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\textsubscript{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\textsubscript{11}, GA\textsubscript{3} and GA\textsubscript{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,
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 (sl) in pea has a lesion in GA20 catabolism resulting in increased levels of GA1, 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’ in pea, slender (sl) in barley, spinally (spy) in Arabidopsis, and procerus (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 (h), and Arabidopsis (phyB) (Ross et al., 1997).

A single gene, male sterile mutant 7B-I 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-I 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-I 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-I in tomato (Lycopersicon esculentum Mill.) was isolated for its photoperiod sensitivity (Sawhney, 1997). 7B-I 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-I 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]ethanesulfonic 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-I and WT seeds were germinated, 40–60 seeds per Petri dish, in the BM supplemented with or without different concentrations of ABA (±)-cis, trans, GA3, (Sigma, St Louis, MO), tetcyclacis (BASF Aktiengesellschaft, Germany), uniconazol (Chevron Chemical Company, Bensenville, 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, GA3, BA, 2,4-D (Sigma, St Louis, MO), 24-epibrassinolide (a gift from Dr Miroslav Strnad, Olomouc, Czech Republic), tetcyclacis, uniconazol 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/w), 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. (Hauser et al., 1990) was followed. 7B-I 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 (H₂O: MeOH = 20:80, v/v). Twenty ng each of [17,17-²H₂]GA₃₄, GA₃₉, GA₄₃, GA₆₄, GA₉₆, GA₂₀, and 200 ng each of [³H]ABA and [¹⁴C]IAA were added to the 80% MeOH extract along with 8000 Bq of [1,2(n)-²H₂]GA₃₄ (Amersham, Oakville, Ontario; 37.7 Ci mmol⁻¹), [1,2(n)-²H₂]GA₃₉ (Amersham, 32.2 Ci mmol⁻¹) and [1,2(n)-²H₂]GA₆₄ (Yokota et al., 1976) as internal standards. The 80% MeOH extract was then purified with a C₁₈ preparative column (C₁₈-PC) (Koshioka et al., 1983a) made of a syringe barrel (inside diameter 2 cm) filled with 3 g of C₁₈ 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 C₁₈-PC was then loaded onto a SiO₂ partition column (SiO₂-PC) (Koshioka et al., 1983a) (column i.d.=1.5 cm) made of 5 g of Woelm SiO₂ (32–100 mesh) deactivated by equilibration with 20% water by weight. The SiO₂-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 SiO₂-PC was further purified by high performance liquid chromatography (HPLC) (Koshioka et al., 1983b; 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 [H₂O: MeOH: acetic acid=89:10:1, (v/v)]; pump B: 100% MeOH. A reversed phase C₁₈ Radial-Pak (-Bondapak column (8 mm x 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 operated with 0–10 min (pump A, 100%; pump B, 70%).

The HPLC fraction residues (fractions grouped based on elution of [³H]-labelled GAs) were further purified with an Alltech Associates Nucleosil N(CH₃)₂ HPLC column (4.6 mm x 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(CH₃)₂ HPLC were methylated by ethereal CH₃N₂. 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 derivatised 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 diameter, 15 m DB-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, GA₃, tetracyclis (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 x 10⁻⁶ M to 3 x 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

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<td>WT</td>
<td>7B-1</td>
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<td>Seed diameter (mm)</td>
<td>2.2 ± 0.1</td>
<td>2.5 ± 0.1*</td>
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<td>Weight of 20 seeds (mg)</td>
<td>62.2 ± 2.6</td>
<td>82.0 ± 7.4*</td>
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Statistical analyses were accomplished separately for each of LD- and SD-grown seeds. * Significantly different from WT at P ≤ 0.05.
Fig. 1. (a, b, c) Effects of ABA, tetacyclis (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), tetacyclis (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 GA3. 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 GA3. 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 ≤ 0.01) from WT and 7B-1 controls, respectively. +, Significantly different (P ≤ 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 GA3 for 5 d. GA3 (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). GA3 partly overcame the uniconazole-induced inhibition of WT seed, but it did not improve 7B-1 seed germination (Fig. 1c). GA3 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 tetcyclacis, ABA, or tetcyclacis and GA3 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 GA3 to this mixture further increased germination (Fig. 2b). In 7B-1 mutant, fluridone alone, or fluridone and GA3, restored germination of tetcyclacis-treated seeds to the control level (Fig. 2b). Similar results were obtained in SD conditions (data not shown).

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) tetcyclacis 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), GA₃ or TCY + GA₃. (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 GA₃ (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 ≤ 0.01) from control, and TCY alone, respectively. * and +, Significantly different (P ≤ 0.05) from control, and TCY alone, respectively.
7B-1 (data not shown). GA₃ (10 μM) had no significant effect on the growth of WT or 7B-1 roots in LD (Fig. 3d). However, GA₃ partially overcame the inhibitory effect of tetcyclacis (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 tetcyclacis (data not shown).

The effects of an auxin (2,4-D), a cytokinin (6-N-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 ± 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 GA₃ 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 GA₃ and tetcyclacis was relatively less for 7B-1 (Fig. 5e; for absolute growth values see Fig. 5f). GA₃ (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 GA₃ (with or without tetcyclacis) is, however, complicated by 7B-1 mutant’s reduced de-etiolation response in LD (Fig. 4).

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.](image-url)
cotyledons and light purple hypocotyls. In SD, 7B-1 seedlings were more sensitive to fluridine than in LD, and with 10 μM fluridine they developed pale yellow and deformed cotyledons (not shown). Interestingly, fluridine plus ABA, or fluridine plus tetacyclacin, or fluridine plus tetacyclacin and GA3 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 tetacyclacin (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

![Image](image_url)

**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).

![Image](image_url)

**Fig. 7.** Effect of tetcyclacis (TCY) and ABA on transpiration over 24 h by WT and 7B-1 seedlings. Seedlings were grown in vitro 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 ≤ 0.05. **, Significantly different (P ≤ 0.01) from control in SD.

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**Fig. 5.** WT and 7B-1 hypocotyl growth in the absence or presence of fluridone, GAs, or tetcyclacis (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 ≤ 0.05) from WT. (b) Hypocotyl growth as a function of fluridone application (10^{-5} to 10^{-3} 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 GA3 (d) and tetcyclacis (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 GA3. 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 GA3 (10 μM) on hypocotyl growth in the presence of tetcyclacis (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 GA3 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 ≤ 0.01) from control, and TCY alone, respectively.
Table 2. Effect of photoperiod on the ratios of endogenous ABA:IAA, IAA:GA_{1}+GA_{3}+GA_{4}, and ABA: growth-active GAs in wild-type (WT) and 7B-1 hypocotyls

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<td>LD SD</td>
<td>LD SD</td>
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<tr>
<td>ABA:IAA</td>
<td>Experiment 1</td>
<td>1.00 0.22</td>
</tr>
<tr>
<td></td>
<td>Experiment 2</td>
<td>1.00 0.78</td>
</tr>
<tr>
<td>IAA:GAs*</td>
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<td>1.00 7.80</td>
</tr>
<tr>
<td></td>
<td>Experiment 2</td>
<td>1.00 12.70</td>
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<tr>
<td>ABA:GAs*</td>
<td>Experiment 1</td>
<td>1.00 1.70</td>
</tr>
<tr>
<td></td>
<td>Experiment 2</td>
<td>1.00 10.03</td>
</tr>
</tbody>
</table>

*GAs = GA_{1} + GA_{3} + GA_{4}.

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.

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 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 tetacyclac 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-I 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 GA₃ had negligible effects on germination of either WT or 7B-I seeds. A pre soak with uniconazole, an early-stage inhibitor of GA-biosynthesis, reduced the germination of WT seeds and this inhibition could be partly overcome by GA₃. 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-I 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 GA₃. However, GA₃ 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 tetcyclacis and uniconazole are also known to elevate ABA levels in growing plants (Zeevaart and Creelman, 1988; Zeevaart et al., 1988; Hauer 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 Waton, 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; Third et al. 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 GA₃ (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-I seeds were more sensitive to exogenous ABA in LD than were WT seeds (Fig. 1a) and fluridone promoted, whereas tetcyclacis inhibited, germination in 7B-I 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 GA₃ + fluridone + tetcyclacis was supplied (Fig. 2b). Together, these observations suggest that 7B-I 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-I and WT seeds.

Differential sensitivity of WT and 7B-I 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-I 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-I roots. Although ABA levels were not analysed in roots, the elevated ABA levels in 7B-I hypocotyls (Fig. 8a) supports this view.

Hypocotyl growth in WT and 7B-I 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-I hypocotyls (Fig. 5b). This observation is consistent with higher levels of ABA in 7B-I hypocotyls (Fig. 8a), and suggests that high ABA levels tend to mute the (presumed) effect of fluridone in reducing the ABA content.

Exogenous GA₃ 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 GA₃ 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-I and WT seedlings grown on a fluridone-containing medium further supports the view that 7B-I has elevated levels of endogenous ABA. Fluridone inhibits pigment biosynthesis, mainly carotenoids, resulting in chlorosis of leaves (Bartels and Waton, 1978; Popova and Riddle, 1996). In these experiments, fluridone (10 µM) bleached the cotyledons in WT seedlings, but the 7B-I 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; Arceci et al., 1985a, b). Both tetracyclac and ABA reduced transpiration in 7B-1 disproportionate to WT (Fig. 7). Tetracyclac 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 tetracyclac 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. flacula, sitiens or notabilis (Sal et al. 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 photomorphogenic 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 photomorphogenic mutant, especially since light is known to reduce etiolation and enhance anthocyanin biosynthesis (Kerkhoff 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 (Kraepel and Miginiac, 1997). The pewl mutant in Arabidopsis, which is deficient in the biosynthesis of the phytochrome A chromophore, contains higher levels of ABA relative to WT (Kraepel 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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References


