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Progesterone: Its occurrence in plants and involvement in plant growth

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Abstract

Progesterone is a mammalian gonadal hormone. In the current study, we identified and quantified progesterone in a range of higher plants by using GC–MS and examined its effects on the vegetative growth of plants. The growth of Arabidopsis (*Arabidopsis thaliana*) seedlings was promoted by progesterone at low concentrations but suppressed at higher concentrations under both light and dark growth conditions. The growth of the gibberellin-deficient mutant *lh* of pea (*Pisum sativum*) was also promoted by progesterone. An earlier study demonstrated that progesterone binds to MEMBRANE STEROID BINDING PROTEIN 1 (MSBP1) of Arabidopsis. In this work, we cloned the homologous genes of Arabidopsis, *MSBP2* and *STEROID BINDING PROTEIN* (*SBP*), as well as of rice (*Oryza sativa*), *OsMSBP1*, *OsMSBP2* and *OsSBP* and examined their expression in plant tissues. All of these genes, except *OsMSBP1*, were expressed abundantly in plant tissues. The roles of progesterone in plant growth were also discussed.

Keywords: *Arabidopsis thaliana*; Cruciferae; *Pisum sativum*; Leguminosae; *Oryza sativa*; Gramineae; Occurrence of progesterone; Steroid; Membrane steroid binding protein; Progesterone-binding protein

1. Introduction

Progesterone **1** is a gonadal steroid hormone essential for continuation of early pregnancy, and plays important roles in ovulation, atresia and luteinization in mammals. Progesterone **1** has also been reported to be present in *Holarrhena floribunda* leaves (Leboeuf et al., 1964) and apple seeds (Gawienowski and Gibbs, 1968). However, these reports may not be reliable because identifications were based on data obtained using TLC and GC. Simons and Grinwich (1989) also detected progesterone **1**, estrogen, androstenone and androgen in a range of plant species using radioimmunoassay (RIA) where ethanol extracts of plant tissues were analyzed without any purification. The level of progesterone **1** determined in some plant tissues exceeded 2600 ng g^{-1} dry wt. Sandberg et al. (1985) found, when RIA was used to analyze the IAA content in extracts of pine seedlings, that extensive sample purification was necessary before accurate RIA data could be obtained. This was because plants contain interfering substances which can overestimate IAA amounts. So most laboratories switched from fairly unspecific immunoassays to mass spectrometry which is both sensitive and accurate (Ljung et al., 2004). To our knowledge, mass spectrometric analysis of progesterone **1** has been done only in the case of

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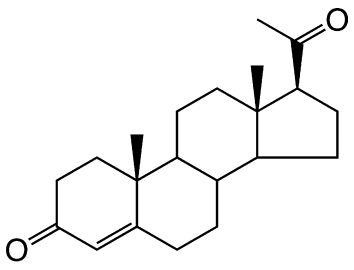
Vitex agnus-castus, where a direct inlet system was used (Saden-Krehula et al., 1991). However, it seems that mass spectrometry has not been applied to determine the content of progesterone **1** in plant tissues (Janeczko and Skoczowski, 2005). Therefore, currently, the occurrence of progesterone **1** seems not to be generally accepted.

The effects of estrogens and androgens on the vegetative and reproductive growth of plants have been studied by many researchers (see Geuns, 1978). Later, the biological activities of progesterone **1** in plants were demonstrated. Ylstra et al. (1995) found that animal hormones, including progesterone **1**, stimulate germination and the tube growth of tobacco pollen. Furthermore, progesterone **1** has been characterized as one of the animal steroid hormones that can induce flowering or generative development in wheat (Janeczko and Filek, 2002) and Arabidopsis (Janeczko et al., 2003). On the other hand, progesterone **1** is known to inhibit growth of human pathogenic bacteria and fungi (Yotis and Stanke, 1966; Clemons et al., 1988), as well as of the saprophytic fungus *Rhizopus nigricans* that contains progesterone-binding membrane receptors coupled to G proteins (Lenasi et al., 2002). However, little has been known about the effects of progesterone **1** on vegetative growth of plants.

Mammalian receptors for steroid hormones are well known transcription factors (Beato, 1989). Although such genomic reactions are slow events, more rapid, non-genomic effects have been demonstrated for steroid hormones (Bramley, 2003; Sutter-Dub, 2002). During the past decade a number of non-genomic events of progesterone **1** have been described. In 1996, a putative progesterone-binding protein with a single membrane spanning domain was identified from porcine (Meyer et al., 1996; Falkenstein et al., 1996), and subsequently, analogous genes were cloned from rat, cattle and human (Bramley, 2003). These putative progesterone-binding proteins were localized to the ER (Falkenstein et al., 1998) or the plasma membrane (Krebs et al., 2000). These membrane proteins show no significant identity to the classical cytosolic progesterone receptor (Falkenstein et al., 1996; Gerdes et al., 1998). The genome DNA databases of higher plants, including Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*), have suggested the presence of multiple genes highly homologous to mammal putative progesterone-binding proteins with a single-membrane spanning domain. Recently, one such binding protein, termed the membrane steroid binding protein (MSBP1) was identified in Arabidopsis and characterized as a negative regulator of cell elongation (Yang et al., 2005).

In the current study, we identified and quantified progesterone **1** levels in a range of higher plants using GC–MS, and investigated its effects on vegetative growth of plants. Furthermore, we cloned several genes homologous to *MSBP1* from Arabidopsis and rice, and examined the expression of these genes. Possible physiological roles of progesterone **1** in plants are also discussed (Structure 1).



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Structure 1. Chemical structure of progesterone.

2. Results and discussion

2.1. Progesterone *1* occurs widely in higher plants

Progesterone **1** was detected in a variety of dicot and monocot species by GC–MS (Table 1). Its mass spectrum obtained from shoots of pea (*Pisum sativum*), closely matched that of authentic progesterone **1** (Fig. 1). Further evidence for its presence was also obtained by selective ion monitoring in an Arabidopsis inflorescence extract (Fig. 2). Similar evidence was obtained for the presence in other plant extracts from Adzuki bean (*Phaseolus angularis*), mung bean (*Vigna radiata*), tomato (*Lycopersicon esculentum*), potato (*Solanum tuberosum*), apple (*Malus domestica*), rice and onion (*Allium cepa*). Furthermore, progesterone **1** was detected in all organs examined including shoots, roots, tubers, inflorescences and seeds. The richest source was shoots of rice although reproductive tissues such as inflorescences, seeds and ears generally contained higher levels. The present work demonstrated that the amounts of progesterone **1** in plant tissues were generally of the order of ca. 1 μg or less per kg fr. wt of tissue (Table 1). Its actual levels may be somewhat higher than the estimated amounts because recovery rates in our analytical procedures were estimated using an authentic specimen to range from ca. 70% to ca. 90%. Unfortunately, we could not compensate exactly for the losses encountered during purification due to the unavailability of adequate internal standards such as deuterium-labeled progesterone **1**. [$^2H_9$]-Progesterone, which was prepared by means of basic catalytic exchange, could not be used as an internal standard because the deuterium atoms were easily eliminated during the extraction and purification procedures employed.

An earlier work demonstrated, by means of GC with FID, that apple seeds contain ~500 mg kg^{-1} fr wt of progesterone **1** (Gawienowski and Gibbs, 1968), three orders of magnitude higher than the current datum obtained using GC–MS. Similarly, the presence of high levels of progesterone **1** had been demonstrated in various plants using RIA by Simons and Grinwich (1989). The estimates obtained in these studies would thus appear to be over-estimates because of non-specific detection techniques used.

The available evidence indicates that progesterone **1** is synthesized from cholesterol via pregnenolone in *H. floribunnda* (Benett and Heftmann, 1965) and from sitosterol

Table 1
Endogenous levels of progesterone **1** in various plants determined by selected ion monitoring

Plant species	Tissue analyzed	Progesterone **1** content (ng kg^{-1} fr. wt)
Dicot		
Arabidopsis thaliana	Shoot	160
	Inflorescence	400
Pisum sativum	Shoot	190
	Root	260
	Mature seed	410
Phaseolus angularis	Etiolated seedling	48
Vigna radiata	Etiolated seedling	21
Lycopersicon esuculentum	Leaf	25
	Immature green fruit	280
	Mature red fruit	6
Solanum tuberosum	Tuber	24
Malus domestica	Flesh	150
	Seed	430
Monocot		
Oryza sativa	Shoot	1540
	Ear	440
Allium cepa	Bulb	68

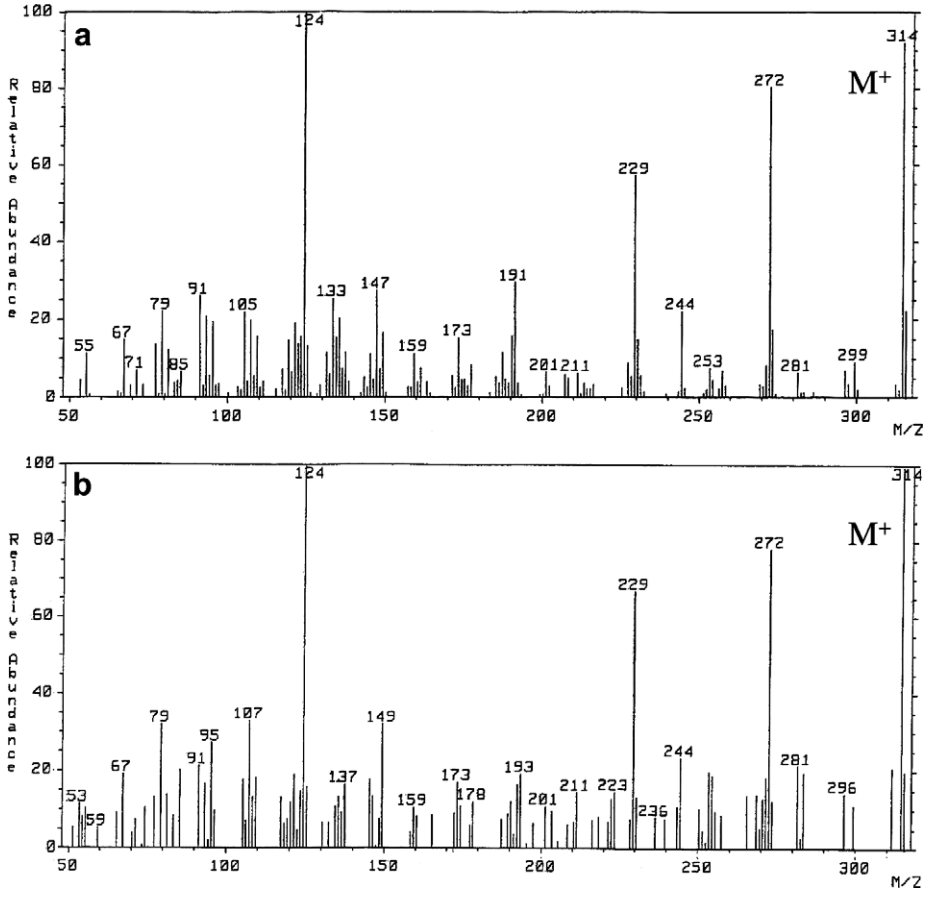

Fig. 1. Mass spectra of progesterone **1** (a) and endogenous progesterone **1** in pea shoots (b). The spectra were determined by GC–MS as described in Section 4. The R_t of progesterone **1** was 6.40 min in both a and b.

in *Digitalis lanata* (Bennett et al., 1969; Geuns, 1978). Progesterone **1** has been shown to be an intermediate in the synthesis of cardenolide from sterols in *Digitalis* spp. (Caspi and Lewis, 1967; Bennett et al., 1968; Herl et al., 2006). Its biosynthesis from pregnenolone has been demonstrated to be catalyzed by Δ^5-3β-hydroxysteroid dehydrogenase from *Digitalis lanata* (EC 1.1.1.51; AJ345026, AY844960) (Frinsterbusch et al., 1999). BLAST search

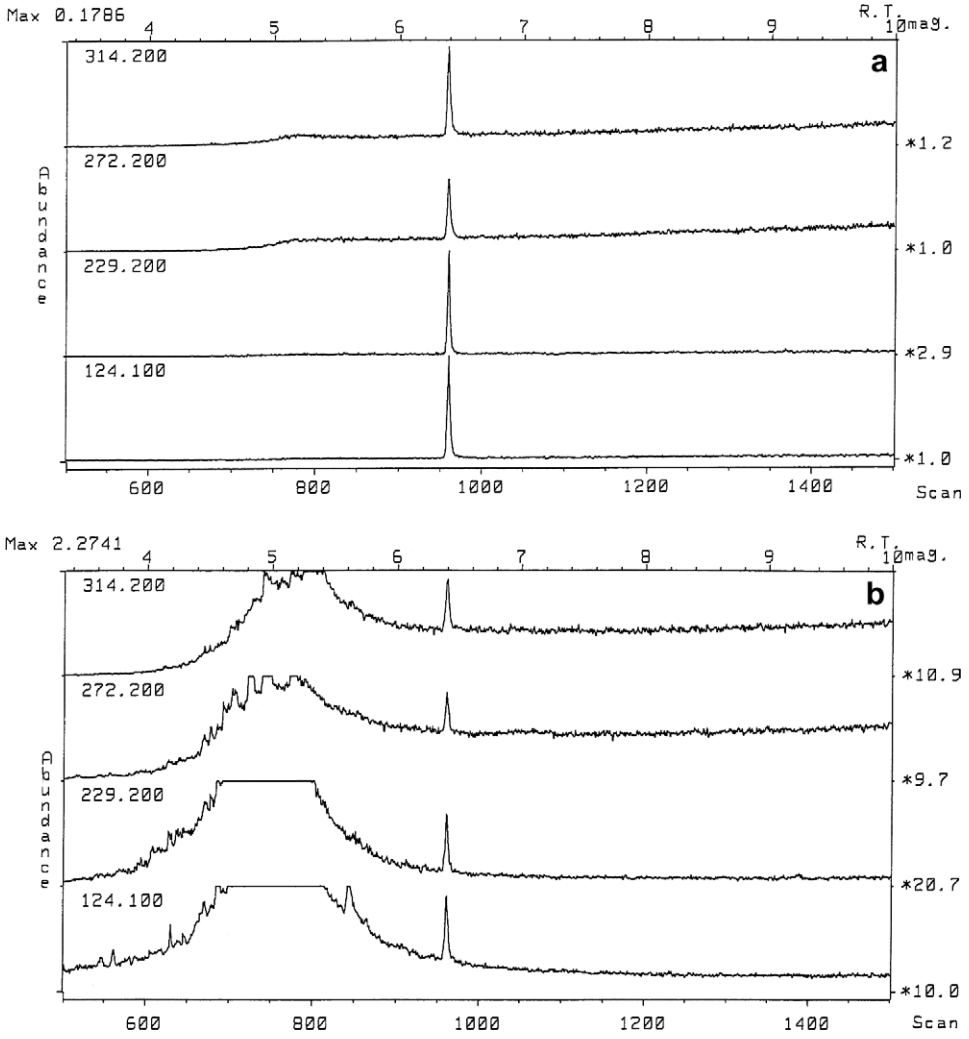

Fig. 2. Selected ion monitoring traces of *m/z* 314 [M^+], 272, 229 and 124 ions of progesterone **1**. (a) Authentic progesterone **1**. (b) Endogenous progesterone **1** in Arabidopsis inflorescence.

shows the presence of a number of homologous enzymes in Arabidopsis and rice genomes. However, it remains undetermined which enzymes are responsible for the synthesis of progesterone **1**.

2.2. Progesterone *1* elicits plant growth promotion

Brassinosteroids are plant steroid hormones that elicit a variety of biological activities such as cell expansion, cell division, vascular differentiation and modulation of stress responses (Clouse and Sasse, 1998). The effects of brassinosteroids on the growth of Arabidopsis (Li et al., 1996), pea (Nomura et al., 1997) and rice (Takeno and Pharis, 1982) have well been studied. So these plants were used to evaluate the biological activities of progesterone **1** in comparison with those of brassinosteroids.

First, we examined the effects of progesterone **1** on light-grown Arabidopsis seedlings. To this end, Arabidopsis seedlings were grown in dim light so that elongation of hypocotyls was easily measured. Thus, application of 0.01–1 μM of progesterone **1** resulted in weak but distinct hypocotyl elongation while at higher concentrations growth was retarded markedly (Fig. 3a and b). The current data were reproducible in three independent experiments and are in keeping with those of Yang et al. (2005). However, the growth promoting activity of progesterone **1** was much lower than brassinolide as shown by Li et al. (1996). Similar effects of progesterone **1** were also observed with dark-grown 4-day-old seedlings (Fig. 3c and d). However, the growth-promoting effect in darkness was transient, and was not observed in 7-day-old seedlings. This explains why Yang et al. (2005) did not observe biological activity in 7-day-old, dark-grown seedlings. Thus, progesterone **1** may have physiological roles in the early stages of seedling growth. The possibility cannot be ruled out that the effect of progesterone **1** may be associated with modification of membranous properties. However, the mode of action will be a subject of future research.

In rice seedlings, dark-grown in deep water, progesterone **1** did not enhance shoot growth although coleoptile

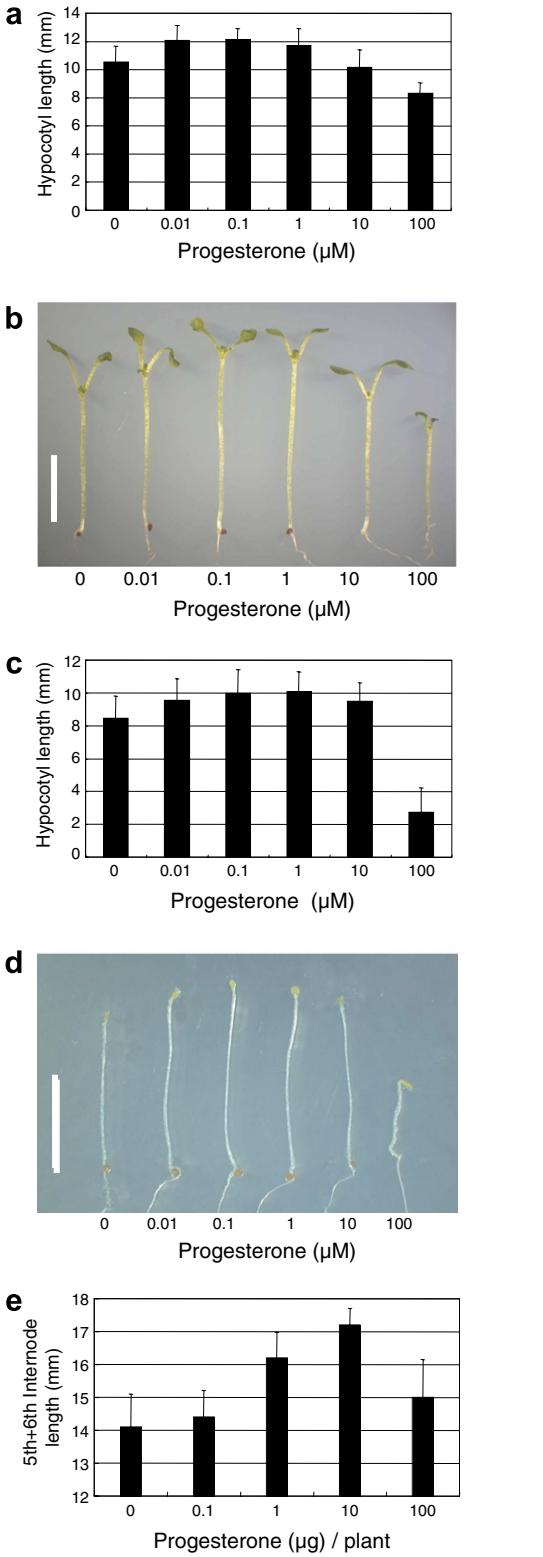

Fig. 3. Biological activity of progesterone **1**. (a) Effect of progesterone **1** on hypocotyl elongation of 10-day-grown Arabidopsis seedlings in the light. The error bars indicate s.d. ($n = 30$). (b) Typical seedlings obtained in A. The bar denotes 5 mm. (c) Effects of progesterone **1** on hypocotyl elongation of 4-day-grown Arabidopsis seedlings in the dark. The error bars indicate s.d ($n = 30$). (d) Typical seedlings obtained in c. The bar denotes 5 mm. (e) Effect of progesterone **1** on stem elongation of the pea mutant *lh*. Data were recorded 4 days after treatment. The error bars indicate s.d ($n = 10$).

elongation was suppressed by ca. 30% when 10 μM progesterone **1** was used (data not shown). Similarly, brassinosteroids have been known to cause no shoot elongation of dwarf rice seedlings (Takeno and Pharis, 1982). We also examined whether progesterone **1** causes leaf lamina bending of rice like brassinosteroids. However, no such biological activity was observed (data not shown).

Progesterone **1** elicited a weak growth promotion for the gibberellin-deficient pea mutant *lh* (Davidson et al., 2004) with the optimum dosage being 10 μg plant^{-1} (Fig. 3e). Similar results were obtained in three independent experiments. In contrast, progesterone **1** did not promote growth of the gibberellin-deficient pea mutants *ls* (Ait-Ali et al., 1997) and *le* (Martin et al., 1997) or that of wild-type pea (cv. Torsdag). Similarly, the brassinosteroid-deficient pea mutant *lkb*, that is highly sensitive to brassinosteroids (Nomura et al., 1997) and the brassinosteroid-insensitive mutant *lka* (Nomura et al., 2003) did not respond to progesterone **1** (data not shown). We could not investigate the effect of progesterone **1** on the Arabidopsis *ga3* mutant that is an ortholog of pea *lh* because the *ga3* mutant cannot germinate without exogenous gibberellin and this makes it difficult to determine the biological activity of progesterone itself. The *lh* mutant has a lesion in kaurene oxidase, a microsomal cytochrome P450 enzyme. Thus it seems that some metabolic and/or physiological change accompanied by the lesion may be associated with the action of progesterone **1**.

Kopcewicz (1969) observed growth-promoting effects of estrone and 17β-estradiol with dwarf pea (var. Cud Kelwedonu). However, we found that 17β-estradiol was inactive when applied to the *lh*, *le*, *ls*, *lka* and *lkb* mutants as well as wild type plants (data not shown). As in mammals, plants may convert progesterone **1** to estrogens, which then elicits a biological response. However, our results suggest that progesterone **1** may be biologically active without transformation to estrogens.

2.3. Genes encoding putative progesterone-binding proteins are present in Arabidopsis and rice

BLAST searches using Hpr6.6 (Gerdes et al., 1998), one of the human progesterone-binding membrane proteins, showed three homologous genes in both Arabidopsis and rice. The Arabidopsis genes were *MEMBRANE STEROID BINDING PROTEIN 1, 2* (*MSBP1, 2*) and soluble *STEROID BINDING PROTEIN* (*SBP*), while the rice genes were *OsMSBP1*, 2 (*Oryza sativa MSBP 1, 2*) and *OsSBP*. We cloned full-length cDNAs of these genes (Fig. 4). The possible binding motifs of all these proteins had high similarities to that of Hpr6.6 (Mifsud and Bateman, 2002): 64% (*MSBP*1), 65% (*MSBP*2), 58% (*SSBP*), 66% (*OsMSBP*1), 66% (*OsMSBP*2) and 61% (*OsSSBP*). Furthermore, a tryptophan residue that is likely required for the specific binding of progesterone **1** is well conserved in all of these proteins, suggesting the binding ability of progesterone **1**. In support of this, Yang et al. (2005) have

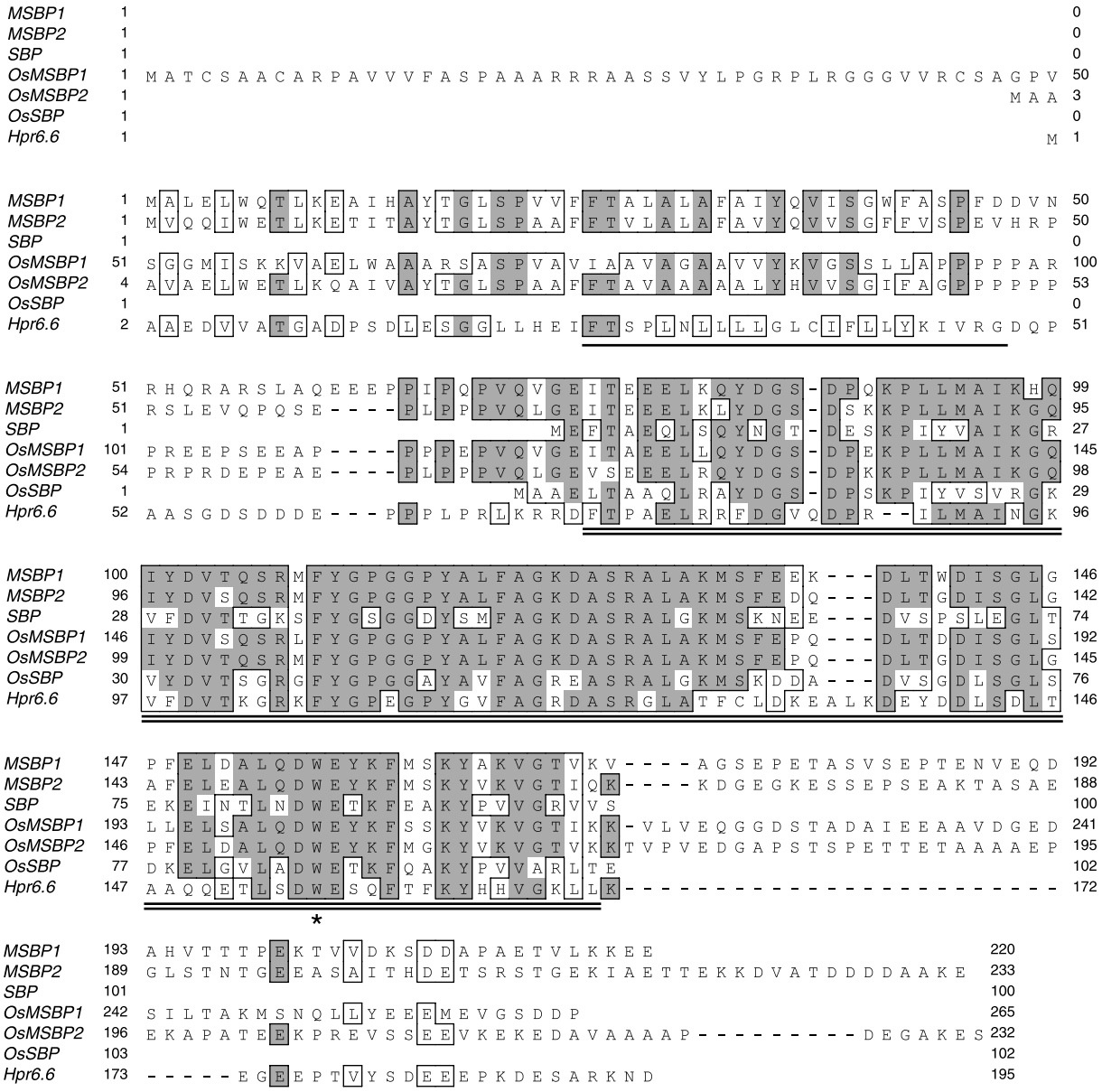

Fig. 4. Alignment of putative progesterone-binding proteins of Arabidopsis and rice with human Hpr6.6. Conserved residues are boxed and identical residues are shown by dark shade. The putative transmembrane domains are underlined. The double-underline indicates a putative ligand-binding domain which is similar to the heme-binding domain of cytochrome *b*5. The asterisk indicates the tryptophan residue required for the specific binding of the ligand.

demonstrated that MSBP1 protein binds progesterone **1**. However, two cysteine molecules required for a single intramolecular disulfide bridge in mammalian progesterone-binding membrane proteins (Falkenstein et al., 2001) are not conserved (Fig. 4).

MSBP1 protein is localized in plasma membrane (Yang et al., 2005). MSBP2, OsMSBP1 and OsMSBP2 have hydrophobic membrane anchors similar to that of MSBP1 and hence appear to be localized in the plasma membrane. The presence of soluble SBP in Arabidopsis was demonstrated by Yang et al. (2005). In this study, we estimated that its homolog, OsSBP is also present in rice. These soluble proteins without membrane anchors do not exist in human and mouse genomes, indicating SBP and OsSBP are plant-specific.

The available plant EST data indicate that putative progesterone-binding membrane proteins are present in various organs of a variety of plant species including *Glycine max*, *L. esculentum*, *S. tuberosum*, *Lactuca sativa*, *Brassica napus*, *Lotus japonicus*, *Medicago truncatula*, *Zea mays*, *Triticum aestivum*, *Gossypium arboreum*, *Citrus sinensis* and *Vitis vinifera*. It is thus evident that these receptor-like proteins are widely distributed in the plant kingdom. The phylogenetic relationships (Fig. 5) suggest that this protein family is widely conserved in animals, insects, yeast and plants. Quite close to putative progesterone-binding

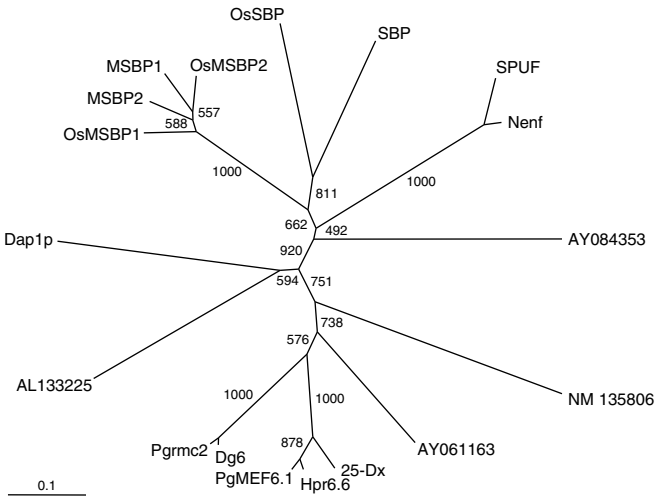

Fig. 5. Phylogenetic relationship among putative progesterone-binding proteins. The phylogenetic tree was constructed by the neighbor-joining method and P-distance mode of CLUSTAL W using the deduced full-length sequences of the following proteins: Arabidopsis (MSBP1, At5g52240; MSBP2, At3g48890; SBP, At2g24940; AY084353, At4g14965), rice (OsMSBP1, AK098881; OsMSBP2, AK105774; OsSBP, AK062552), human (Hpr6.6, Y12711; Dg6, AJ002030; SPUF, AF173937), rat (25-Dx, U63315; Nenf; NM_001002851; Pgrmc2, NM_001008374), pig (PgMEF6.1, X99714), fruit fly (AY061163; NM_135806), baker's yeast (Dap1p, Z73526) and fission yeast (AL133225). Bootstrap mode (1000 replications) was used for estimating the confidence that can be assigned to particular nodes in the tree.

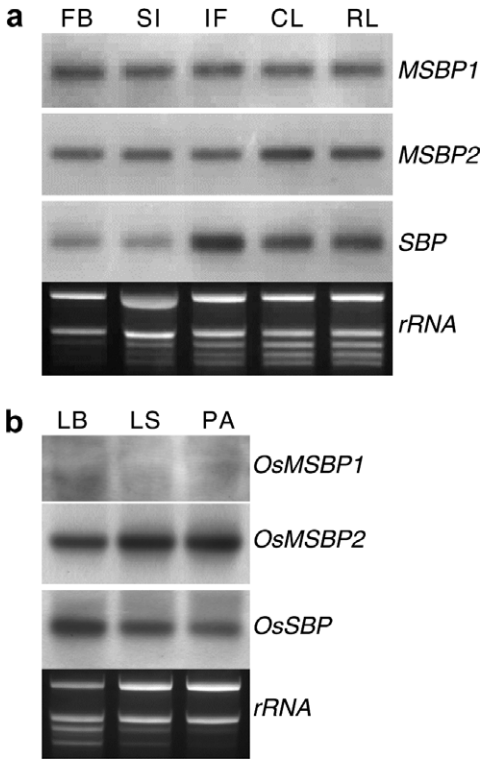

Fig. 6. Northern blot analysis of putative progesterone-binding protein gene transcripts in light-grown Arabidopsis and rice. Ribosomal RNA stained with ethidium bromide was shown as loading controls. (a) Expression of Arabidopsis genes *MSBP1*, *MSBP2* and *SBP* in apical flower buds (FB), siliques (SI), inflorescences (IF), cauline leaves (CL) and rosette leaves (RL) of Arabidopsis. (b) Expression of rice genes *OsMSBP1*, *OsMSBP2* and *OsSBP* in leaf blades (LB), leaf sheaths (LS) and panicles (PA) of rice.

proteins of Arabidopsis and rice is the secreted protein of unknown function (SPUF) of human. Its homologous proteins are distributed in a range of animals, although their function is not known. Also similar are the putative progesterone-binding membrane proteins of yeast (Dap1p and AL133225), fruit fly (AY061163 and NM135806), rat (25-Dx, Nenf and Pgrmc2), pig (PgMEF6.1) and human (Hpr6.6 and Dg6). Human putative progesterone-binding membrane protein, Hpr6.6, regulates cell death by oxidative damage (Hand and Craven, 2003). In yeast, Dap1p is involved in DNA damage repair and sterol synthesis (Hand et al., 2003). Interestingly, overexpression of MSBP1 alters gene expression related to sterol metabolism and signaling (Yang et al., 2005). In rat, 25-Dx is induced by treatment with dioxin (Selmin et al., 1996). Therefore, it may be possible that, in plants, putative progesterone-binding membrane proteins are involved in various physiological events.

2.4. Genes encoding putative progesterone-binding proteins are expressed throughout plant tissues of Arabidopsis and rice

Using full-length cDNAs of the *MSBP* and *SBP* genes, their expression in Arabidopsis was investigated by RNA gel blot analysis in three independent experiments. In light-grown Arabidopsis seedlings, the *MSBP1*, *MSBP 2* and *SBP* genes were all expressed in apical flower buds, siliques, inflorescences, cauline leaves and rosette leaves (Fig. 6a). Yang et al. (2005) showed that *MSBP1* is expressed in various tissues. In keeping with this finding, expression of the *MSBP1* and *MSBP2* mRNAs was similar in all the tissues examined. However, *SBP* was expressed higher in inflorescence, cauline leaves and rosette leaves, suggesting that its function may be distinct from other membranous binding proteins.

In light-grown adult rice plants, the *OsMSBP2* and *OsSBP* genes were highly expressed in leaf blades, leaf sheaths and panicles (Fig. 6b), but the expression of the *OsMSBP1* gene in these tissues was not observed. However, its transcript must be present at very low level because we could clone cDNA of the *OsMSBP1* gene using rice mRNAs (Fig. 6b). Expression of *OsMSBP2* and *OsSBP* in roots of 10-day-old rice seedlings was at the same level as that in shoots (data not shown). Light-grown and dark-grown seedlings were compared with respect to the expression of *OsMSBP2* and *OsSBP* in the roots and shoots. However, no effects of light on their expression were observed (data not shown) in contrast to the observation in Arabidopsis (Yang et al., 2005). In rat, the transcript level of 25-Dx (Fig. 5) is repressed by exogenous progesterone **1** (Krebs et al., 2000). However, such a negative regulation due to progesterone **1** was not observed in either Arabidopsis or rice (data not shown).

To examine the functions of *MSBP1*, *MSBP2* and *SBP* genes in more detail, we searched their expression levels using *Arabidopsis* microarray database in the Genevestigator (http://www.genevestigator.ethz.ch/). The expressions of *MSBP1* and *MSBP2* are highly and constantly observed in Arabidopsis plants from vegetative to reproductive stages. Furthermore, these genes are expressed in most organs including cotyledon, hypocotyl, stem, root, leaf, inflorescence, and flower. However, within the flower, the expression of *MSBP1* and *MSBP2* is extremely suppressed in pollen in contrast to other flower tissues such as carpel, petal, sepal, stamen and stigma. Therefore, *MSBP1* and *MSBP2* may function throughout the growth, but may not be involved in pollen physiology. On the other hand, the expression of *SBP* in Arabidopsis plants is about one-order of magnitude lower than those of *MSBP1* and *MSBP2* throughout the growth, but, interestingly, a marked build-up of *SBP* mRNAs was observed in the early bolting stage. Anatomically, a high level of *SBP* mRNAs is observed in the hypocotyl of adult plants where xylem and cork are rich sources of *SBP* mRNAs, suggesting that *SBP* might be involved in xylem differentiation. Interestingly, another plant steroid hormone, brassinosteroid, is known to regulate xylem differentiation. It should be noted that the expression patterns of *SBP* clearly differ from those of *MSBP1* and *MSBP2*, again suggesting *SBP* has distinct physiological roles from *MSBP1* and *MSBP2*.

3. Concluding remarks

Mass spectrometric evidence indicates that progesterone **1** occurs widely in plants. It also elicits a weak but distinct growth promoting response in some biological systems. Genes encoding putative progesterone-binding proteins were shown to be expressed ubiquitously in Arabidopsis and rice. Thus, we postulate that progesterone **1** may be an endogenous growth regulator. However, very little is known about how endogenous progesterone **1** elicits biological activity and, furthermore, its biosynthesis and metabolism in plants remains to be investigated.

4. Experimental

4.1. Plant materials

Plant materials for quantitation of progesterone **1** were obtained as follows. Arabidopsis (*A. thaliana* ecotype Columbia-0) were sown on pots filled with a 1:1 mixture of vermiculite–soil mix (Sakatanotane, Japan), placed in a cold room at 4 °C for 1 day and then grown for 37 days in a growth chamber at 23 °C under continuous fluorescent light (60 μmol m^{-2} s^{-1}) with 1000× Hyponex fed every week. Pea seedlings (*P. sativum* cv. Torsdag) were grown for 37 days as described by Nomura et al. (1997). Etiolated seedlings of Adzuki (*P. angularis*) and mung bean (*V. radiata*) were obtained by growing in darkness for 5 days at 25 °C. Tomato (*L. esculentum*, cv. Saturn) was grown under natural conditions and top three leaflets, green fruit (two) and mature red fruit (one) were harvested. Tubers of potato (*S. tuberosum* cv. Danshaku) were obtained locally. Fruit of apple (*M. domestica* cv. Tsugaru) was obtained locally and separated into flesh and core, from which twelve seeds were collected. Rice (*O. sativa* cv. Nipponbare) was grown for 5 months under natural conditions.

To obtain plant materials for gene expression assays, Arabidopsis (ecotype Columbia-0) was grown for 50 days as described above. Rice (cv. Nipponbare) was grown for 120 days in pots under natural conditions.

4.2. Extraction and purification of progesterone *1*

Plant tissues were extracted using a homogenizer with 10 vol. of MeOH for 2 h at room temperature. The mixture was filtered and the residual solid was re-extracted with 5 vol. of MeOH overnight. The filtrates were combined, evaporated and partitioned between 0.5 M K_2HPO_4 (150 ml) and EtOAc (150 ml). The EtOAc phase was washed with 0.5 M K_2HPO_4 (150 ml), evaporated to dryness and then partitioned between hexane (50 ml) and MeOH–H_2O (4:1, v/v, 50 ml). The hexane phase was further partitioned against MeOH–H_2O (4:1, v/v, 50 ml). The MeOH–H_2O phases were then combined, diluted 2-fold with H_2O and partitioned two times against hexane (200 ml). The hexane extracts were subjected to further analysis. Typically, the hexane extract of Arabidopsis inflorescence (110 g) was absorbed onto Celite 1 g, then placed on a column of silica gel (Wakogel C-300, 3.8 g). Elution was carried out sequentially with 76 ml volumes each of hexane and hexane–EtOAc mixtures (9:1, 8:2, 7:3, 6:4, 3:7). Progesterone **1** was eluted in an 8:2 mixture of hexane–EtOAc. The eluate was evaporated to dryness, dissolved in MeOH and passed through a small column of octadecylsilica (76 mg) and diethylaminosilica (76 mg). Eluates with MeOH 5 ml were evaporated to dryness and purified by HPLC using a SenshuPak ODS-3251-D column (8 × 250 mm; Senshu Scientific Co., Japan) at 40 °C. Elution was at the flow rate of 2.5 ml min^{-1} using a gradient system: 0–5 min, MeOH–H_2O (1:1 v/v); 5–30 min, MeOH–H_2O (1:1, v/v) to 100% pure MeOH. Progesterone **1** had a 26–28 min R_t.

4.3. Quantitative analysis of progesterone *1*

GC–MS was conducted using a JEOL JMS-AX505W spectrometer (EI, 70 eV) equipped with a DB-5 capillary column (15 m × 0.25 mm; 0.25 μm film thickness, Agilent Technologies, USA). The temp. of the inlet, interface and ionization chamber was 280 °C , 280 °C and 200 °C , respectively. The column oven temp. was first maintained at 170 °C (1.5 min), elevated to 280 °C at a 37 °C min^{-1} gradient, then to 300 °C at a 1.5 °C min^{-1}gradient. The amount of progesterone **1** was calculated from the area

of M^+ ion peak using a calibration curve constructed with authentic progesterone **1**. To determine its recovery rates of **1**, 10 μg of progesterone **1** was subjected to the analytical procedures used in this work. Three independent experiments resulted in 70%, 76% and 90% recoveries of progesterone **1**.

4.4. Administration of progesterone *1* to plants

Pea seeds of wild type (cv. Torsdag) and the mutants *lh*, *ls*, *le*, *lka* and *lkb* were originally supplied from James B. Reid, Tasmania University. Seeds of the Arabidopsis *ga3* mutant were originally derived from Arabidopsis Biological Resource Center at Ohio State. Pea seedlings were grown and treated as described by Nomura et al. (1997). Progesterone **1** dissolved in EtOH (5 μl) containing 0.05% Tween 20 was applied to the 4th internodes of 8-day-old seedlings. Arabidopsis seeds were sown on half-strength Murashige–Skoog media containing 0.8% agar, 1.5% sucrose and a certain amount of progesterone **1**. After a two-day cold treatment at 4 °C, seedlings were grown at 22 °C under continuous dim light (13 $\mu mol\, m^{-2}\, s^{-1}$) for 10 days or in the dark for 4 days. Rice seedlings were grown either in H_2O or H_2O containing progesterone **1** in a dark growth chamber at 30 °C. In one experiment, 10 plants (pea) and 30 plants (Arabidopsis) were examined and three replicates were done. One typical experiment was included in Fig. 3.

4.5. Isolation of cDNA encoding putative progesterone-binding proteins from Arabidopsis and rice

Total RNA was extracted from shoots of 29-day-old Arabidopsis and 8-day-old rice by using RNeasy Plant Mini Kit with RNase-Free DNase Set (QIAGEN). A first-strand cDNA was synthesized by M-MLV Reverse Transcriptase (Invitrogen) using Oligo-d(T) primer at 37 °C. The full-length cDNAs coding putative progesterone-binding proteins of Arabidopsis and rice were PCR amplified by Expand High Fidelity PCR System (Roche) using the following primers: *MSBP1*, CACACTACTGATCTTGAGAATCC and CTGGTCTCTATGACAAATCAGAG; *MSBP2*, TCGCTTCTCGATAGCTTCTC and CTGTGCCTCTCATTACATTGAC; *SBP*, TTCCAGAAACAATTGATCAGAG and TGCAATCTCAGAAGAGAGACC; Os*MSBP1*, CGAGAGGTGTGTTTGGTAAG and CGACAGCAGTAATGCAGTATC; *OsMSBP2*, CGAAGCTGTGAAAAGACCAG and ATAAGCACTAGCACCTCCAG; *OsSBP*, CGAGGAGAGAGAAAGAGAGAG and CACTGACACACAGGGAGAAG. The cDNAs of *OsMSBP1* and *OsMSBP2* were amplified in H_2O-DMSO (95:5, v/v) because of their high GC content. The amplified cDNA was integrated into pCR2.1-TOPO vector using TOPO TA Cloning Kit (Invitrogen) and sequenced by Long-Read Tower Sequencer (Amersham). Sequence analysis was performed using MacVector software (Oxford Molecular, Oxford, UK). The phylogenetic tree was constructed by using a ClustalW program in the DDBJ homepage (http://www.ddbj.nig.ac.jp/Welcome.html).

4.6. Northern blot analysis

Total RNA was extracted from plant tissues by using RNeasy Plant Mini Kit (QIAGEN), and a portion (0.5 μg) was subjected to electrophoresis in a 1% agarose-0.41 M formaldehyde gel. RNA blot analyses were performed with a DIG-labeled RNA probe containing the ORF regions of the genes to be examined as described by Nomura et al. (2003).

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