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The Arabidopsis *deetiolated2* Mutant Is Blocked Early in Brassinosteroid Biosynthesis

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The Arabidopsis *DEETIOLATED2* (*DET2*) gene has been cloned and shown to encode a protein that shares significant sequence identity with mammalian steroid 5α-reductases. Loss of *DET2* function causes many defects in Arabidopsis development that can be rescued by the application of brassinolide; therefore, we propose that *DET2* encodes a reductase that acts at the first step of the proposed biosynthetic pathway—in the conversion of campesterol to campestanol. Here, we used biochemical measurements and biological assays to determine the precise biochemical defect in *det2* mutants. We show that DET2 actually acts at the second step in brassinolide biosynthesis in the 5α-reduction of (24*R*)-24-methylcholest-4-en-3-one, which is further modified to form campestanol. In feeding experiments using $^{2}H_{6}$-labeled campesterol, no significant level of $^{2}H_{6}$-labeled campestanol was detected in *det2*, whereas the wild type accumulated substantial levels. Using gas chromatography–selected ion monitoring analysis, we show that several presumed null alleles of *det2* accumulated only 8 to 15% of the wild-type levels of campestanol. Moreover, in *det2* mutants, the endogenous levels of (24*R*)-24-methylcholest-4-en-3-one increased by threefold, whereas the levels of all other measured brassinosteroids accumulated to <10% of wild-type levels. Exogenously applied biosynthetic intermediates of brassinolide were found to rescue both the dark- and light-grown defects of *det2* mutants. Together, these results refine the original proposed pathway for brassinolide and indicate that mutations in *DET2* block the second step in brassinosteroid biosynthesis. These results reinforce the utility of combining genetic and biochemical analyses to studies of biosynthetic pathways and strengthen the argument that brassinosteroids play an essential role in Arabidopsis development.

INTRODUCTION

Since the discovery of brassinolide (Grove et al., 1979), >40 natural analogs, called collectively brassinosteroids (BRs), have been isolated and characterized (reviewed in Fujioka and Sakurai, 1997). BRs are ubiquitously distributed in the plant kingdom, and when applied exogenously at nanomolar to micromolar levels, they exhibit a wide spectrum of physiological effects, including promotion of cell elongation and division, enhancement of tracheary element differentiation, retardation of abscission, enhancement of gravitropic-induced bending, promotion of ethylene biosynthesis, and enhancement of stress resistance. Although it has been proposed that BRs be considered a new class of plant hormones (Yokota and Takahashi, 1986; Sasse, 1991; Sakurai and Fujioka, 1993; Clouse, 1996), their specific physiological functions and essential roles in plant growth have remained obscure. In addition, the biosynthesis and metabolism of BRs in plants have not been completely elucidated.

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Using cultured cells and seedlings of *Catharanthus roseus*, we have proposed two alternative biosynthetic pathways of brassinolide (Figure 1). One is an "early C6 oxidation pathway," in which oxidation at C6 occurs before the introduction of vicinal hydroxyls at C22 and C23 of the side chain, as shown in Figure 1 (Yokota et al., 1990; Suzuki et al., 1993, 1994a, 1994b, 1995a, 1995b; Fujioka et al., 1995a, 1995b). The second is a "late C6 oxidation pathway," in which C6 is oxidized after the introduction of hydroxyls at the side chain and C2 of the A ring (Choi et al., 1996, 1997). The occurrence of intermediates from both the early (6-oxo) and late (6-deoxo) pathways has been shown in a variety of plants, suggesting wide operation of either or both pathways (reviewed in Sakurai and Fujioka, 1997). Although both early and late C6 oxidative intermediates exist in a variety of plants, it remains unclear whether both of these pathways should be considered biosynthetic pathways or whether the 6-deoxo intermediates are breakdown products of brassinolide metabolism.

During the course of studies on light-controlled developmental pathways, a number of Arabidopsis mutants, known

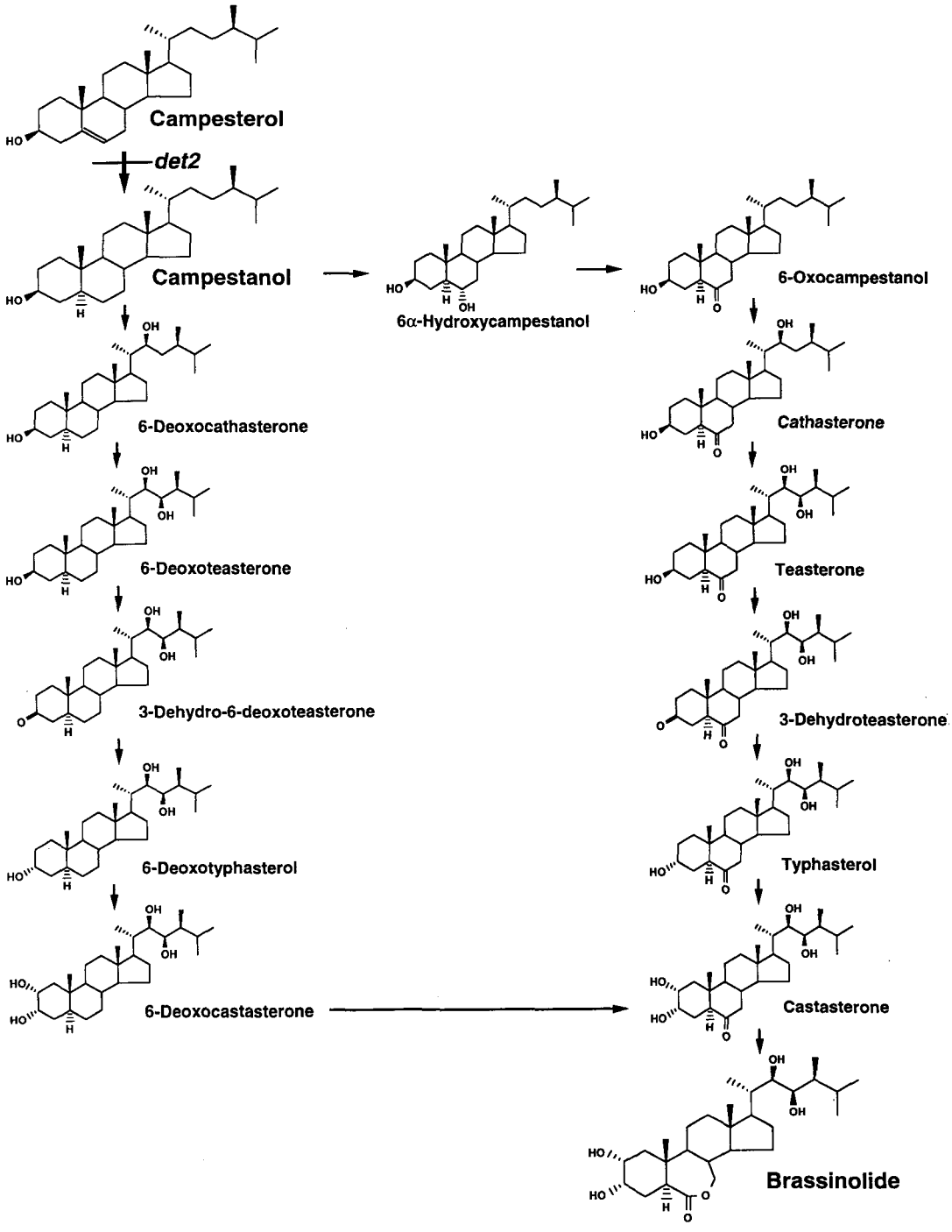

Figure 1. Proposed Biosynthetic Pathways of Brassinolide.

Most of the biosynthetic steps shown here were established by feeding experiments using cultured cells of *C. roseus*.

as *deetiolated* (*det*; Chory et al., 1989, 1991), *constitutive photomorphogenic* (*cop*; Deng et al., 1994), *fusca* (*fus*; Castle and Meinke, 1994; Misera et al., 1994), *diminuto* (*dim*; Takahashi et al., 1995), *constitutive photomorphogenic and dwarf* (*cpd*; Szekeres et al., 1996), and *cabbage* (*cbb*; Kauschmann et al., 1996), have been identified. They develop as light-grown plants when grown in the dark. One such mutant, *det2*, has many characteristics of light-grown plants when grown in the dark, including inhibition of hypocotyl growth, expansion of cotyledons, development of primary leaf buds, accumulation of anthocyanin, and derepression of light-regulated gene expression (Chory et al., 1991). When grown in the light, *det2* is a dwarf, having dark green leaves, reduced male fertility and apical dominance, and delayed senescence and flowering (Chory et al., 1991, 1994).

The *DET2* gene has been cloned and encodes a protein that is predicted to share significant sequence identity with mammalian steroid 5α-reductases (Li et al., 1996). Loss of *DET2* function leads to defects in light-regulated development that can be ameliorated by the application of brassinolide (Li et al., 1996). Using a human kidney cell culture system, we recently showed that DET2 catalyzes the reduction of several animal steroids in vitro (Li et al., 1997). In experiments conducted simultaneously, we also showed that the human steroid reductases can rescue *det2* mutant phenotypes (Li et al., 1997). These results suggest that DET2 is indeed a steroid 5α-reductase with biochemical properties similar to human enzymes. These findings strongly support the original proposal that *DET2* encodes a steroid 5α-reductase involved in BR biosynthesis.

Determination of the precise role that *DET2* plays in BR biosynthesis is essential for elucidating the physiological roles of brassinolide in the growth and development of plants as well as for molecularly characterizing BR biosynthesis. Perusal of the proposed biosynthetic pathway from *C. roseus* suggests that DET2 acts at the first step of BR biosynthesis in the formation of campestanol from campesterol. This is the only step in the proposed BR pathway that would involve a reduction, as opposed to an oxidative conversion. Arabidopsis appears to use the same biosynthetic intermediates as *C. roseus* in the synthesis of brassinolide, because our previous studies investigating endogenous BRs in Arabidopsis indicate that castasterone, typhasterol, 6-deoxocastasterone, and 6-deoxotyphasterol accumulate to detectable levels in Arabidopsis shoots (Fujioka et al., 1996). Here, we undertook quantitative analyses of endogenous sterols and BRs in the wild type and *det2* mutants of Arabidopsis. We also used wild-type and *det2* seedlings to examine the metabolic conversion of campesterol to campestanol directly and undertook *det2* rescue experiments using all of the proposed biosynthetic intermediates of brassinolide. The results show clearly that *det2* mutants are blocked at an early step of BR biosynthesis and that campestanol is converted to brassinolide via branched, parallel biosynthetic pathways that involve both 6-deoxoBRs and 6-oxoBRs. Moreover, the detailed analyses presented here suggest that at least one additional enzyme is involved in the early steps of BR biosynthesis.

RESULTS

Metabolism of $^2H_6$-Labeled Campesterol in Wild-Type and *det2* Seedlings

The only step in the proposed BR biosynthetic pathway that might be catalyzed by a steroid reductase is the initial step, that is, the reduction of campesterol to campestanol (Figure 1). Metabolism of campesterol to campestanol in the wild type and *det2* was examined using aseptically grown seedlings. Wild-type and *det2* seedlings were fed $^2H_6$-labeled campesterol and incubated for 4 days. The campestanol fraction obtained from wild-type seedlings was analyzed by using gas chromatography–mass spectrometry (GC-MS) after conversion to the trimethylsilyl derivative. In the mass spectrum obtained from the metabolites, ions due to $^2H_6$-labeled campestanol were clearly observed at an *m/z* of 480 (M^+), 465, 423, 390, and 375 along with endogenous campestanol (*m/z* of 474 [M^+], 459, 417, 384, and 369) (Figure 2A). Figures 2B and 2C show mass chromatograms of monitoring ions at an *m/z* of 480 (M^+) and 465 ($M^+ - 15$). In the wild type, both ions were clearly observed (Figure 2B), whereas no peak was found in *det2* (Figure 2C). The experiments were repeated three times, with similar results. In all experiments, the wild type accumulated substantial levels of $^2H_6$-labeled campestanol, whereas no or trace amounts of $^2H_6$-labeled campestanol were detected in *det2*. Based on the detection limits of this type of experiment, these results indicate that the *det2* mutation reduces the ability of Arabidopsis to convert campesterol to campestanol by at least an order of magnitude (S. Fujioka, unpublished data).

Quantitative Analyses Indicate That *det2* Mutants Are Blocked in the Formation of Campestanol from (24*R*)-24-Methylcholest-4-en-3-one

To ascertain endogenous sterols in Arabidopsis, we analyzed sterols in the seedlings of the wild type and *det2* mutants. Eight sterols—sitosterol, campesterol, cholesterol, stigmasterol, brassicasterol, isofucosterol, fucosterol, and sitostanol—were identified in Arabidopsis seedlings by using full-scan GC-MS analysis. In both the wild type and *det2*, sitosterol was the most abundant sterol, accounting for ~50% of the total sterols. Campesterol comprised 20% of the total sterols, and cholesterol was the third most abundant sterol, accumulating to ~10% of the total (data not shown). These results are in agreement with previously reported data (Patterson et al., 1993).

We measured quantitatively the endogenous levels of both campesterol and campestanol in wild-type and *det2*

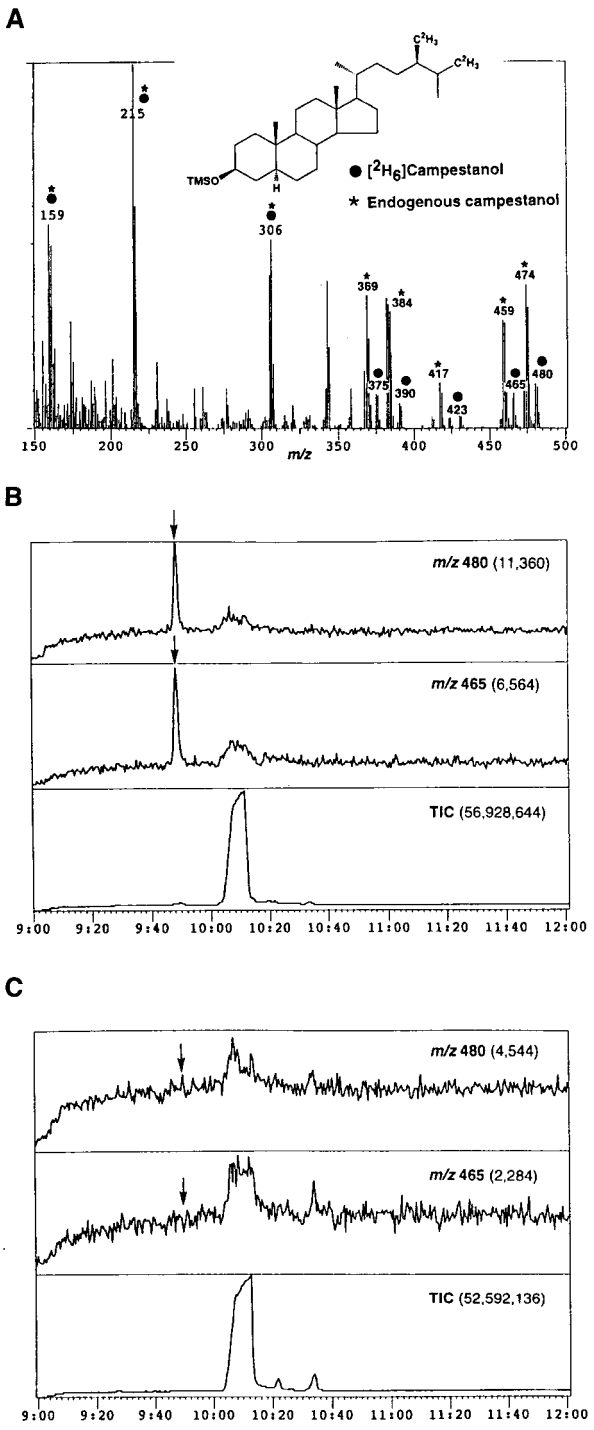

Figure 2. Identification of $^2H_6$-Labeled Campestanol Converted from $^2H_6$-Labeled Campesterol.

(A) The mass spectrum of $^2H_6$-labeled campestanol obtained by feeding $^2H_6$-labeled campesterol in the wild type. The dots indicate the ions derived from $^2H_6$-labeled campestanol; the asterisks indicate ions derived from endogenous campestanol.

seedlings by using GC–selected ion monitoring (GC-SIM) with a deuterium-labeled internal standard. As indicated in Table 1, this more sensitive GC-SIM analysis revealed that *det2-1* actually accumulated ~10% of the wild-type levels of campestanol, although the full-scan GC-MS analysis failed to detect its presence in *det2* mutants. Because the molecular basis of *det2-1* is a single nucleotide change, resulting in the nonconservative substitution of a glutamate by a lysine, the 10% level of campestanol detected in *det2-1* mutants could be accounted for by the low activity of the mutated DET2 protein or by the existence of an alternative pathway. We then measured campestanol accumulation in two other null alleles of *det2—det2-2* (containing a nonsense mutation at codon 53) and *det2-3* (containing a seven-nucleotide deletion). As shown in Table 1, both of these presumed null mutants accumulated a level of campestanol similar to that of the *det2-1* mutants, suggesting that an alternative pathway or a second steroid 5α-reductase operates in the synthesis of minor amounts of campestanol in all three *det2* mutants analyzed. Indeed, low-stringency hybridization blots suggest the possibility of a second *DET2*-like gene (J. Li and J. Chory, unpublished data).

If campesterol is a direct substrate of DET2, then its levels should accumulate in *det2* mutants. Unexpectedly, we observed a reduction in campesterol levels in the *det2* mutants to that of 33 to 45% of wild-type levels (Table 1). Together with the data presented by Li et al. (1997) for the substrate specificity of recombinant DET2, our data suggest that campestanol is not formed directly from campesterol. We then looked for the presence of a 3-oxo,$\Delta^{4,5}$ steroid as a possible intermediate in the formation of campestanol from campesterol. As a result, (24*R*)-24-methylcholest-4-en-3-one (a 3-oxo,$\Delta^{4,5}$ steroid) was identified by full-scan GC-MS analysis (*m/z* 398 [M^+, 48], 383 [15], 356 [25], 341 [9], 313 [14], 275 [44], 229 [100], 187 [23], and 159 [19]). The quantitative levels of (24*R*)-24-methylcholest-4-en-3-one were analyzed in the wild type and *det2* by using GC-SIM with a deuterium-labeled internal standard. In the wild type, its level was 0.39 μg/g fresh weight of tissue, whereas the *det2* mutant accumulated 1.4 μg/g fresh weight of tissue. The accumulation of this 3-oxo,$\Delta^{4,5}$ steroid rather than campesterol in *det2* suggests that this steroid is the substrate for the DET2 reductase.

(B) Mass chromatogram of the ions of $^2H_6$-labeled campestanol in the metabolites of the wild type. TIC, total ion chromatogram.

(C) Mass chromatogram of the ions of $^2H_6$-labeled campestanol in the metabolites of *det2*.

The arrows in **(B)** and **(C)** indicate the peak of $^2H_6$-labeled campestanol. The values on the x-axes in **(B)** and **(C)** are the retention times, where 9:20 indicates 9 min and 20 sec. The numeric designations in parentheses are arbitrary units.

Table 1. Campesterol and Campestanol Content in Wild-Type and *det2* Seedlings

Tissue	Campesterol[a]	Campestanol[b]
Wild type	34.0	1170
det2-1	18.6	132
det2-2	19.5	97
det2-3	22.9	180

[a] Campesterol content is expressed as micrograms per gram fresh weight of tissue.
[b] Campestanol content is expressed as nanograms per gram fresh weight of tissue.

Quantification of BRs in the Shoots of the Wild Type and *det2*

We have previously identified castasterone, 6-deoxocastasterone, typhasterol, and 6-deoxotyphasterol in the shoots of wild-type Arabidopsis plants by using GC-MS (Fujioka et al., 1996; see Figure 1). To confirm that *det2* is deficient in BRs, endogenous levels of these BRs from wild-type and *det2* shoots were determined by using GC-SIM with internal standards. Castasterone and 6-deoxocastasterone were detected in *det2*, but their levels in *det2* were <10% of those in the wild type (Table 2). Moreover, typhasterol and 6-deoxotyphasterol found in the wild type were not detected in *det2*, indicating that their levels were <0.05 ng/g fresh weight of tissue.

Brassinolide and Its Biosynthetic Precursors Rescue *det2* Mutant Phenotypes

The BR biosynthetic pathway shown in Figure 1 has been proposed based on feeding experiments and the natural occurrence of various intermediates in both cultured cells and seedlings of *C. roseus* as well as in several other plant systems (Park et al., 1989; Yokota et al., 1990; Choi et al., 1993, 1996, 1997; Suzuki et al., 1993, 1994a, 1994b, 1995a, 1995b; Fujioka et al., 1995a, 1995b, 1996); however, there is no genetic evidence for such a pathway in any plant. With the biochemical basis of the *det2* mutation determined to be at an initial step in the proposed pathway, we could easily test whether this pathway is indeed responsible for brassinolide biosynthesis in Arabidopsis. If this hypothetical pathway actually operates in Arabidopsis, we would expect that not only brassinolide but also biosynthetic intermediates beyond campestanol would rescue *det2* mutants to a wild-type phenotype.

Table 2. BR Content in Wild-Type and *det2* Shoots[a]

BRs	Wild Type	*det2*
Castasterone	0.75	0.06
6-Deoxocastasterone	0.71	0.07
Typhasterol	0.11	ND[b]
6-Deoxotyphasterol	0.95	ND

[a] BR content is expressed as nanograms per gram fresh weight of tissue.
[b] ND, not detected.

To test this idea, we first examined the effect of these compounds on hypocotyl elongation of etiolated seedlings of both the wild type and *det2*. As shown in Figure 3A, 1 μM BRs, including cathasterone, teasterone, 3-dehydroteasterone, typhasterol, castasterone, and brassinolide, had little or no effect on wild-type seedlings; however, this concentration of BRs was effective in rescuing the defective hypocotyl growth of dark-grown *det2* mutants. The hypocotyl elongation of *det2* mutants was increased by 2.5- to fourfold by these BRs (Figure 3B). Consistent with earlier results (Fujioka et al., 1995b), biological activities of these BRs tended to increase with their order in the hypothesized biosynthetic pathway (Figures 3A and 3B). When applied at higher concentrations, 10 μM cathasterone was equivalent to 1 μM brassinolide (Figure 3C), implying that there is no major rate-limiting step between cathasterone and brassinolide in the dark.

The three intermediates after the *det2* block—campestanol, 6α-hydroxycampestanol, and 6-oxocampestanol—showed virtually no activity in rescuing the *det2* phenotype, even at 10 μM (Figure 3C). Such an observation was not surprising, however, because it has been reported that the biological activity of 6-oxocampestanol is only one-five hundredth that of cathasterone (Fujioka et al., 1995b) and that the endogenous level of 6-oxocampestanol was at least 500-fold higher than that of cathasterone in cell cultures of *C. roseus* (Fujioka et al., 1995a). This suggests that the introduction of a hydroxyl group in the side chain is a rate-limiting step in the BR biosynthetic pathway. Therefore, a 500 μM concentration of 6-oxocampestanol and an even higher concentration of either campestanol or 6α-hydroxycampestanol are necessary to have a biological effect on hypocotyl growth of *det2* seedlings similar to that of 1 μM cathasterone. In fact, 50 μM 6α-hydroxycampestanol or 6-oxocampestanol had substantial biological activity, although we were unable to test their activities at higher concentrations because of poor solubility of the intermediates (data not shown).

Similar results were obtained when these compounds were tested in the suppression of the light phenotypes of *det2* mutants, as shown in Figure 4. Whereas 1 μM campestanol or 6α-hydroxycampestanol had little or no effect on *det2* mutants, 0.05 μM brassinolide was sufficient to rescue the *det2* mutant phenotype. A 1 μM concentration of various intermediates in the middle of the pathway could rescue *det2* defects to a lesser degree. It is interesting that whereas 1 μM 6-oxocampestanol had no activity in rescuing the hypocotyl growth of *det2* mutants in the dark, it had some biological activity in light-grown *det2* plants.

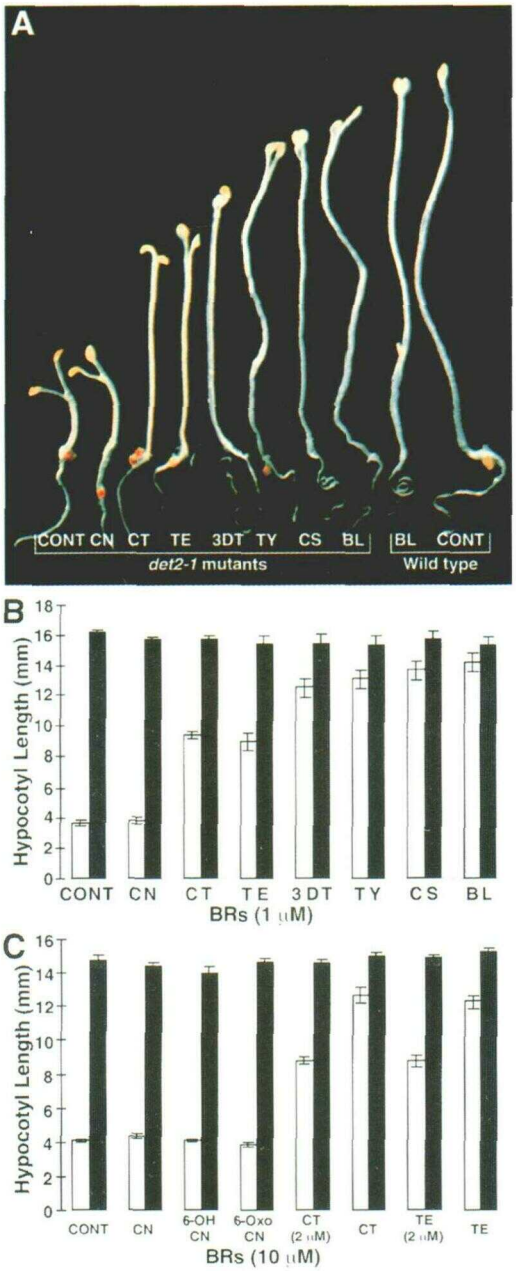

Figure 3. Effect of BRs on the Hypocotyl Growth of Dark-Grown *det2-1* Mutants.

(A) Dark-grown 10-day-old seedlings treated with or without 1 μM 6-oxoBRs.

(B) Hypocotyl elongation of dark-grown 10-day-old *det2-1* seedlings induced by 1 μM 6-oxoBR.

(C) Hypocotyl elongation of dark-grown 7-day-old *det2-1* seedlings induced by 6-oxoBR.

The concentration of BRs used for **(C)** is 10 μM, except where indicated on the x-axis. Data in **(B)** and **(C)** are the mean ± SE obtained from a sample size of 20 seedlings; black bars represent the wild type, and white bars represent *det2-1*. CONT, control; CN, campestanol; 6-OH CN, 6α-hydroxycampestanol; 6-Oxo CN, 6-oxocampestanol; CT, cathasterone; TE, teasterone; 3DT, 3-dehydroteasterone; TY, typhasterol; CS, castasterone; BL, brassinolide.

Recently, Choi et al. (1996, 1997) proposed an alternative pathway for the biosynthesis of brassinolide, namely, the late C6 oxidation pathway in which C6 is oxidized after the introduction of hydroxyls at the side chain and C2 of the A ring. Previously, 6-deoxoBRs were considered to be dead-end products rather than precursors in the biosynthesis of brassinolide. Both 6-oxoBRs and 6-deoxoBRs were detected in Arabidopsis shoots (Fujioka et al., 1996). If two pathways are operating in Arabidopsis, we would expect that these 6-deoxoBRs are also capable of rescuing the *det2* mutant phenotype because the two pathways are proposed to branch after DET2 action. To test this idea, similar studies with these 6-deoxoBRs were performed. As shown in Figures 5A and 5C, these compounds had little or no effect on wild-type seedlings but were quite active in the dark in rescuing the defective hypocotyl growth of *det2* mutants. They are, however, slightly less active than their corresponding 6-oxidized forms. When tested in the light, 1 μM 6-deoxoBRs was sufficient to restore a wild-type phenotype to *det2* mutants (Figure 5B). Interestingly, these 6-deoxoBRs showed stronger activity than did their corresponding 6-oxoBRs in the light.

DISCUSSION

The recent description of a number of different Arabidopsis BR biosynthetic mutants has pointed to a pivotal role for BRs in plant growth and development (reviewed in Clouse, 1996). Here, we present biochemical and physiological data that the Arabidopsis DET2 steroid 5α-reductase acts early in the proposed BR biosynthetic pathway in the formation of campestanol from (24*R*)-24-methylcholest-4-en-3-one (Figure 6). We could not detect significant formation of $^2H_6$-labeled campestanol from $^2H_6$-labeled campesterol in feeding experiments with *det2* mutants, whereas the formation of $^2H_6$-labeled campestanol from $^2H_6$-labeled campesterol was clearly observed in the wild type. Moreover, *det2* mutants accumulate ~10% of wild-type levels of campestanol and other brassinolide biosynthetic intermediates. *det2* mutants are also rescued to wild-type stature by the proposed intermediate and late biosynthetic intermediates, suggesting that the pleiotropic phenotypes of *det2* mutants are caused by a 10-fold reduction of BRs in this mutant. Recently, Nomura et al. (1997) reported that the dwarf mutant (*lkb*) of pea is BR deficient. The endogenous levels of BRs in *lkb* are reduced by nine- to 23-fold from that of the wild type. *lkb* may be blocked in the biosynthetic pathway before the formation of

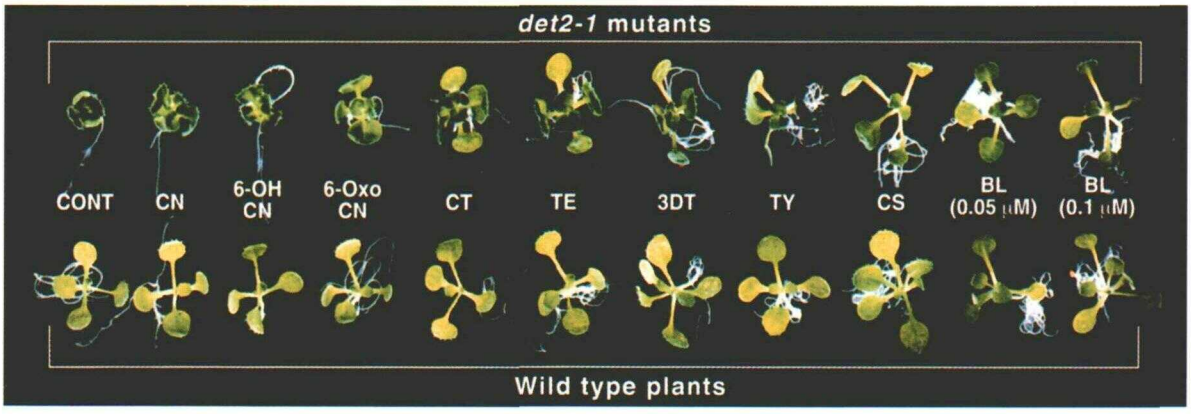

Figure 4. Rescue of Light-Grown Phenotypes of *det2-1* Seedlings with 6-OxoBR.

Shown are 14-day-old light-grown *det2-1* and wild-type plants treated with or without 6-oxoBRs. Abbreviations are as given in the legend to Figure 3.

teasterone. To date, there has been no direct genetic evidence for the existence of the proposed biosynthetic pathways shown in Figure 1. The detection of some of these intermediates in wild-type Arabidopsis and pea and the correlated reduction of these intermediates in the *det2* and *lkb* mutants now provide firm biochemical evidence for the existence of such a pathway in these two plants.

Although the data that we have accumulated indicate that DET2 acts at the primary step in the previously proposed pathway, the data point to the fact that the proposed BR biosynthetic pathway is missing at least one key intermediate at the primary step. If the proposed reaction for DET2 were correct, one might expect to observe an increased accumulation of the substrate, campesterol, in the *det2* mutants. However, this was not the case, suggesting that an additional intermediate exists between campesterol and campestanol. This proposed intermediate was predicted to have a 3-oxo,$\Delta^{4,5}$ structure, as opposed to the 3β-hydroxyl,$\Delta^{5,6}$ structure of campesterol. In animals, steroid 5α-reductases are known to be inactive toward 3β-hydroxyl,$\Delta^{5,6}$ steroids (Hsia and Voigt, 1974; Russell and Wilson, 1994). We have recently shown that both the type 1 and type 2 human 5α-reductases can rescue the *det2-1* mutation and that recombinant DET2 cannot recognize 3β-hydroxyl,$\Delta^{5,6}$ steroids in vitro (Li et al., 1997). This study shows that the 3-oxo,$\Delta^{4,5}$ intermediate ([24*R*]-24-methylcholest-4-en-3-one) accumulated in *det2* mutants as a direct substrate of DET2, suggesting the existence of three enzymatic steps between campesterol and campestanol in Arabidopsis (Figure 6). Consequently, at least one other enzyme, the plant ortholog of the mammalian 3β-hydroxysteroid dehydrogenase/$\Delta^{5,6}$-$\Delta^{4,5}$ isomerase that catalyzes the oxidation and isomerization of 3β-hydroxyl,$\Delta^{5,6}$ precursors to 3-oxo,$\Delta^{4,5}$ steroids (Lachance et al., 1990), must exist in Arabidopsis. We are currently attempting to detect the other proposed intermediates in the formation of campestanol from campesterol and their metabolic conversions.

Quantitative analysis of campestanol in three presumed null alleles of *det2* indicates that these mutants still accumulate between 8 and 15% of the wild-type levels of campestanol. This suggests that there is a second steroid 5α-reductase in Arabidopsis that plays a minor role in BR biosynthesis. Monkeys, rats, and humans each contain two steroid 5α-reductase isoforms, called type 1 and type 2 (reviewed in Russell and Wilson, 1994). In mammals, the genes for these two reductases are differentially expressed, and the enzyme is found in different subcellular membrane fractions (Russell and Wilson, 1994). Low-stringency hybridization gel blots using *DET2* as a probe for Arabidopsis genomic DNA suggest that there is a second homologous gene present in the Arabidopsis genome (J. Li and J. Chory, unpublished data). It is interesting that there are duplicated genes for tryptophan biosynthesis and nitrate reductases in Arabidopsis (Last et al., 1991; Last, 1993; Wilkinson and Crawford, 1993). In both cases, one of the two isoforms is responsible for >90% of the total activity in vivo, whereas the second isoform plays a minor role. Further molecular and genetic studies should aid in the identification of the second, presumably less abundant steroid 5α-reductase.

The existence of the *det2* mutant allowed us to test the possibility that brassinolide is not synthesized via a simple linear biosynthetic pathway. Indeed, these studies now provide strong genetic and biochemical evidence that brassinolide is synthesized in two parallel pathways that branch after the formation of campestanol. Feeding experiments using intermediates in each of the two pathways resulted in differential growth effects between dark- and light-grown seedlings. Although a trivial explanation for this observation is

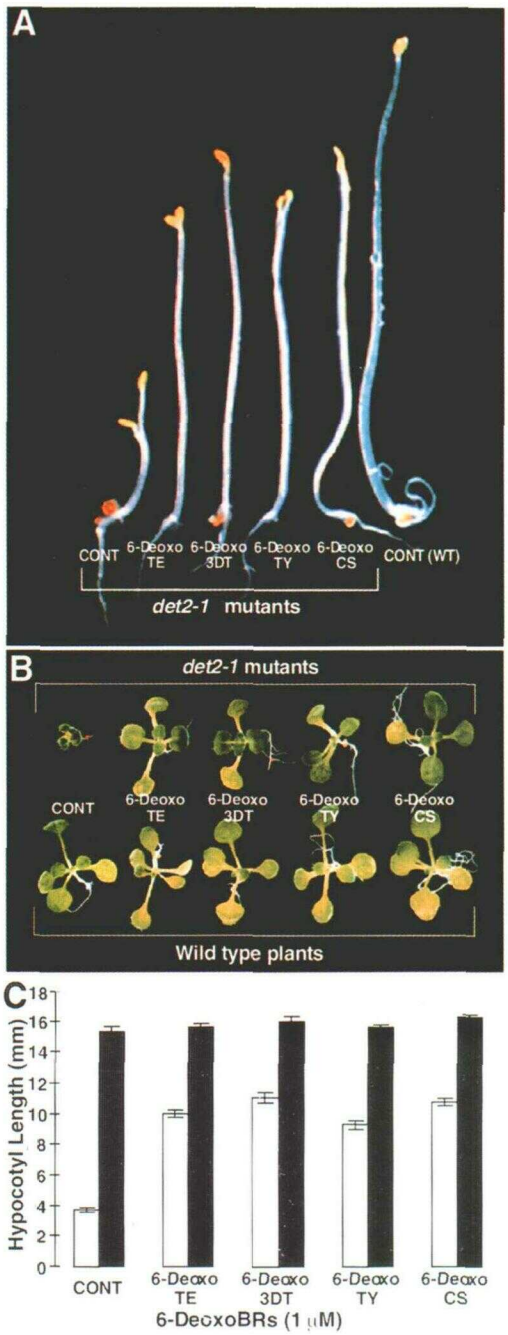

Figure 5. Complementation of *det2-1* Mutation by the Application of 6-DeoxoBRs.

(A) Ten-day-old dark-grown *det2-1* seedlings treated with various 6-deoxoBRs compared with untreated *det2-1* mutant and wild-type seedlings of the same age.

(B) Fourteen-day-old light-grown *det2-1* and wild-type seedlings.

(C) 6-DeoxoBR–induced hypocotyl elongation of dark-grown *det2-1* mutants. Data represent the means ± SE obtained from a sample size of 20 seedlings. Black bars represent the wild type, and white bars represent *det2-1*.

that the 6-oxo intermediates are less stable in the light than are the 6-deoxo intermediates, it is possible that the late oxidation pathway plays a predominant role in light-grown plants, whereas the early oxidation pathway is dominant in dark-grown seedlings. It will be interesting to determine whether the brassinolide biosynthetic pathways are regulated by light, as has been shown for gibberellin biosynthesis (Xu et al., 1995; Wu et al., 1996). Further measurments of endogenous brassinosteroid intermediates and their interconversion under dark and light conditions should clarify this hypothesis.

In summary, we have shown that the Arabidopsis *det2* mutant is blocked early in BR biosynthesis by biochemical, physiological, and molecular studies. This mutant therefore provides an important tool for investigating the physiological roles as well as biosynthetic pathways of BRs in higher plants. Future studies should help to elucidate the regulation of the biosynthesis of this important hormone by environmental conditions.

METHODS

Plant Material

deetiolated2 (*det2*) mutants were isolated originally after mutagenesis of wild-type seeds from the Columbia ecotype (Chory et al., 1991; Li et al., 1996). Three (*det2-1*, *det2-2*, and *det2-3*) of eight *det2* alleles were used for the analyses described here (Li et al., 1996). The *det2-1* allele has a nonconservative substitution of lysine for glutamate at position 204. *det2-2* causes premature termination at position 53. *det2-3* contains a frameshifting deletion at position 711 (Li et al., 1996). If not specified, it is assumed that the mutant allele number is 1 (e.g., *det2-1* is referred to as *det2*).

Authentic Sterols

Campesterol, sitosterol, and brassicasterol were obtained from Tama Biochemical Co., Ltd. (Tokyo, Japan). Stigmasterol and cholesterol were obtained from Sigma. Campestanol and sitostanol were obtained by catalytic hydrogenation (palladium–charcoal) of campesterol and sitosterol, respectively. Isofucosterol was extracted from orange (Takatsuto et al., 1992). Fucosterol was obtained from Wako Pure Chemical Co., Ltd. (Osaka, Japan). The synthesis of (24*R*)-24-methylcholest-4-en-3-one and its deuterium-labeled compound will be described elsewhere (S. Takatsuto, T. Noguchi, and S. Fujioka, unpublished data).

CONT, control; 6-Deoxo TE, 6-deoxoteasterone; 6-Deoxo 3DT, 3-dehydro-6-deoxoteasterone; 6-Deoxo TY, 6-deoxotyphasterol; 6-Deoxo CS, 6-deoxocastasterone; WT, wild type.

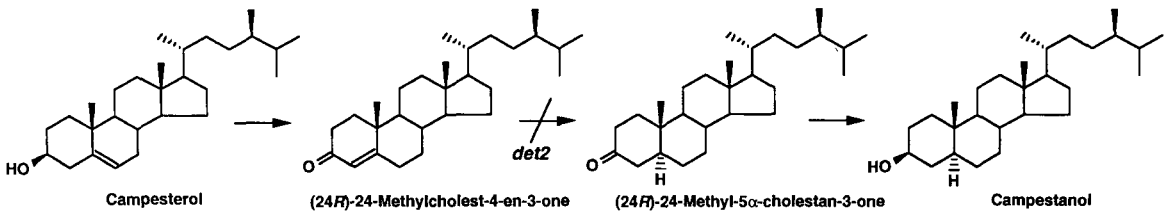

Figure 6. Modified Biosynthetic Sequence between Campesterol and Campestanol.

DET2 acts in the 5α reduction of (24*R*)-24-methylcholest-4-en-3-one.

Synthesis of Brassinosteroids

6-Deoxobrassinosteroids (6-deoxoBRs; 6-deoxoteasterone, 3-dehydro-6-deoxoteasterone, 6-deoxotyphasterol, and 6-deoxocastasterone) were synthesized from (20*S*)-20-formyl-6β-methoxy-3α,5-cyclo-5α-pregnane (Takatsuto et al., 1997). Cathasterone was synthesized from (22*R*,23*R*,24*S*)-3β-acetoxy-22,23-epoxy-5α-ergostan-6-one (Fujioka et al., 1995a). 6-Oxocampestanol and 6α-hydroxycampestanol were synthesized according to the method of Suzuki et al. (1995b). Teasterone, typhasterol, castasterone, and brassinolide were synthesized as described previously (Takatsuto et al.,1984; Takatsuto, 1986).

Synthesis of $^2H_6$-Labeled BRs

To synthesize $^2H_6$-labeled campestanol, a solution of $^2H_6$-labeled crinosterol (6 mg) in ethyl acetate (2 mL) was hydrogenated over 5% palladium–charcoal (15 mg) at room temperature at atmospheric pressure for 3 hr. After filtration to remove the catalyst, the filtrate was concentrated and subjected to column chromatography (Wakogel C-300; Wako). Elution with hexane–ethyl acetate (5:1 [v/v]) resulted in colorless crystals of $^2H_6$-labeled campestanol (5.4 mg).

To obtain purified $^2H_6$-labeled campesterol, a solution of $^2H_6$-labeled crinosterol (2.4 mg) in ethyl acetate (0.5 mL) was hydrogenated over 5% palladium–charcoal (6 mg) at atmospheric pressure at 0°C for 0.5 hr and then at room temperature for 0.5 hr. After filtration to remove the catalyst, the filtrate was concentrated and subjected to column chromatography (Wakogel C-300; Wako). Elution with hexane–ethyl acetate (5:1 [v/v]) afforded a mixture of $^2H_6$-labeled crinosterol, $^2H_6$-labeled campesterol, $^2H_6$-labeled Δ^{22}-campestanol, and $^2H_6$-labeled campestanol (total 2.2 mg) as a colorless solid, with the ratio being estimated by the ^1H-nuclear magnetic resonance spectrum 30:50:5:15. This mixture was purified by octadecylsilane (ODS)–HPLC twice to yield pure $^2H_6$-labeled campesterol (0.4 mg). Their structures and purity were confirmed rigorously by mass spectrometry and ^1H-nuclear magnetic resonance.

$^2H_6$-labeled typhasterol and $^2H_6$-labeled castasterone were synthesized according to methods previously reported (Takatsuto and Ikekawa, 1986). $^2H_6$-labeled deoxotyphasterol and $^2H_6$-labeled 6-deoxocastasterone were synthesized according to Choi et al. (1996, 1997).

Gas Chromatography–Mass Spectrometry and Gas Chromatography–Selected Ion Monitoring Analyses

Gas chromatography–mass spectrometry (GC-MS) and GC–selected ion monitoring (GC-SIM) analyses were performed on a JEOL (Akishima, Tokyo, Japan) Automass JMS-AM 150 mass spectrometer connected to a Hewlett-Packard (Wilmington, DE) 5890A-II gas chromatograph with capillary column DB-5 (0.25 mm × 15 m; 0.25-μm film thickness). The temperature and analytical conditions were the same as previously described (Suzuki et al., 1994a).

The sterol fractions were trimethylsilylated with 10 to 20 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) at 80°C for 30 min. The samples corresponding to castasterone and 6-deoxocastasterone were treated with pyridine containing methaneboronic acid (20 μg per 10 μL). The samples corresponding to 6-deoxotyphasterol and typhasterol were treated with pyridine containing methaneboronic acid (20 μg per 10 μL) at 80°C for 30 min and then with 10 μL of MSTFA at 80°C for 30 min.

Sterol Analysis

Seedlings of the wild type and *det2* were germinated and grown for 12 days on half-concentrated Murashige and Skoog medium (Murashige and Skoog, 1962) containing 0.8% agar and 1% sucrose under a 16-hr light and 8-hr-dark regime at 22°C. Wild-type (2 g fresh weight of tissue) and *det2-1* (2 g fresh weight) seedlings were collected for analysis. The two samples were extracted three times with 100 mL of MeOH–$CHCl_3$ (4:1 [v/v]). The extract (100 mg fresh weight equivalent) was partitioned between $CHCl_3$ and H_2O, and the $CHCl_3$-soluble fraction was purified on silica gel and ODS cartridges and subjected to ODS-HPLC (Suzuki et al., 1995b). The sterol fractions were subjected to GC-MS analysis after trimethylsilylation.

For the identification of (24*R*)-24-methylcholest-4-en-3-one, the shoots (10 g fresh weight) of 6-week-old greenhouse-grown *det2* plants were used. The extraction and purification were performed as described above. The (24*R*)-24-methylcholest-4-en-3-one fraction was subjected to GC-MS without derivatization.

For the quantitative analysis of endogenous campesterol and campestanol, seedlings were grown as described above. Wild-type (420 mg fresh weight), *det2-1* (630 mg fresh weight), *det2-2* (980 mg fresh weight), and *det2-3* (710 mg fresh weight) seedlings were collected and extracted with 100 mL of MeOH–$CHCl_3$ (4:1 [v/v]) twice. $^{13}C_5$-labeled campesterol (400 ng) (Suzuki et al., 1995b) and $^2H_6$-labeled campestanol (20 ng) were added to each extract (20-mg fresh weight equivalent) as internal standards. The extract was purified by silica gel and ODS cartridges and ODS-HPLC, as described above. The campesterol and campestanol fractions were subjected to GC-SIM analysis after trimethylsilylation. The endogenous levels of campesterol and campestanol were determined as the ratio of the peak areas of molecular ions for the endogenous sterol and for the internal standard.

For the quantitative analysis of endogenous (24*R*)-24-methylcholest-4-en-3-one, the 500-mg fresh weight equivalent of the extracts from the wild type (2 g fresh weight) and *det2-1* (2 g fresh weight) described above was used. $^2H_6$-labeled (24*R*)-24-methylcholest-4-en-3-one (1 μg) was added to each extract as an internal standard, and the purification was performed as described above. The (24*R*)-24-methylcholest-4-en-3-one fraction was subjected to GC-SIM analysis, and the endogenous level was determined as the ratio of the peak areas of molecular ions for the endogenous one and for the internal standard.

Metabolism of $^2H_6$-Labeled Campesterol

An MeOH solution (20 μL) of $^2H_6$-labeled campesterol (10 μg) was added to a 200-mL flask containing Arabidopsis seedlings grown in 30 mL of Murashige and Skoog medium supplemented with 3% sucrose. The seedlings were incubated for 4 days at 22°C in the light on a shaker (110 rpm). After incubation, the seedlings were extracted with MeOH, and the extract was partitioned between $CHCl_3$ and H_2O. Purification was performed under the same conditions as previously described (Suzuki et al., 1994a). The fraction corresponding to campestanol was subjected to GC-MS analysis after trimethysilylation.

Quantification of Endogenous BRs

Wild-type and *det2* plants were grown in 16-hr light and 8-hr dark conditions in a greenhouse and in 8-hr light and 16-hr dark conditions in a growth chamber, respectively. The shoots of 3-week-old plants (wild type) and 6-week-old plants (*det2*) were harvested and lyophilized immediately after the harvest. Lyophilized shoots (50-g fresh weight equivalent) were extracted with 250 mL of MeOH-$CHCl_3$ (4:1 [v/v]) twice, and deuterium-labeled internal standards (1 ng/g fresh weight) were added. The extract was partitioned between $CHCl_3$ and H_2O after evaporation of the solvent in vacuo. The $CHCl_3$-soluble fraction was subjected to silica gel chromatography (Wakogel C-300; Wako; 15 g). The column was subsequently eluted with 150 mL of $CHCl_3$, 2% MeOH in $CHCl_3$, and 7% MeOH in $CHCl_3$. Each of 2% MeOH and 7% MeOH fractions was purified by Sephadex LH-20 column chromatography (column volume of 200 mL; column eluted with MeOH–$CHCl_3$ [4:1 (v/v)]). The effluents of elution volume/total column volume (V_e/V_t) of 0.6 to 0.8 were collected as BR fractions. After prepurification on an ODS cartridge column (10 × 50 mm [inner diameter × column length]) with MeOH, the eluates were subjected to ODS–HPLC at a flow rate of 8 mL/min with the solvents of 65% acetonitrile for the eluate derived from the 2% MeOH fraction and of 80% acetonitrile for the eluate derived from the 7% MeOH fraction. HPLC purification from the 7% MeOH fraction yielded a castasterone fraction (retention times from 10 to 15 min), typhasterol fraction (retention times from 25 to 30 min), and 6-deoxocastasterone fraction (retention times from 40 to 45 min), and HPLC purification from the 2% MeOH fraction yielded a 6-deoxotyphasterol fraction (retention times from 55 to 60 min). Each fraction was derivatized and analyzed by GC-SIM. The endogenous levels of BRs were determined as the ratio of the peak areas of prominent ions for the endogenous one and for the internal standard.

Complementation of *det2* with Brassinolide Biosynthetic Intermediates

For the rescue experiments, a 1 μM concentration of the various intermediates was used, unless otherwise noted. The seedlings were germinated and grown on BR-containing Murashige and Skoog medium both in the light and dark at 22°C (25 mL per dish). For dark-grown seedlings, the hypocotyl lengths were measured, and their photographs were taken after 10 days. For light-grown seedlings, the photographs were taken after 14 days. Approximately 100 seeds were sown on 100 × 15 mm Petri dishes; from these, the hypocotyls of 20 seedlings were chosen randomly and measured.

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