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Reduced de-etiolation of hypocotyl growth in a tomato mutant is associated with hypersensitivity to, and high endogenous levels of, abscisic acid

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Abstract

A recessive single gene mutant, *7B-1*, in tomato was originally selected for its photoperiod-dependent male sterility. The *7B-1* mutant also has some pleiotropic effects including reduced light-induced inhibition, i.e. de-etiolation, of the hypocotyl in long days (LD), increased seed size and weight, and reduced transpiration rate. These traits led us to investigate the sensitivity of *7B-1* to exogenous hormones and the interaction of these responses with daylength. In LD, but not in short days (SD), *7B-1* was more sensitive than wild-type (WT) to exogenous abscisic acid (ABA) for inhibition of seed germination, root elongation and transpiration rate. *7B-1* mutant also exhibited reduced responses to exogenous gibberellin (GA_3) for hypocotyl elongation, and to inhibitors of GA biosynthesis for seed germination and root and hypocotyl elongation. *7B-1* hypocotyls contained a higher level of endogenous ABA than WT in both photoperiods, although ABA levels were higher in LD than in SD. In contrast, growth-active GAs, i.e. GA_1, GA_3 and GA_4, and IAA were low in the mutant hypocotyls. The *7B-1* mutant appears to be an ABA-overproducer, and the photoperiod-regulated ABA levels may be responsible for the hypersensitivity of the mutant to exogenous ABA.

Key words: Abscisic acid, elongated mutant, fluridone, gibberellins, tetcyclacis, tomato.

Introduction

Plant hormones are important internal factors which control developmental processes in interaction with various environmental stimuli, such as light and temperature (Davies, 1995). A genetic approach, such as the isolation and characterization of mutants affected in response to hormones, is a powerful tool for the study of the role of plant hormones in gene-controlled growth and development. Many of the mutants shown to be defective in the biosynthesis or action of hormones were originally selected on the basis of changes in shoot (hypocotyl, stem) growth. For example, a number of dwarf mutants have been isolated that are affected either in endogenous GA content, or in GA-action (for review, see Ross *et al*., 1997). Also, mutants affected in auxin responses are known which display alterations in elongation capacity, including dwarf stature (reviewed in Reid and Howell, 1995). Recently, several dwarf mutants have been reported which have a defect in different steps of brassinosteroid biosynthesis and action (Clouse *et al*., 1996; Altmann, 1999; Ephritikhine *et al*., 1999). However,

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Abbreviations: ABA, abscisic acid; BA, ^{6}*N*-benzyladenine; DW, dry weight; FLU (fluridone), 1-methyl-3-phenyl-5-[3-trifluoromethyl-(phenyl)]-4(1*H*)-pyridinone; FW, fresh weight; GA(s), gibberellin(s); IAA, indole-3-acetic acid; LD, long day(s); SD, short day(s); TCY (tetcyclacis), 5-(4-chlorophenyl)-3,4,5,9,10-pentaza-tetra-cyclo 4,5,1,$O^{2,6}$,$O^{8,11}$ dodeca-3, 9-diene; UNI (uniconazole), (E)-(*p*-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol; WT, wild-type; 2,4-D, 2,4-dichlorophenoxyacetic acid.

fewer hormone biosynthesis or action mutants are known which exhibit greater than normal growth (Reid and Howell, 1995; Ross *et al*., 1997).

Mutants exhibiting extended growth have been shown to be either GA-biosynthesis/metabolism or GA-response mutants. For example, the mutant *slender* (*sln*) in pea has a lesion in GA_{20} catabolism resulting in increased levels of GA_1, which causes seedlings to develop exceptionally long internodes (Reid *et al*., 1992; Ross *et al*., 1997). Another elongated mutant in *Brassica* (*ein*) (Rood *et al*., 1990) is a GA-overproducer and it has a phytochrome lesion (Devlin *et al*., 1992). Other elongated mutants, such as *lacry*^{s} in pea, *slender* (*sln*) in barley, *spindly* (*spy*) in *Arabidopsis*, and *procera* (*pro*) in tomato (Chandler, 1988; Lanahan and Ho, 1988; Jacobsen and Olszewski, 1993; Potts *et al*., 1985; Jones, 1987; Jupe *et al*., 1988) have been classified as constitutive GA response mutants. Finally, elongated mutants have been reported which have an enhanced response to applied GA, such as the phyB-deficient long hypocotyl mutants in cucumber (*lh*), pea (*lv*), and *Arabidopsis* (*phyB*) (Ross *et al*., 1997).

A single gene, male sterile mutant *7B-1* in tomato (*Lycopersicon esculentum*) is photoperiod sensitive (Sawhney, 1997). In LD it produces male sterile flowers, but in SD most of the flowers are male fertile. The *7B-1* mutant also has some pleiotropic effects, including reduced light-induced inhibition, or de-etiolation, of hypocotyls in LD, and increased seed size and weight. The objectives of this study were (1) to examine the sensitivity of the *7B-1* mutant to exogenous hormones, i.e. ABA and GA, and their biosynthetic inhibitors, (2) to determine whether endogenous levels of ABA, GAs and IAA, are affected in the mutant, and (3) to determine whether the photoperiod-dependent mutant responses are related to changes in endogenous hormones. The differential sensitivity of the mutant to exogenous hormones and its relationship with photoperiod is discussed.

Materials and methods

Plant material and growth conditions

The spontaneous recessive male sterile mutant *7B-1* in tomato (*Lycopersicon esculentum* Mill.) was isolated for its photoperiod sensitivity (Sawhney, 1997). *7B-1* plants are male sterile under LD (12–16 h), but under SD (8–10 h) most of the flowers are male fertile. Thus, for all experiments, *7B-1* and WT seeds were obtained from plants grown in SD.

For seed germination and sterile culture of plants, seeds were sterilized in 50% (v/v) Javex-5 solution (3% hypochlorite sodium) for 20 min, rinsed extensively with sterile distilled water, and were sown on 0.7% (w/v) agar medium in Petri dishes (100×15 mm). The basal medium (BM) consisted of Murashige and Skoog salts (Murashige and Skoog, 1962), 1% (w/v) sucrose and 1 mM MES (2-[*N*-morpholino]ethanesulphonic acid) buffer (pH adjusted to 6.1 by KOH). Petri dishes with germinated seeds were placed in an incubator at 25/23 °C, light/dark under either a 16/8 h, light/dark (LD), or an 8/16 h, light/dark (SD) photoperiod. Illumination was provided by white fluorescent tubes (F20T12/CW, Sylvania, USA) with the photon fluence rate of 25–40 μmol m^{-2} s^{-1}. Older plants were grown in soil (Sunshine No. 1, Sun Gro Horticulture, USA) at a temperature regime of 25/18 °C, light/dark in a growth chamber with LD or SD photoperiod under high-intensity fluorescent tubes (F72T12/CW/VHO, Sylvania, USA) and incandescent bulbs (Long Life 7500 h, Litemor, Canada) at an illumination of 90–200 μmol m^{-2} s^{-1}.

Germination tests

7B-1 and WT seeds were germinated, 40–60 seeds per Petri dish, in the BM supplemented with or without different concentrations of ABA (±)-*cis*, *trans*, GA_3 (Sigma, St Louis, MO), tetcyclacis (BASF Aktiengesellschaft, Germany), uniconazole (Chevron Chemical Company, Benseville, IL) or fluridone (Eli Lilly, Indianapolis, IN). All plant growth regulating substances were added to the medium via sterile filtration while medium was cooling after autoclaving. Seed germination, defined as radicle protrusion, was scored from 2–6 d after sowing.

Root and hypocotyl elongation

Four-day-old seedlings grown on the BM were transferred to Petri dishes that were vertically aligned. Here, a new medium was used supplemented with different concentrations of ABA, GA_3, BA, 2,4-D (Sigma, St Louis, MO), 24-epibrassinolide (a gift from Dr Miroslav Strnad, Olomouc, Czech Republic), tetcyclacis, uniconazole or fluridone. After 3 d of incubation, new growth of hypocotyls and roots was measured.

Measurements of cell and organ dimensions

Epidermal cell lengths were measured on hypocotyls excised from 7-d-old seedlings grown *in vitro*. Hypocotyls were stained with methyl-blue dye (0.01% w/v), gently squashed on a microscope slide and examined with a compound microscope (Optiphot, Nikon, Japan). Four hypocotyls of uniform size were chosen from each treatment, and 30 epidermal cells were measured for each hypocotyl. Root and hypocotyl lengths were measured with a graduated ruler to the nearest millimetre.

Transpiration measurements

The method of Häuser *et al*. (Häuser *et al*., 1990) was followed. *7B-1* and WT seedlings with a second leaf (14–20 d after seed sowing) were chosen. Roots were excised, and seedlings with hypocotyls, cotyledons and leaves were placed upright in glass vials (25 mm in diameter×55 mm height) with 10.0 ml of distilled water supplemented either with ABA (100 μM) or tetcyclacis (50 μM). To restrict evaporation of the water to a minimum, the vials were covered with plastic lids which had slits into which hypocotyls were fitted. The vials (six replicates per treatment) were then placed in a growth chamber (25/23 °C, light/dark; 24–40 μmol m^{-2} s^{-1}) for 24 h and incubated under LD or SD. Transpiration losses were determined by weighing the vials (without seedlings) at the beginning and at the end of the experiment. All values were then corrected to account for evaporation that took place in the absence of seedlings. Transpiration was expressed as grams of water lost over 24 h g^{-1} of tissue dry weight.

Analysis of endogenous hormones

The tomato hypocotyls were ground and extracted with 80% MeOH (H_2O : MeOH = 20 : 80, v/v). Twenty ng each of [17,17-$^2H_2$]GA_1, GA_3, GA_4, GA_7, GA_9, GA_{20}, and 200 ng each of [$^2H_6$]ABA and [$^{13}C_6$]IAA were added to the 80% MeOH extract along with 8000 Bq of [1,2(*n*)-^{3}H]GA_1 (Amersham, Oakville, Ontario; 37.7 Ci mmol^{-1}), [1,2(*n*)-^{3}H]GA_4 (Amersham, 32.2 Ci mmol^{-1}) and [1,2(*n*)-^{3}H]GA_9 (Yokota *et al*., 1976) as internal standards. The 80% MeOH extract was then purified with a C_{18} preparative column (C_{18}-PC) (Koshioka *et al*., 1983*a*) made of a syringe barrel (inside diametre 2 cm) filled with 3 g of C_{18} preparative reversed-phase material (Waters Ltd). The 80% MeOH eluate was taken to dryness *in vacuo* at 35 °C. The 80% MeOH residue from the C_{18}-PC was then loaded onto a SiO_2 partition column (SiO_2-PC) (Koshioka *et al*., 1983*a*) (column i.d.=1.5 cm) made of 5 g of Woelm SiO_2 (32–100 mesh) deactivated by equilibration with 20% water by weight. The SiO_2-PC was eluted with EtOAc : hexane (95 : 5, v/v; saturated with 0.5 M formic acid) and the eluate was taken to dryness *in vacuo* at 35 °C. The EtOAc : hexane residue from SiO_2-PC was further purified by high performance liquid chromatography (HPLC) (Koshioka *et al*., 1983*b*; Pearce *et al*., 1994).

The HPLC (Waters Ltd) apparatus consisted of two pumps (model M-45), an automated gradient controller (model 680), and a Rheodyne injector (model 7125). The solvents were, pump A: 10% MeOH in 1% acetic acid [H_2O : MeOH : acetic acid=89 : 10 : 1, (v/v)], pump B: 100% MeOH. A reversed phase C_{18} Radial-PAK (-Bondapak column (8 mm × 10 cm) was used with a 10–73% linear gradient programme at a flow rate of 2 ml min^{-1}. The manually implemented 10–73% linear gradient programme was 0–10 min (pump A, 100%; pump B, 70%). The HPLC fractions were taken to dryness *in vacuo*. The C_{18} HPLC fraction residues (fractions grouped based on elution of [^{3}H]-labelled GAs) were further purified with an Alltech Associates Nucleosil $N(CH_3)_2$ HPLC column (4.6 mm × 15 cm) eluted with 99.9% MeOH in 0.1% acetic acid (Pearce *et al*., 1994), 1 min fractions being taken to dryness *in vacuo*. The GA/ABA/IAA-containing fractions from the Nucleosil $N(CH_3)_2$ HPLC were methylated by ethereal CH_2N_2. The methylated sample was then silylated by BSTFA with 1% TMCS (Hedden, 1987; Gaskin and MacMillan, 1991).

The identification and quantification of GAs was carried out on a gas chromatograph (GC-MS) in -selected ion monitoring (-SIM) mode. The derivatized sample was injected onto a capillary column installed in a Hewlett-Packard 5890 GC with a capillary direct interface to a HP 5970 mass selective detector (MSD). The capillary column was a 0.25 (mm film thickness, 0.25 mm internal diametre, 15 m DB1-15N column (J&W Scientific, Inc.). The capillary head pressure was 4 psi with a He carrier gas flow rate of 1.1 ml min^{-1}. The GC temperature programme was as follows: 0.1 min at 60 °C, then up to 200 °C at 20 °C min^{-1}, then up to 250 °C at 4 °C min^{-1} and finally to 300 °C at 25 °C min^{-1}, and stayed at 300 °C for 5 min before returning to 60 °C. The interface temperature was maintained at 300 °C and the MSD was operated with the electron multiplier at 1600 V. Three m/z ions from each deuterated GA and endogenous GA were monitored. The dwell time was 10 s. The data were processed using the HP G1034C MS ChemStation Software. Based on the capillary GC Rts of stable isotope internal standards and relative abundance of the characteristic m/z ions monitored, endogenous GAs were identified and amounts estimated based on the isotope dilution technique (Fujioka *et al*., 1988).

Statistical analysis

Statistical significance of the treatment differences was assessed using Student's *t*-test when only two components were compared. Two-way analysis of variance was utilized in all other comparisons.

Results

Seed germination

7B-1 seeds that developed on plants under both SD and LD were larger in size, darker in color and weighed more than WT seeds (Table 1). On the BM, WT and *7B-1* seeds showed similar germination percentages under both photoperiods. In both genotypes germination started after 2–4 d, and after 5–6 d it was 80% germination on average (from at least 40 independent experiments) in LD and in SD. Because of the variability in germination between seed batches (data not shown), germination comparisons between the two genotypes in response to growth regulators were calculated relative to control values on the basal medium.

Seed germination was assessed in the presence of various concentrations of ABA, GA_3, tetcyclacis (a GA-biosynthesis inhibitor of the norbornanodiazetine type), uniconazole (a GA-biosynthesis inhibitor of triazole type) (Rademacher *et al*., 1987; Grossmann, 1990), or fluridone, an inhibitor of ABA biosynthesis (Gamble and Mullet, 1986; Saab *et al*., 1990). Germination of WT seeds was inhibited by ABA from 3×10^{-6} M to 3×10^{-5} M under both LD and SD, although inhibition was more pronounced under SD than in LD (Fig. 1a). In LD seed germination in *7B-1* was 2–3 times more

Table 1. *Diameter and weight of wild-type (WT) and* 7B-1 *mutant seeds that developed on plants grown in LD or SD*

Values are means ± SE of four independent experiments. In each experiment 20 seeds were randomly chosen from each of four fruits (also randomly chosen), one fruit from each of four plants.

	LD		SD	
	WT	*7B-1*	WT	*7B-1*
Seed diametre (mm)	2.2 ± 0.1	2.5 ± 0.1*	1.7 ± 0.1	2.1 ± 0.1*
Weight of 20 seeds (mg)	62.2 ± 2.6	82.0 ± 7.4*	36.6 ± 0.6	48.4 ± 1.7*

Statistical analyses were accomplished separately for each of LD- and SD-grown seeds. * Significantly different from WT at $P \leq 0.05$.

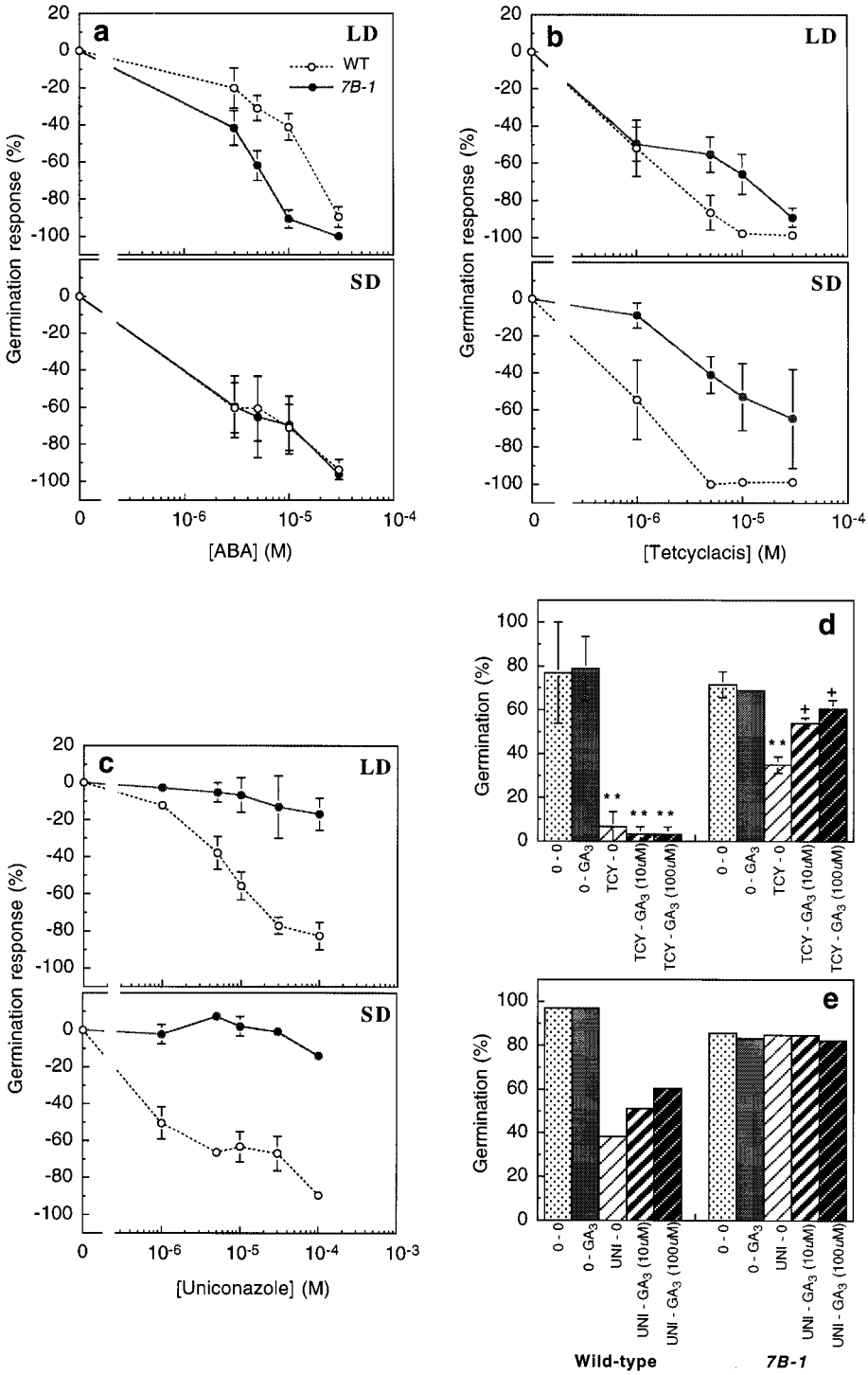


Fig. 1. (a, b, c) Effects of ABA, tetcyclacis (TCY), and uniconazole (UNI) on seed germination in WT (○) and the *7B-1* mutant (●) in LD and SD. Inhibition of seed germination by ABA (a), tetcyclasis (b) and uniconazole (c) relative to the mean germination on basal medium (BM), 6 d after seed sowing. 40 seeds of each genotype were sown for each treatment in each experiment. Each value represents mean germination ±SE of six independent experiments for ABA and TCY in LD, and of three experiments for ABA and TCY in SD and uniconazole in both LD and SD. (d, e) Effects of TCY (d) or UNI (e) on seed germination with or without GA_3. Seeds were soaked for 2 d in TCY solution (100 μM; +5 °C, darkness) and then incubated in LD for another 5 d on the BM, with or without 10 μM or 100 μM of GA_3. 40 seeds of each genotype were sown for each treatment for each experiment. Bars represent means ±SE of three independent experiments (d), or one representative experiment of two (e). **, Significantly different ($P \leq 0.01$) from WT and *7B-1* controls, respectively. +, Significantly different ($P \leq 0.05$) from TCY alone.

inhibitory to ABA than that in WT seeds (Fig. 1a). However, under SD, *7B-1* and WT showed similar response to a range of ABA concentrations (Fig. 1a). Germination of WT seeds was strongly inhibited by tetcyclacis over a range of concentrations in both photoperiods (Fig. 1b). In contrast, *7B-1* seeds showed a much reduced sensitivity to tetcyclacis inhibition, especially in SD (Fig. 1b). Uniconazole also strongly inhibited WT seed germination in both photoperiods over a wide range of concentrations, but germination of *7B-1* seeds was essentially unaffected by uniconazole (Fig. 1c).

The question of whether exogenous GA would restore germination in tetcyclacis- or uniconazole-treated WT seeds was tested by transferring seeds that had been imbibed in the inhibitor solution for 2 d to a medium containing GA_3 for 5 d. GA_3 (10 or 100 μM) was unable to restore germination in tetcyclacis-treated WT seeds, but it did increase germination of *7B-1* seeds (Fig. 1d). GA_3 partly overcame the uniconazole-induced inhibition of WT seed, but it did not improve *7B-1* seed germination (Fig. 1e). GA_3 alone had no promotive effect on seed germination of both genotypes. Similar results were obtained in SD conditions (data not shown).

Since tetcyclacis is also known to stimulate ABA accumulation in actively growing plants by inhibiting its degradation (Grossmann, 1990), the effect of fluridone (an inhibitor of ABA synthesis), alone or in combination with tetcyclasis, ABA, or tetcyclasis and GA_3 was tested. Fluridone alone (10 μM) enhanced the rate of seed germination of both WT and *7B-1* seeds (Fig. 2a). ABA (30 μM) together with fluridone reduced germination of both WT and *7B-1* seeds to the level of ABA alone (Fig. 2b). Fluridone also partly overcame the inhibitory effect of tetcyclacis on WT seed germination and the addition of GA_3 to this mixture further increased germination (Fig. 2b). In *7B-1* mutant, fluridone alone, or fluridone and GA_3, restored germination of tetcyclacis-treated seeds to the control level (Fig. 2b). Similar results were obtained in SD conditions (data not shown).

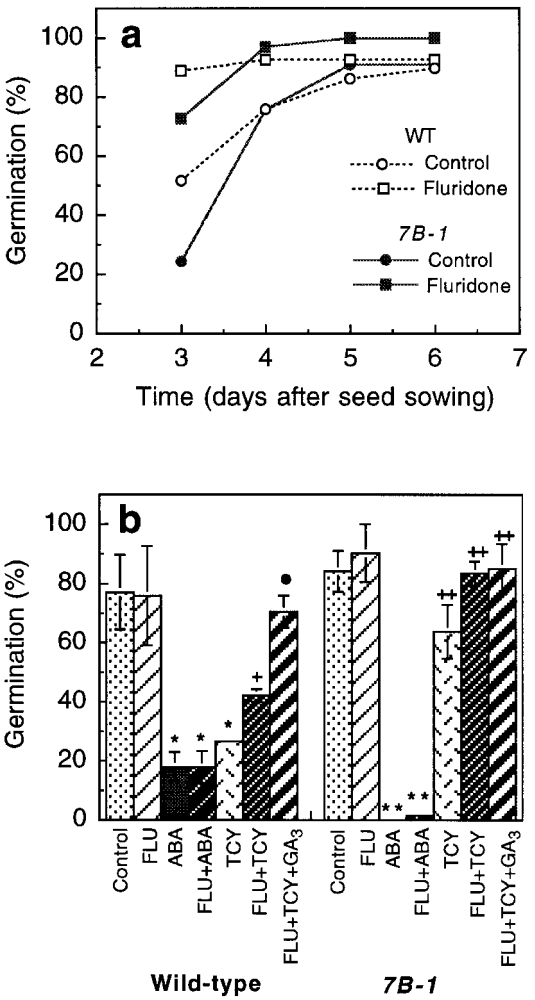


Fig. 2. Germination of WT and *7B-1* seeds in the presence of fluridone, ABA, tetcyclacis (TCY), and GA_3 in LD. (a) Kinetics of seed germination in WT and the *7B-1* mutant in the presence of fluridone (10 μM) in LD. For each genotype and treatment at least 40 seeds were scored for germination. Data from one representative experiment are shown. (b) Effects of fluridone (FLU) (10 μM), ABA (30 μM), TCY (30 μM) or GA_3 (10 μM) alone or in combination on germination of WT and *7B-1* seeds in LD. At least 40 seeds of each genotype and treatment were scored for germination 6 d after sowing. Values represent means ±SE of three independent experiments. *, + and ●, Significantly different ($P \leq 0.05$) from the control, TCY alone, and FLU + TCY, respectively. ** and + +, Significantly different ($P \leq 0.01$) from the control, and FLU + ABA, respectively.

Root elongation

Root growth on BM did not differ in *7B-1* or WT seedlings in LD or SD (Fig. 3a). Nor were there any differences between the two genotypes with regard to the formation of lateral roots or root hairs (data not shown). ABA inhibited the root growth of WT and *7B-1* seedlings in both photoperiods, but *7B-1* roots were 10 times more sensitive to ABA under LD, across a range of ABA concentrations, than were WT roots (Fig. 3b). For example, 50% inhibition of *7B-1* root elongation occurred at an ABA dose 10 times lower than that required for WT roots. In contrast, under SD, *7B-1* and WT roots showed similar responses to exogenous ABA (Fig. 3b). Fluridone applied at low concentrations (0.1–1 μM) under LD tended to stimulate WT root growth (approximately 20%), but inhibited *7B-1* root growth by 20% (data not shown). At higher doses (10–100 μM) root elongation was inhibited by about 60% for both genotypes and fluridone had similar inhibitory effects under SD (data not shown).

Tetcyclacis inhibited the root growth of WT and *7B-1* seedlings under both photoperiods at 10^{-6} M or higher. However, *7B-1* seedlings were less sensitive to tetcyclacis-induced inhibition in LD, though not in SD (Fig. 3c). At high concentrations (10–30 μM) tetcyclasis caused root necrosis and this was more evident for WT than for

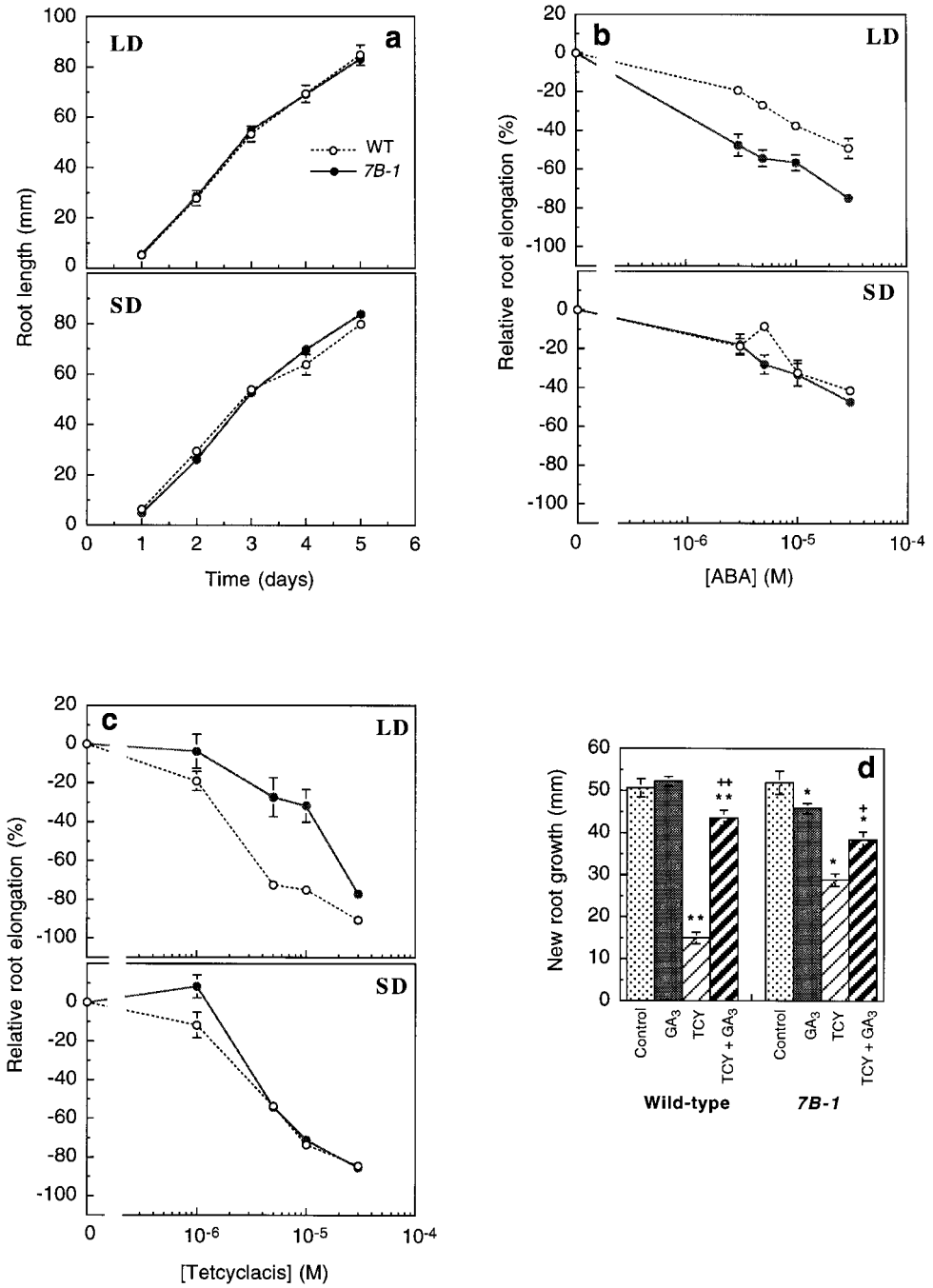


Fig. 3. WT and *7B-1* root elongation in the absence or presence of ABA, tetcyclacis (TCY), GA_3 or TCY + GA_3. (a) Root growth kinetics of LD- and SD-grown WT (○) and *7B-1* mutant (●) seedlings grown on the BM. Root growth inhibition by ABA (b) or TCY (c) of 7-d-old WT (○) and *7B-1* (●) seedlings in LD and SD. For (b) and (c), 4-d-old seedlings developed on the BM were transferred onto ABA- or TCY-supplemented medium and new root growth was measured after 3 d. Similar results were observed in three additional LD, and two additional SD experiments. Inhibition of root growth is expressed relative to the mean growth of the same genotype on BM. Dose–response curves (b) and (c) are shown for one representative experiment out of three independent experiments for ABA in LD out of two for ABA in SD, out of six for TCY in LD and out of two for TCY in SD. (d) The ability of GA_3 (10 μM) to counteract the inhibition of root elongation induced by TCY (10 μM) in 7-d-old WT and in *7B-1* seedlings grown in LD. Four-day-old seedlings were grown on the BM, then transferred onto the growth regulator-supplemented medium. New root growth was measured 3 d after the transfer. In (a–d), values represent the means ± SE of at least 12 seedlings from one representative experiment. ** and + +, Significantly different ($P \leq 0.01$) from control, and TCY alone, respectively. * and +, Significantly different ($P \leq 0.05$) from control, and TCY alone, respectively.

7B-1 (data not shown). GA_3 (10 μM) had no significant effect on the growth of WT or *7B-1* roots in LD (Fig. 3d). However, GA_3 partially overcame the inhibitory effect of tetcyclasis (10 μM) (Fig. 3d). Similar results were observed for SD-grown seedlings (data not shown). Uniconazole (100 μM) was appreciably less inhibitory, giving only a 40% reduction of root elongation for both genotypes, compared to 80–90% inhibition by 30 μM tetcyclasis (data not shown).

The effects of an auxin (2,4-D), a cytokinin (^6N-benzyladenine), and a brassinosteroid (24-epibrassinolide) were also examined across a wide range of concentrations, but there were no differences in root growth between the two genotypes (data not shown).

Hypocotyl elongation

Hypocotyls of both genotypes showed more light-induced inhibition of hypocotyl growth (de-etiolation) in LD than in SD. However, *7B-1* hypocotyls consistently showed less de-etiolation than WT in LD (Figs 4, 5a), but were of similar length to WT in SD (Fig. 5a). The epidermal cell lengths of *7B-1* hypocotyls were also longer than WT in LD, but were not different from WT in SD (Fig. 5a). Fully etiolated dark-grown *7B-1* and WT seedlings did not differ from WT in hypocotyl length (means $\pm$SE; WT: 72.5 ± 5.4 mm; *7B-1*: 71.5 ± 2.7 mm). Hypocotyl elongation was inhibited by ABA (3–30 μM) and the response was similar for *7B-1* and WT in both SD and LD (data not shown). Fluridone at 10^{-7} and 10^{-6} M promoted elongation of WT hypocotyls in LD by approximately 35% and 55%, respectively, relative to only 10% and 18%, respectively, promotion of *7B-1* hypocotyl elongation (Fig. 5b). Higher doses of fluridone (10^{-5} and 10^{-4} M) had a nil or weak inhibitory effect on WT hypocotyls elongation (Fig. 5b), but appreciably inhibited *7B-1* hypocotyl elongation under LD (Fig. 5c; data shown for 100 μM). In SD, fluridone at low concentrations (0.01–1 μM) had no effect on hypocotyl elongation for either genotype (data not shown), and at high concentrations (10–100 μM) fluridone had a similar inhibitory effect on WT and *7B-1* hypocotyl growth (Fig. 5c; shown for 100 μM).

Application of GA_3 stimulated hypocotyl growth (Fig. 5d) and tetcyclacis inhibited it (Fig. 5e) for both genotypes and in both photoperiods. However, the hypocotyl growth response to both GA_3 and tetcyclacis was relatively less for *7B-1* (Fig. 5e; for absolute growth values see Fig. 5f). GA_3 (10 μM) partially overcame the inhibitory effect of tetcyclacis (10 μM) on hypocotyl growth for both genotypes (Fig. 5f). A direct comparison of each genotype's response to GA_3 (with or without tetcyclacis) is, however, complicated by *7B-1* mutant's reduced de-etiolation response in LD (Fig. 4). Similar results were observed for seedlings cultured in SD (data not shown).

In both photoperiods, hypocotyl growth of *7B-1* and WT showed a similar sensitivity to application of an auxin, a cytokinin and a brassinosteroid (data not shown).

Seedling morphology after fluridone treatment

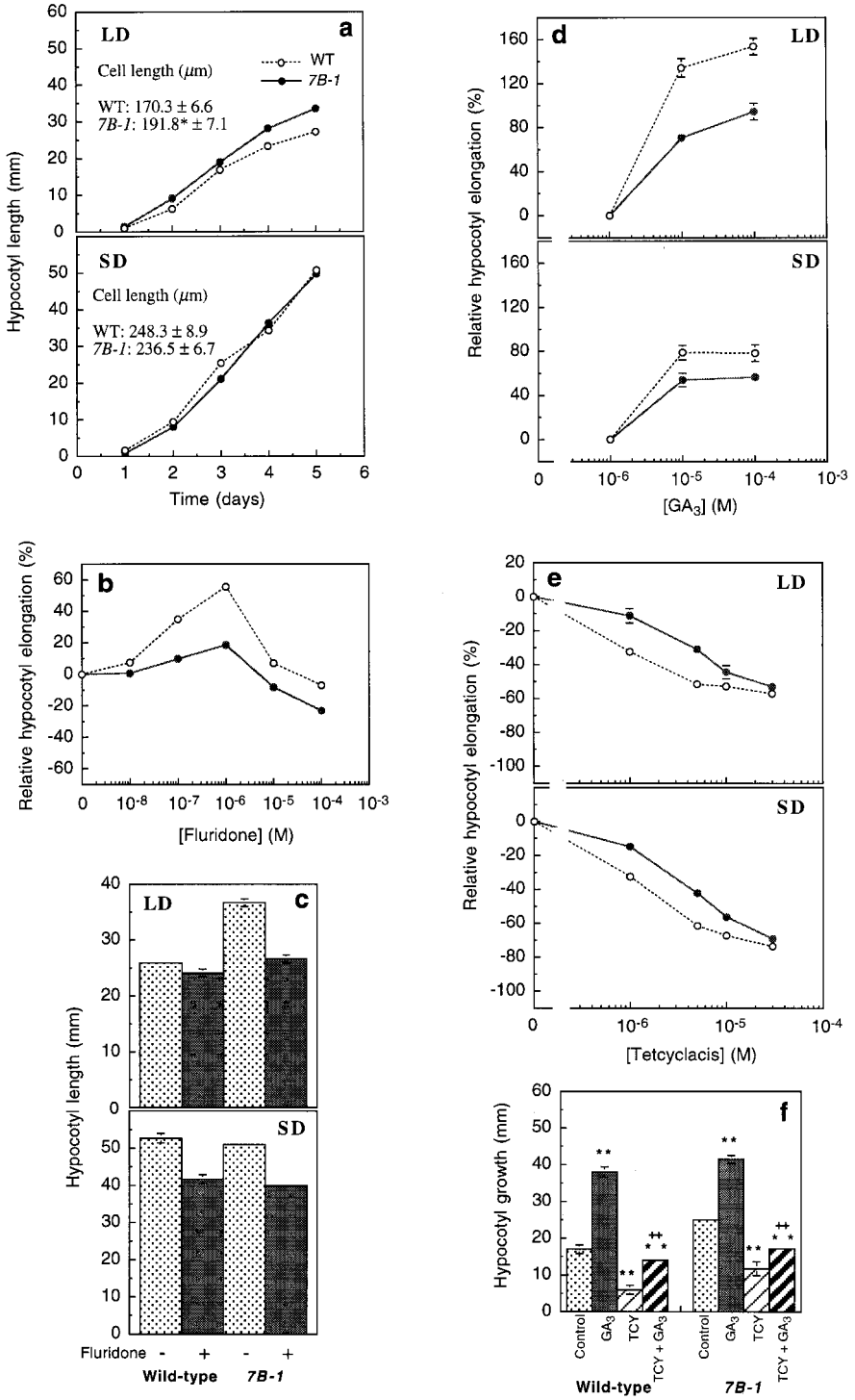
On the BM both the WT and *7B-1* seedlings had green cotyledons and yellow-green hypocotyls and the size and shape of cotyledons were similar for both genotypes in LD and in SD (Fig. 6, upper panel). With the addition of 0.1 μM fluridone, WT seedlings had yellow-green cotyledons and purple hypocotyls (not shown), but with 10 μM fluridone WT seedlings developed pale yellow, deformed cotyledons and light purple hypocotyls (Fig. 6, lower left). In contrast, *7B-1* seedlings had normal green cotyledons with 0.1 μM or 10 μM fluridone, although they had purple hypocotyls (Fig. 6, lower right). With 100 μM fluridone *7B-1* seedlings developed albino

Fig. 4. Morphology of 7-d-old WT (left) and *7B-1* (right) seedlings grown on the BM in LD.

cotyledons and light purple hypocotyls. In SD, *7B-1* seedlings were more sensitive to fluridone than in LD, and with 10 μM fluridone they developed pale yellow and deformed cotyledons (not shown). Interestingly, fluridone plus ABA, or fluridone plus tetcyclacis, or fluridone plus tetcyclasis and GA_3 permitted the development of normal pigmentation in cotyledons and hypocotyls in both genotypes, under either LD or SD (data not shown).

Transpiration experiments

Water loss from seedling shoots (roots excised) of both WT and *7B-1* was measured after 24 h in response to tetcyclacis (50 μM) or ABA (100 μM). The control WT shoots in LD showed greater transpiration than in SD, but the daylength had no effect on transpiration by *7B-1* shoots (Fig. 7). In LD the control WT shoots showed a



greater transpiration than *7B-1* shoots, but there was no difference between the two genotypes in SD. Tetcyclacis is known to reduce transpiration of detached leaves in several plant species (Häuser *et al*., 1990), but it had no significant effect on water loss by shoots of either genotype in LD or in SD (Fig. 7). However, ABA inhibited transpiration of WT shoots in SD and of *7B-1* shoots in LD (Fig. 7).

Endogenous hormones in hypocotyls

Endogenous ABA, IAA and GAs were analysed from 7-d-old hypocotyls of *7B-1* and WT seedlings grown *in vitro* on BM in LD or SD. In both genotypes the relative amount of endogenous ABA was greater in LD than in SD (data from one experiment shown in Fig. 8a). Thus, LD favours ABA accumulation in both genotypes, although the absolute amounts of ABA varied between two independent experiments. The relative levels of endogenous ABA in hypocotyls differed between the mutant and WT. In LD *7B-1* hypocotyls had >2-times more endogenous ABA than WT hypocotyls (Fig. 8a). Here, it should be remembered that *7B-1* hypocotyls are relatively long under LD, i.e. they do not de-etiolate as

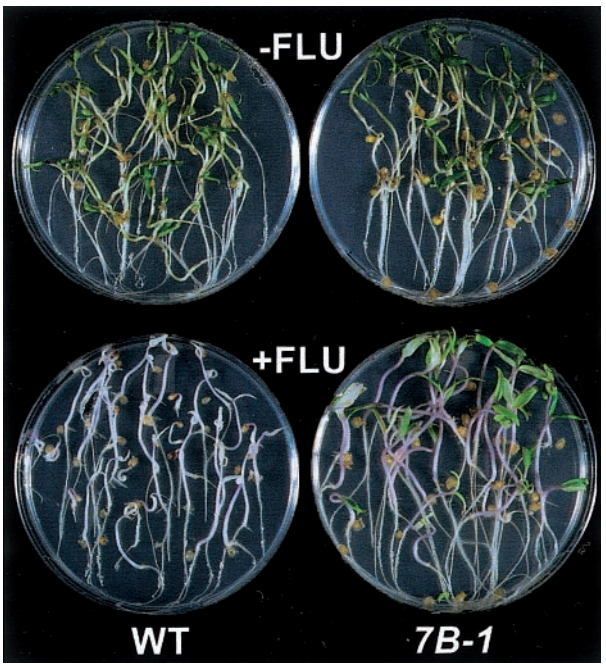


Fig. 6. Comparison of 7-d-old WT (left) and *7B-1* (right) seedlings grown *in vitro* under LD, either on BM (upper panel), or BM supplemented with fluridone (10 μM) (lower panel).

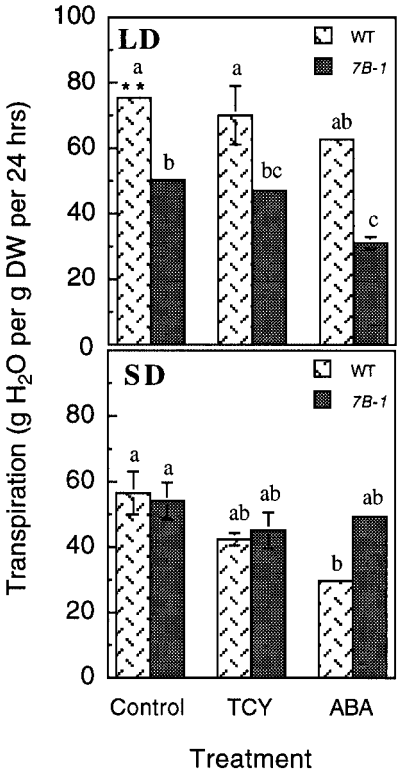


Fig. 7. Effect of tetcyclasis (TCY) and ABA on transpiration over 24 h by WT and *7B-1* seedlings. Seedlings were grown *in vivo* for 14 d in LD or SD. Shoots were then excised and placed in vials with TCY (50 μM) or ABA (100 μM) solution. Six replicates were used per treatment for each genotype. Values are the means ± SE obtained from three independent experiments. Values with different letters are significantly different from each other at $P \leq 0.05$. **, Significantly different ($P \leq 0.01$) from control in SD.

Fig. 5. WT and *7B-1* hypocotyl growth in the absence or presence of fluridone, GA_3, or tetcyclasis (TCY). (a) Growth kinetics of WT (○) and *7B-1* (●) hypocotyls grown on BM in LD and SD. Values represent the means ± SE of at least 12 seedlings for one representative experiment. Values for hypocotyl epidermal cell length are means ± SE of three independent experiments. In each experiment, 120 epidermal cells were measured in four uniform hypocotyls of each genotype. *, Significantly different ($P \leq 0.05$) from WT. (b) Hypocotyl growth as a function of fluridone application (10^{-8}–10^{-5} M) for 7-d-old-WT (○) and *7B-1* (●) seedlings grown in LD. Seeds were germinated on the BM and after 4 d seedlings were transferred to fluridone-supplemented medium. New hypocotyl growth was measured 3 d after transfer. Each value represents the mean of 25 seedlings from one representative experiment. Stimulation or inhibition of hypocotyl growth is expressed as a percentage, relative to hypocotyl growth of the same genotype on BM. (c) Effects of fluridone (100 μM) on hypocotyl elongation of WT and *7B-1* seedlings under LD or SD 7 d after seed sowing. Growth, transfer and measurement details are the same as for (b) above. Values represent mean ± SE of 15 hypocotyls from one representative experiment. Effect of GA_3 (d) and tetcyclasis (e) on hypocotyl growth in 7-d-old WT (○) and *7B-1* mutant (●) seedlings grown in LD or SD. Growth, transfer and measurement details are the same as for (b) above, except the medium contained GA_3. Each value represents the means ± SE of at least 12 seedlings in one representative experiment. Similar results were observed in three additional experiments. (f) Effects of GA_3 (10 μM) on hypocotyl growth in the presence of tetcyclasis (TCY) (10 μM) in 7-d-old WT and *7B-1* seedlings grown in LD. Growth, transfer and measurement details are the same as for (b) above, except the medium contained GA_3 plus TCY. Values represent the means ± SE of at least 12 seedlings in one representative experiment. Similar results were obtained in an additional experiment. ** and ++, Significantly different values ($P \leq 0.01$) from control, and TCY alone, respectively.

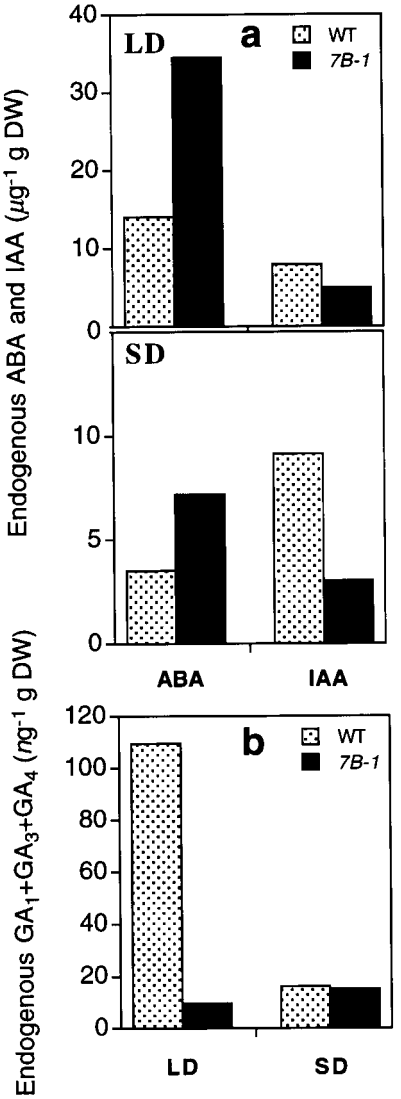


Fig. 8. Concentrations of ABA and IAA (a) and the sum of the growth-active GAs ($GA_1 + GA_3 + GA_4$) (b) in WT and *7B-1* hypocotyls. ABA, IAA and GAs were analysed in hypocotyls of 7-d-old seedlings grown *in vitro* on BM in LD or SD, by GC-MS-SIM, with quantifications made using stable isotope dilution techniques. Values represent the result of one experiment. A similar trend was obtained in one additional experiment for each photoperiod, although absolute amounts varied between the two experiments.

much as WT. In SD *7B-1* hypocotyls also had more ABA than WT, but the relative proportion was less.

The absolute level of endogenous IAA was not influenced by daylength for either genotype (Fig. 8a). However, WT hypocotyls had approximately 2× as much IAA in SD as the mutant, but the values were not different in LD (Fig. 8a).

In two separate experiments only the growth-active GAs, i.e. GA_1, GA_3, GA_4 were consistently identified by GC-MS-SIM in WT and *7B-1* hypocotyls. These were quantified using isotope dilution techniques for seedlings grown under LD and SD. There was a photoperiod effect

Table 2. *Effect of photoperiod on the ratios of endogenous ABA : IAA, IAA : growth-active GAs, and ABA : growth-active GAs in wild-type (WT) and* 7B-1 *hypocotyls*

For comparison of the hormone ratios in each genotype and experiment, the absolute values of endogenous hormone levels in LD were set to 1.00, and relative the values (ratios) in SD were thus calculated.

		WT		*7B-1*	
		LD	SD	LD	SD
ABA : IAA	Experiment 1	1.00	0.22	1.00	0.34
	Experiment 2	1.00	0.78	1.00	0.92
IAA : GAs[a]	Experiment 1	1.00	7.80	1.00	0.39
	Experiment 2	1.00	12.70	1.00	0.96
ABA : GAs[a]	Experiment 1	1.00	1.70	1.00	0.13
	Experiment 2	1.00	10.03	1.00	0.90

[a]GAs = $GA_1 + GA_3 + GA_4$.

on endogenous GA levels in WT hypocotyls; growth-active GAs were drastically reduced under SD, compared to LD (Fig. 8b). However, photoperiod had no effect on growth-active GA levels in *7B-1* hypocotyls (Fig. 8b). As well, *7B-1* hypocotyls had only about 1/10 the amount of growth-active GAs as WT in LD, but the GA content did not differ between the two genotypes in SD (Fig. 8b).

The relative ratios of the various hormones for hypocotyls grown in LD and SD were as follows. The ratio of ABA : IAA in WT hypocotyls was consistently lower in SD relative to LD across two replicate experiments (Table 2). A similar trend was observed for *7B-1* hypocotyls (Table 2). The ratio of IAA : growth-active GAs was exceptionally elevated in WT hypocotyls in SD, but it was low in *7B-1* (Table 2). The ratio of ABA : growth-active GAs was enhanced 1.7–10.00-fold for WT in SD, relative to LD. In contrast, for *7B-1* the ratio of ABA : growth-active GAs tended to be reduced (0.1–0.9-fold; Table 2). Thus, relatively high IAA/GAs and ABA/GAs ratios in *7B-1* hypocotyls in LD, compared to WT, are correlated with the reduced de-etiolation (increased growth) in the mutant in LD (Fig. 4).

Discussion

The recessive mutant *7B-1* in tomato was originally isolated for its photoperiod-dependent male sterility (Sawhney, 1997). Here, it is shown that in LD the mutant has longer hypocotyl than the WT, i.e. it shows reduced de-etiolation, and increased sensitivity to exogenous ABA for inhibition of seed germination and root elongation. In contrast *7B-1* is less sensitive to tetcylacis in the inhibition of germination, root elongation and hypocotyl elongation relative to WT seedlings. The mutant hypocotyls also have relatively high levels of endogenous

ABA and low levels of growth-active GAs in LD. Additionally, they have altered ratios of ABA : GAs and IAA : GAs. These results will be discussed in terms of the *7B-1* mutant being affected in ABA biosynthesis and/or metabolism.

Seed germination experiments suggest accumulation of endogenous ABA in 7B-1 *mutant*

Tomato seeds germinate well in both light and dark (Mancinelli *et al*., 1966) and applied GA_3 had negligible effects on germination of either WT or *7B-1* seeds. A presoak with uniconazole, an early-stage inhibitor of GA-biosynthesis, reduced the germination of WT seeds and this inhibition could be partly overcome by GA_3. This implies that active GA biosynthesis is required for tomato seed germination, although effects of uniconazole on ABA metabolism cannot be ruled out (see below). However, uniconazole did not inhibit germination of *7B-1* seeds. This suggests that levels of pre-existing GAs in the mutant were sufficient to allow for near-normal germination. Tetcyclacis, another early-stage GA biosynthesis inhibitor, also inhibited germination of WT seeds, but this inhibition could not be overcome by subsequent treatment with GA_3. However, GA_3 did partially overcome the inhibitory effect of tetcyclacis on *7B-1* seed germination. While these contrasting responses of the mutant and WT seeds to the two inhibitors are intriguing, they are not readily explained. That said, both tetcyclasis and uniconazole are also known to elevate ABA levels in growing plants (Zeevaart and Creelman, 1988; Zeevaart *et al*., 1988; Häuser *et al*., 1990; Grossmann, 1990) and ABA is a potent inhibitor of seed germination for both genotypes (Fig. 1a). Hence, the explanation may lie with ABA : GA balances within the imbibed seeds, ignoring the possible toxic effects of applied inhibitors.

Fluridone, an inhibitor of carotenoid and ABA biosynthesis (Bartels and Watson, 1978; Gamble and Mullet, 1986; Saab *et al*., 1990; Xu and Bewley, 1995), promoted seed germination in both genotypes (Fig. 2a). This response is similar to that in corn, alfalfa, mung-bean, and lettuce (Hole *et al*., 1989; Xu *et al*., 1990; Thind *et al*., 1997; Yoshioka *et al*., 1998). Interestingly, fluridone also partly restored germination in tetcyclacis-treated WT seeds, and completely restored it when it was supplied together with GA_3 (Fig. 2b). This strongly suggests that tetcyclacis-induced inhibition in WT seeds is caused not only by inhibition of GA biosynthesis but also by increases in ABA levels.

7B-1 seeds were more sensitive to exogenous ABA in LD than were WT seeds (Fig. 1a) and fluridone promoted, whereas tetcyclacis inhibited, germination in *7B-1* seeds. However, when fluridone was supplied together with tetcyclacis full germination occurred in *7B-1*, but not in WT seeds. Full germination did occur for WT seeds, though, when GA_3 + fluridone + tetcyclacis was supplied (Fig. 2b). Together, these observations suggest that *7B-1* seeds have high endogenous ABA levels and that elevated ABA, in a fine balance with endogenous GAs, confers an increased sensitivity to exogenous ABA. However, this suggestion needs to be confirmed by an analysis of ABA and GAs in *7B-1 and WT seeds.*

Differential sensitivity of WT and 7B-1 *roots and hypocotyls to exogenous growth regulators is also related to elevated endogenous ABA in the mutant*

Exogenous ABA generally inhibits elongation of most plant organs (Pilet and Barlow, 1987), although in a few cases low concentrations of ABA can have a stimulatory effect on root growth (Collet, 1970; Yamaguchi and Street, 1977). *7B-1* root growth was more sensitive to the inhibitory effects of ABA in LD than were WT roots (Fig. 3b). This differential response to exogenous ABA may be related to the possible higher levels of ABA in *7B-1* roots. Although ABA levels were not analysed in roots, the elevated ABA levels in *7B-1* hypocotyls (Fig. 8a) supports this view.

Hypocotyl growth in WT and *7B-1* showed a similar sensitivity to exogenous ABA. However, fluridone at low concentrations (0.1 and 1 μM) stimulated greater growth in WT than in *7B-1* hypocotyls (Fig. 5b). This observation is consistent with higher levels of ABA in *7B-1* hypocotyls (Fig. 8a), and suggests that high ABA levels tend to mute the (presumed) effect of fluridone in reducing the ABA content.

Exogenous GA_3 partially overcame the inhibitory effect of tetcyclacis on root (Fig. 3d) and hypocotyl elongation (Fig. 5f). This suggests that GAs are possibly involved as causal factors in root and hypocotyl growth in tomato. Exogenous GA_3 had no effect on root elongation, which implies that endogenous GA levels in roots may be at, or near, optimal levels, a suggestion consistent with earlier work (Tanimoto, 1991) which showed that GA-promotion of root growth was obtained only after use of a GA biosynthesis inhibitor.

Morphology of fluridone-treated seedlings supports the notion that the 7B-1 *mutant has high ABA content*

The morphology of *7B-1* and WT seedlings grown on a fluridone-containing medium further supports the view that *7B-1* has elevated levels of endogenous ABA. Fluridone inhibits pigment biosynthesis, mainly carotenoids, resulting in chlorosis of leaves (Bartels and Watson, 1978; Popova and Riddle, 1996). In these experiments, fluridone (10 μM) bleached the cotyledons in WT seedlings, but the *7B-1* mutant cotyledons remained green (Fig. 6), indicating that the mutant has high ABA content. However, when fluridone was administered with

ABA, normal, green cotyledons developed in WT as well as in *7B-1* seedlings (data not shown), implying that optimal levels of ABA are required for normal pigment development.

Hypocotyls of *7B-1* treated with fluridone (10 μM) had an intense purple colour, relative to WT, suggesting high levels of anthocyanin. Interestingly, *7B-1* seeds were also darker in colour than WT seeds (Table 1), and this may also be due to high anthocyanin. Anthocyanin is formed in response to light, but it can also be produced in response to stress and hormones, including ABA (Mol *et al*., 1996). The presumed increase in anthocyanin in *7B-1* hypocotyls may thus be related to higher ABA level in the mutant.

ABA accumulation in 7B-1 *seedlings results in reduced transpiration rate*

Reduced transpiration under LD in *7B-1* seedlings relative to WT (Fig. 7) can also be explained on the basis of elevated ABA in *7B-1* hypocotyls (Fig. 8a), assuming that hypocotyl ABA levels also reflect ABA levels in the cotyledons and/or influence stomatal opening. The ability of ABA to induce stomatal closure and thus regulate transpiration is well documented (Jones and Mansfield, 1970; Kriedemann *et al*., 1972; Arteca *et al*., 1985*a*, *b*). Both tetcyclacis and ABA reduced transpiration in *7B-1* disproportionately to WT (Fig. 7). Tetcyclacis has also been reported to reduce transpiration in oilseed rape and this effect was associated with increased levels of endogenous ABA (Häuser *et al*., 1990). Hence, the already elevated levels of endogenous ABA in *7B-1* appear to sensitize the mutant to additional ABA whether applied directly, or indirectly, i.e. from tetcyclasis treatment.

A positive effect of endogenous IAA on stomatal opening (Pemadasa, 1982; M Fellner, unpublished results) may also be a factor, and an interaction between ABA and IAA in stomata function (Irving *et al*., 1992) is possible. Thus, the high ABA/IAA ratio in *7B-1* under LD may result in reduced transpiration by the mutant. In contrast, elevated IAA levels, relative to ABA, in SD (Fig. 8a) could be responsible for *7B-1* maintaining a relatively high level of transpiration under SD (Fig. 7).

7B-1 *as an ABA-biosynthesis/metabolism mutant*

The *7B-1* mutant has some phenotypic traits which resemble GA-response or GA-overproducing mutants (Ross *et al*., 1997). For example, bigger *7B-1* seeds relative to WT suggest increased level of endogenous GAs during seed development (Swain *et al*., 1995). Relative to WT, there was less de-etiolation in *7B-1* hypocotyls in LD (Figs 4, 5a) and *7B-1* greenhouse-grown plants were taller at late stages (after floral bud anthesis) of development (data not shown). These phenotype characters all suggest increased GA levels in *7B-1*. However, the mutant hypocotyls had relatively low levels of growth-active GAs in LD relative to WT (Fig. 8b).

Unlike the GA mutants, ABA-biosynthesis or ABA-response mutants are not always drastically affected in elongation growth. ABA-deficient mutants in tomato, e.g. *flacca*, *sitiens* or *notabilis* (Tal and Nevo, 1973) show relatively small or no changes in stem elongation relative to WT. Similarly, the ABA-supersensitive *era1* mutant in *Arabidopsis* shows relatively normal growth and development (Cutler *et al*., 1996), but the ABA-deficient (*aba*) mutant in *Arabidopsis* has reduced growth (Koornneef *et al*., 1982). The phenotypic traits of *7B-1* are consistent with the notion that it is an ABA-overproducing mutant. It is therefore hypothesized that a product of the *7B-1* gene lesion influences ABA biosynthesis or catabolism. Greater accumulation of endogenous ABA in the *7B-1* mutant, especially in LD relative to WT, may also explain the amplified responses of the mutant to applied ABA in LD.

The *7B-1* mutant shows phenotypic similarities to the *sl-2* mutant in tomato (background cv. Rutgers). The *sl-2* mutant contains higher ABA concentrations in vegetative and floral parts compared to WT tissues (Singh and Sawhney, 1998). However, unlike the *7B-1* mutant, *sl-2* does not show the photoperiod-dependent male sterility. None the less, a genetic complementation test is needed to rule out the possibility that the two mutations are allelic.

7B-1 *as photomorphogenetic mutant*

The reduced de-etiolation of the hypocotyl, the tall stature of older plants (not shown) and the putative increased chlorophyll and anthocyanin content in *7B-1* raises the question of whether *7B-1* is a photomorphogenetic mutant, especially since light is known to reduce etiolation and enhance anthocyanin biosynthesis (Kerckhoffs and Kendrick, 1997).

The possible roles played by four hormones, or hormone classes, IAA, ABA, GAs, and cytokinins, in light signal transduction have been reviewed recently (Kraepiel and Miginiac, 1997). The *pew1* mutant in *Arabidopsis*, which is deficient in the biosynthesis of the phytochrome A chromophore, contains higher levels of ABA relative to WT (Kraepiel *et al*., 1994). Given the reduced de-etiolation of *7B-1* hypocotyl in LD, it is possible that ABA and/or IAA are involved in light signalling. In WT hypocotyls, there was a low IAA : growth-active GAs ratio in LD, compared to SD (Table 2). This corresponds with the greater hypocotyl de-etiolation of WT in LD and suggests that hypocotyl de-etiolation induced by light is regulated by auxin, or more specifically by a lowered IAA : GA balance (Fig. 8a; Table 2). The fact that *7B-1* has higher IAA : GAs ratio

in LD than in SD again suggests a stimulatory role for auxin in the increased elongation (reduced de-etiolation) of the *7B-1* hypocotyl in LD. That said, the greater de-etiolation in WT in LD, relative to SD, is also positively associated with higher ABA : IAA ratio in LD than in SD. However, in *7B-1*, no such relationship exists. This suggests either that ABA does not play a dominant role in tomato hypocotyl elongation, or that *7B-1* (which already has elevated levels of ABA) is less sensitive to the inhibitory effect of elevated endogenous ABA levels on hypocotyl growth.

These results thus suggest that *7B-1* could be a mutant affected in light perception or signal transduction. If so, it would be an excellent system with which to investigate the complex interactions between hormone biosynthesis on the one hand and light and hormone signalling on the other.

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