-Overexpressing Plants through Microarray Analysis

Seven-day-old etiolated seedlings of the *MSBP1*-overexpressing (two independent lines) and wild-type plants were harvested and frozen quickly in liquid nitrogen. The RNA samples were processed as recommended by Affymetrix (Santa Clara, CA). Briefly, total RNA was extracted using TRIzol reagent (Invitrogen) and RNeasy mini kits (Qiagen). Two micrograms of poly(A)⁺-mRNA was converted to double-stranded cDNA using the SuperScript polymerase II (Invitrogen) with a T7-(dT)₂₄ primer incorporating a T7 RNA polymerase promoter (Genset, La Jolla, CA). Biotin-labeled cRNA was synthesized in vitro from 1 μg of double-stranded cDNA using the Enzo RNA transcript labeling kit (Affymetrix). Fifteen micrograms of biotin-labeled cRNA was purified and fragmented into 35 to 300 base pieces and then hybridized with an Arabidopsis genome array (Affymetrix ATH1). After removal of the hybridization mixture, the arrays were washed and then stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR) and biotinylated goat anti-streptavidin antibody (Vector Laboratories, Burlingame, CA) in the Affymetrix fluidics stations using standard procedures supplied by the manufacturer. Arrays were scanned using an Agilent GeneArray scanner (Affymetrix G2500A GeneArray Scanner; Palo Alto, CA). The microarray data were normalized using the Affymetrix Microarray Suit program (version 5.0) and set the algorithm absolute call flag indicating the reliability of the data points according to P (present), M (marginal), and A (absent). The significant difference (P-value ≤ 0.0025) of each gene between the control and *MSBP1*-enhanced plants was examined using Wilcoxon rank test of the Affymetrix Microarray Suit program. The genes with the consensus significant difference in two independent lines were selected and analyzed by a further restrict screening criteria (in the two independent lines, at least onefold variation of ≥2.0 and the other of ≥1.5 for upregulated genes; at least onefold of ≤0.5 and the other ≤0.67 for downregulated ones). Finally, the annotations for the selected probe sets ID were rechecked at the Affymetrix Web site. The experiments were repeated twice in different times and the results were combined.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under the following accession numbers: *MSBP1* (NM_124603); *PGC1_PIG* (Q95250); *AtExp1* (ATU30476); *β-tubulin2* (M20405); *KOR* (AA585915); *TCH-4* (AF051338); *Meri-5* (AA042665), and *Actin2* (NM_112764).

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Arabidopsis Membrane Steroid Binding Protein 1 Is Involved in Inhibition of Cell Elongation

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