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Viewpoint

Occurrence of brassinosteroids in non-flowering land plants, liverwort, moss, lycophyte and fern

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ABSTRACT

Endogenous brassinosteroids (BRs) in non-flowering land plants were analyzed. BRs were found in a liverwort (*Marchantia polymorpha*), a moss (*Physcomitrella patens*), lycophytes (*Selaginella moellendorffii* and *S. uncinata*) and 13 fern species. A biologically active BR, castasterone (CS), was identified in most of these non-flowering plants but another biologically active BR, brassinolide, was not. It may be distinctive that levels of CS in non-flowering plants were orders of magnitude lower than those in flowering plants. 22-Hydroxycampesterol and its metabolites were identified in most of the non-flowering plants suggesting that the biosynthesis of BRs via 22-hydroxylation of campesterol occurs as in flowering plants. Phylogenetic analyses indicated that *M. polymorpha*, *P. patens* and *S. moellendorffii* have cytochrome P450s in the CYP85 clans which harbors BR biosynthesis enzymes, although the P450 profiles are simpler as compared with Arabidopsis and rice. Furthermore, these basal land plants were found to have multiple P450s in the CYP72 clan which harbors enzymes to catabolize BRs. These findings indicate that green plants were able to synthesize and inactivate BRs from the land-transition stage.

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1. Introduction

Brassinosteroids (BRs) are steroidal hormones involved in the growth and development of plants (Clouse and Sasse, 1998). Physiological roles of BRs have been investigated intensively in flowering plants such as Arabidopsis, rice, pea and tomato. However, there is only limited knowledge on the roles of BRs in non-flowering plants. Information on the occurrence of endogenous BRs in such plants, may provide important clues on evolutional aspects and physiological functions of BRs.

It has been demonstrated that the biosynthesis of BRs begins with C-22 hydroxylation of campestanol (**6**) in Arabidopsis (Fujioka and Yokota, 2003) (Fig. 1). Later, Fujita et al. (2006) and Ohnishi et al. (2012) showed that campesterol (CR) (**1**) is also hydroxylated more efficiently than campestanol (**6**) at C-22 by CYP90B1. The route via 22-hydroxycampesterol (22-OH-CR) (**2**) is called the campestanol (**6**)-independent pathway (Fig. 1) and in the current study whether such a pathway also operates in non-flowering plants was investigated.

Evolutionary relationships of non-flowering and flowering plants are illustrated in Fig. 2. Bryophytes consisting of liverworts, mosses and hornworts are basal lineages of land plants, and diverged from the common ancestor of land plants more than 430 million year ago (Qiu et al., 2006). They have distinct features from other land plants, such as the dominant haploid life cycle and a lack of vascular tissues. Bryophytes, therefore, constitute a key group to understand the molecular and physiological basis of land plant evolution (Bowman et al., 2007; Qiu et al., 1998; Wickett et al., 2014). However, evolutionary relationships among liverworts, mosses and hornworts remain controversial (Wickett et al., 2014). Lycophytes are the most primitive group in extant vascular plants which appeared ~410 million years ago (Banks et al., 2011) and are sometimes called fern allies. Ferns and flowering plants diverged ~380 million years ago. In the current study, *Equisetum arvence* (horsetail) is classed as a member of the fern family, according to the proposal of Smith et al. (2006).

Complete genome sequences are available for a lycophyte *Selaginella moellendorffii* (Banks et al., 2011) and a moss *Physcomitrella patens* (Rensing et al., 2008), while genome sequencing of a liverwort *Marchantia polymorpha* is currently ongoing (http://marchantia.info/genome/index.php/Main_Page). Ferns are the second largest group of vascular plants. However, there is currently only limited knowledge of the fern genome (Li and Pryer, 2014; Sessa et al., 2014).

This paper reports on the occurrence of castasterone (CS) (**17**) and its biosynthetic intermediates in non-flowering plants including *M. polymorpha, P. patens*, two *Selaginella* species and 13 species of ferns belonging to 7 families. Phylogenetic analysis with respect to enzymes of BR biosynthesis and catabolism was also carried out.

2. Results and discussion

2.1. BRs in dicots and monocots

The occurrence of the campestanol (**6**)-independent pathway (Fig. 1) in monocots was predicted based on genetic and enzymological studies with rice (*Oryza sativa*) (Sakamoto et al., 2006). However, to-date, no chemical analysis of diagnostic intermediates, including 22-OH-CR (**2**), 22-hydroxy-5α-ergostan-3-one (22-OH-3-one) (**8**) and 3-epi-6-deoxocathasterone (3-epi-6-deoxoCT) (**9**), has been conducted with monocots. Endogenous BRs were, therefore, analyzed, including such key intermediates, in rice and maize (*Zea mays*) by GC-MS. Basically, the data obtained were comparable to those of *Arabidopsis* (Table 1), indicating that the campestanol (**6**)-independent pathway is also operative in monocots. These findings were used as a reference when considering quantitative BR data obtained with non-flowering plants.

Brassinolide (BL) (**18**) and CS (**17**) are biologically active BRs. In Arabidopsis, CYP85A1 is responsible for the conversion of 6-deoxocastasterone (6-deoxoCS) (**13**) to CS (**17**), while CYP85A2 converts 6-deoxoCS (**13**) to BL (**18**) via CS (**17**) (Fig. 1) (Ohnishi et al., 2009). However, as shown in Table 1, BL (**18**) is not present in detectable amounts in Arabidopsis seedlings. This may be because, as Bancos et al. (2006) showed, BL (**18**) accumulates to a level of 125 pg g fr. wt^{-1} 6 h after the start of light irradiation, but thereafter disappears within 6 h. On the other hand, only CYP85A1 occurs in rice which as a consequence synthesizes CS (**17**) but not BL (**18**) (Table 1) (Kim et al., 2008). Maize also has a single C-6 oxidase, Brd1, designated CYP85A1 (Makarevitch et al., 2012), in keeping with our finding that CS (**17**) but not BL (**18**) is endogenous in maize (Table 1).

2.2. Phylogenetic analysis of cytochrome P450s

Phylogenetic analysis of cytochrome P450 enzymes involved in the biosynthesis and metabolisms of BRs, as well as their precursor sterols, was conducted with respect to Arabidopsis, rice, *S. moellendorffii*, *P. patens* and *M. polymorpha* (Fig. 3).

CYP51 (sterol 14α-demethylase) and CYP710 (sterol C-22 desaturase) are paired enzymes important in sterol synthesis in planta (Nelson and Werck-Reichhart, 2011). Occurrence of these enzymes has been reported in *S. moellendorffii* (Umate, 2015) and *P. patens* (Morikawa et al., 2009; Umate, 2015). The present study showed that these P450s are also present in *M. polymorpha* (Fig. 3). In keeping with this, sterols such as cholesterol, campesterol (**1**), stigmasterol and sitosterol have been identified in *S. moellendorffii* (Chiu et al., 1988)), *P. patens* (Morikawa et al., 2009) and *M. polymorpha* (Kim et al., 2001; Matsuo et al., 1973). Occurrence of sterols in ferns has also been reported (Chiu et al., 1988).

The CYP85 clan comprises P450s involved in BR biosynthesis. In Arabidopsis and rice these P450s are functionally grouped into C-3 oxidase, C-22 hydroxylase, C-23 hydroxylase and C-6 oxidase (Fig. 3) (Mizutani and Ohta, 2010). Non-flowering plants were also found to have P450s in the CYP85 clan (Table 2). The functions of the P450s have not yet been demonstrated biochemically, but are assumed to have similar functions to those of flowering plants from the homology. Nonetheless, functional diversification of the P450s seems to be less evident in non-flowering plants (Table 2). Especially, *M. polymorpha* has only one CYP85 homolog in the CYP85 clan. This suggests that P450 enzymes involved in CYP85 clan are at the middle stages of functional development in basal land plants.

The CYP72 clan of Arabidopsis comprises CYP734A1 and CYP72C1 which are responsible for catabolism or deactivation of BRs (Fig. 3). CYP734A1 degrades CS (**17**) and BL (**18**) through C-26 hydroxylation followed by further oxidation (Neff et al., 1999; Turk et al., 2003). However, CYP72C1 was shown to catalyze hydroxylation of carbons other than C-26 or degrade precursors of CS (**17**) (Nakamura et al., 2005; Takahashi et al., 2005; Turk et al., 2005). In rice, three P450s, CYP734A2, CYP734A4 and CYP734A6 with high homologies to Arabidopsis CYP734A1 (Sakamoto et al., 2011) are responsible for C-26 hydroxylation. The present study demonstrated that *M. polymorpha*, *P. patens and S. moellendorffii* have multiple P450 enzymes in the CYP72 clan (Fig. 3). It has been shown that *M. polymorpha* converts BL (**18**) to 26-nor-BL and this conversion should accompany 26-hydroxylation of BL (**18**) (Kim et al., 2000). Altogether it is suggested that these ancestral plants are able to deactivate BRs.

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