Treatment of dark-grown *Arabidopsis thaliana* with a brassinosteroid-biosynthesis inhibitor, brassinazole, induces some characteristics of light-grown plants


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**Abstract.** When a brassinosteroid biosynthesis inhibitor, brassinazole (Brz), was applied at concentrations ranging from 0.1 to 2 μM, *Arabidopsis thaliana* (L.) Heynh seedlings grown in the dark exhibited morphological features of light-grown plants, i.e. short hypocotyls, expanded cotyledons, and true leaves, in a dose-dependent manner. Control (non Brz-treated) seedlings grown in the dark for 40 d did not develop leaf primordia. However, treatment with the lowest concentration of Brz induced the development of leaf buds, although it hardly induced any short hypocotyls, and treatment with the highest concentration of Brz induced both short hypocotyls and leaves. Labeling experiments with the thymidine analogue 5-bromo-2′-deoxyuridine revealed that amplification of cell nuclei and organellar nucleoids is activated in the shoot apical meristems of dark-grown Brz-treated seedlings. These results suggest that Brz-treatment induces development of true leaves. Furthermore, condensation and scattering of plastid nucleoids, which is known to occur during the differentiation of etioplasts into chloroplasts, was observed in the plastids of dark-grown Brz-treated cotyledons. In addition, high levels of ribulose-1,5-bisphosphate carboxylase-oxygenase proteins accumulated in the plastids of the cotyledons. Electron microscopy showed that the plastids were etioplasts with a prolamellar body and few thylakoid membranes. These results suggest that Brz treatment in the dark induces the initial steps of plastid differentiation, which occur prior to the development of thylakoid membranes. This is a novel presumed function of brassinosteroids. These cytological changes seen in Brz-treated *Arabidopsis* were exactly the same as those seen in a brassinosteroid-biosynthesis-deficient mutant, *det2*, supporting the hypothesis that Brz has no side-effects except inhibiting brassinosteroid biosynthesis, and should prove a useful tool in clarifying the role of brassinosteroids.

**Key words:** *Arabidopsis* (brassinosteroid, mutant) – Brassinazole – Brassinosteroid biosynthesis – Mutant *Arabidopsis (det2)* – Plastid differentiation

**Introduction**

Brassinosteroids, which are a newly recognized class of plant hormone, have various physiological and morphological effects on plants (Sasse 1999). Microchemical and molecular techniques have revealed details of the biosynthesis and metabolism of brassinosteroids and confirmed predictions of their essential role in normal plant growth (Clouse 1996; Fujioka and Sakurai 1997; Clouse and Sasse 1998). Several brassinosteroid-deficient mutants have been isolated in *Arabidopsis* (Takahashi et al. 1995; Gachotte et al. 1996; Kauschmann et al. 1996; Li et al. 1996; Szekeres et al. 1996; Choe et al. 1998, 1999a, b) and pea (Nomura et al. 1997). These mutants exhibit a common dwarf phenotype, with dark-grown curled leaves when grown in the light and short hypocotyls and expanded cotyledons when grown in the dark. In all of these mutants, the application of brassinolide restores normal growth.

*Deetiolated2* (*det2*), which was the first brassinosteroid biosynthesis-deficient mutant identified, was originally found to be defective in growth regulation by light. When grown in the dark, plants exhibited the phenotype of light-grown plants (Chory et al. 1991). Furthermore, they accumulated high levels of light-regulated RNA and photosynthetic proteins in the absence of light. Subsequently, an increase in the expression of light-regulated genes was also confirmed in seedlings of the constitutive photomorphogenesis and dwarfism (*cpd*) mutant grown in the dark. This mutant is defective in cytochrome P450 (CYP90), which plays an essential role

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Abbreviations: BrdU = 5-bromo-2′-deoxyuridine; Brz = brassinazole; DAPI = 4′,6-diamidino-2-phenylindole

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in the biosynthesis of brassinosteroids (Szekeres et al. 1996). In this context, brassinosteroids seem to affect light-dependent signaling pathways. In some brassinosteroid-biosynthesis-deficient mutants, such as det2 and dwarf4 (dwf4), electron microscopy has shown that etioplasts fail to differentiate to chloroplasts, suggesting that the mutation uncouples the plastid differentiation pathway (Chory et al. 1991; Azpiroz et al. 1998). In contrast, when grown in the dark, several other mutants, such as det1, constitutive photomorphogenic1 (cop1), and cop9, develop a few thylakoid membranes in plastids, which seem to have differentiated from etioplasts into chloroplasts (Deng and Quail 1992; Chory et al. 1989; Wei and Deng 1992). Azpiroz et al. (1998) recently postulated that a brassinosteroid-biosynthesis-deficient mutant, dwf4, may not be a light-regulatory mutant, and photomorphogenesis (i.e., the development of such traits as true leaves in the dark) may be a secondary effect of reduced hypocotyl length and growth conditions. At present, it is unclear whether the morphological changes that occur in brassinosteroid-biosynthesis-deficient mutants grown in the dark can be explained as aberrations of the photomorphogenesis signaling pathway.

Electron microscopy is the most popular method of examining the differentiation of plastids, although it cannot detect DNA in the plastid nucleoids or elsewhere. In previous studies of brassinosteroid-deficient mutants, differentiation from etioplasts to chloroplasts was evaluated using the loss of the prolamellar body and the development of thylakoid membranes as criteria. It was concluded that brassinosteroids are not involved in plastid differentiation (Chory et al. 1991; Azpiroz et al. 1998). However, it has been reported that in the process of differentiation from proplastid to chloroplast during leaf development in Arabidopsis (Fujie et al. 1994) the amplification of plastid DNA and scattering of the plastid nucleoids occur before the appearance of characteristic chloroplast features, such as developed thylakoids or the expression of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) proteins. Furthermore, a change in the distribution of plastid nucleoids was also observed during the greening of etioplasts of dark-grown leaves in Avena sativa (Hashimoto and Possingham 1989). In this context, these changes in the plastid nucleoids seem to be the initial event in plastid differentiation. To examine whether plastids begin to differentiate, it is necessary to observe plastid DNA by fluorescence microscopy, using 4',6-diamidino-2-phenylindole (DAPI) for example, in addition to electron microscopy.

Recently, the first brassinosteroid-biosynthesis inhibitor, brassinazole (Brz), was reported (Asami and Yoshida 1999; Min et al. 1999). The phenotype of Brz-treated plants is very similar to that of brassinosteroid-deficient mutants, and normal growth is restored by the application of brassinolide. Furthermore, it has already been determined by feeding plants brassinosteroid-biosynthesis intermediates that the Brz action site is at least one step upstream from testosterone formation (Asami et al. 2000). In general, plant hormone inhibitors are useful tools, as demonstrated in the study of gibberellin (Yokota et al. 1991). Therefore, Brz should be helpful for clarifying the function of brassinosteroids in plants, as a complement to studies of brassinosteroid-deficient mutants. To be able to use Brz as a tool, it is necessary to confirm in detail that various morphological and cytological changes in Brz-treated plants are due to inhibition of brassinosteroid biosynthesis, and not to side-effects of the inhibitor.

In this work, we first demonstrated that various changes in Brz-treated Arabidopsis are perfectly coincident with those in a brassinosteroid-biosynthesis-deficient mutant, det2, suggesting that Brz has no side-effects except inhibition of brassinosteroid biosynthesis. Second, we took advantage of its effects as an inhibitor and revealed that Brz-treatment (inhibition of brassinosteroid biosynthesis) induces the development of true leaves and short hypocotyls independently in the dark. Third, we examined in detail the cytological changes in Brz-treated Arabidopsis from a different perspective than that of previous studies of brassinosteroid-deficient mutants, and found novel characteristics suggesting that Brz induces a very early step in plastid differentiation.

Materials and methods

Plants and growth conditions

Seeds of Arabidopsis thaliana (L.) ecotype Columbia were sown on solid half-strength Murashige and Skoog (1962) medium containing 1.5% sucrose and 0.8% agar in plant culture jars with filters for aeration (AGRIPOS; 7 cm i.d. × 11 cm). They were incubated in growth chambers at 22 °C under continuous light or dark. For the experiments, brassinazole (Brz) and brassinolide were added to the medium beforehand. Dr. J. Chory provided seeds of detiolated2 (det2) mutant lines, and det2-1 was used here.

Light microscopy

High-magnification photographs of the seedlings were taken with an Olympus IX70 microscope (Olympus, Tokyo, Japan). For sectioning, the samples were fixed in 2% glutaraldehydebuffered with 20 mM sodium cacodylate at pH 7.0 for 20 h at 4 °C, dehydrated through an ethanol series, and then embedded in Technovit 7100 resin (Kulzer and Co., Wehrheim, Germany). The sections (0.7 μm thick) were cut with a glass knife on an ULTRA-CUT UCT ultramicrotome (Leica, Vienna, Austria), placed on coverslips and dried. They were stained with 0.5% toluidine blue O in 0.1 M phosphate-buffered saline (PBS; pH 7.4; Wako, Osaka, Japan) for 30 s and then washed in distilled water for 10 s. The samples were observed with an Olympus IX70 microscope.

Investigation of DNA synthesis using 5-bromo-2'-deoxyuridine (BrdU)

Eight-day-old seedlings grown in continuous dark were transferred to new medium containing 10 μM BrdU, a thymidine analogue, and 1 μM 5-fluorodeoxyuridine to enhance incorporation of BrdU. Then, they were incubated for 48 h in continuous dark, embedded in Technovit 7100 resin, and sectioned as described above. The samples on coverslips were treated with 2 N HCl for 30 min and washed with distilled water and PBS (pH 7.4) for 10 min. After blocking for 20 min at room temperature in blocking buffer (5%