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Organogenetic Response of Photomorphogenic Mutants of Tomato

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Summary

The effect of white (WL) and red (RL) light on organogenesis *in vitro* was studied using explants isolated from seedlings of wild-type (WT) and two photomorphogenic mutants of tomato (*Lycopersicon esculentum* Mill.) – *aurea* (*au*) and *high pigment* (*hp*). Explants excised from hypocotyls and cotyledons of green and etiolated seedlings were cultured on shoot or root inducing media. It was observed that both continuous white (CWL) and red light (CRL) stimulate shoot formation on hypocotyl explants isolated from green seedlings of WT and *hp* plants compared with control ones cultured in darkness. On the other hand, *au* mutant shows very low organogenetic response in spite of light conditions applied. Explants isolated from both green and etiolated seedlings were not able to form shoots when they were cultured in darkness. In contrast to green explants, etiolated ones formed roots in spite of being grown on a shoot inducing medium. Root regeneration from etiolated explants was stimulated by short, 5-min-long daily pulses of RL. This effect was reversed by subsequent far-red light (FRL) irradiation. Stimulation of shoot regeneration from etiolated explants was found when 2-h-long daily irradiation with WL or RL was applied. The highest and the lowest shoot regeneration response was obtained from *hp* and *au* explants, respectively, with an intermediate response from WT. Under the same growth conditions shoot formation was accompanied by root formation, which also occurred in a light dependent manner. The highest number of roots regenerated from *au*-derived explants. The results that we have obtained may suggest that shoot formation is strongly dependent on the light sensitivity of plants and light conditions applied. It also seems that the pattern of organ (shoot and root) development in tomato is affected by the etiolated/deetiolated phenotype of explant. Therefore, we believe that the organogenetic response of tomato *in vitro* is at least partly regulated by phytochrome.

Key words: In vivo, Organogenesis, Photomorphogenesis, Photomorphogenic mutants, Phytochrome, Root regeneration, Shoot regeneration, Tomato.

Abbreviations: au = *aurea* mutant; BAP = benzylaminopurine; CWL = continuous white light; CRL = continuous red light; D = darkness; FR = far red light; *hp* = *high pigment* mutant; IAA = indole-3-acetic acid; RIM = root-inducing medium; RL = red light; SIM = shoot inducing medium; WL = white light; WT = wild type.

Introduction

Light affects plant growth and development in two different ways. It provides energy for the production of organic compounds in the process of photosynthesis. It also regulates plant growth and development independently of photosynthesis in the process of photomorphogenesis (Kendrick and Kronenberg, 1994). Light is the most important physical factor affecting morphogenesis *in vitro* (Thorpe, 1994).

Light conditions were obligatory for shoot regeneration from tobacco pith tissue, with the highest stimulation of the process observed on white and blue light. Red light prevented

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shoot formation (Weis and Jaffe, 1969). Saitou et al. (1992) have shown that long time irradiation with white light was required for shoot regeneration from hairy roots of horseradish induced by inoculation with *Agrobacterium rhizogenes*. Shoot formation from hairy roots was photoreversibly regulated by the phytochrome system (Saitou et al., 1992). Callus of *Actinidia deliciosa* formed shoots in darkness and under white light, but the highest number of shoots was produced after red light treatment (Muleo and Morini, 1990). The callus tissue of *Actinidia* contains spectrophotometrically dectectable, photoreversible phytochrome, as the callus of olive (*Olea europaea*) (Muleo et al., 1994). In cotyledonary explants of tomato bud formation (Lercari et al., 1986) was absent in darkness but promoted by red and low irradiance of white light. The reversibility of a 10-min pulse of red light by subsequently applied pulses of far-red light indicates the involvement of the phytochrome system in the control of tomato regeneration (Lercari et al., 1986). Phytochrome-dependent enhancement of shoot formation by 5-min-long pulses of red light applied daily was also observed in cotyledon cultures of lettuce (Kadkade and Seibert, 1977).

In this paper we analyse the effect of light and etiolated/deetiolated phenotype on the organogenetic response of tomato explants cultured *in vitro* on shoot- or root-inducing media. Besides the wild type (WT) plants, two photomorphogenic mutants of tomato were used: *aurea* (*au*) and *high pigment* (*hp-1*). The first one was chosen because of its inability to synthesize the chromophore group of phytochrome. Therefore, it is insensitive to red and far-red light and exhibits reduced responsiveness to white light (Kendrick et al., 1994, 1997). *hp-1* mutant shows exaggerated photoresponse to light treatment. However, analysis of the total spectrophotometrically detectable phytochrome in *hp-1* showed that it contains a similar phytochrome level, comparable to the WT (Kerckhoffs et al., 1997). Nevertheless, the molecular nature of the *hp-1* mutation is still not recognised (Kerckhoffs et al., 1997).

Materials and Methods

Plant material

Seeds of *aurea* (*au*) and *high pigment* (*hp*) mutants of tomato (*Lycopersicon esculentum* Mill.) and their isogenic wild type (cv. Ailsa Craig) were a generous gift from Dr. R. Kendrick (Wageningen Agricultural Institute, Wageningen, The Netherlands).

In all experiments seeds were surface-sterilized for 10 min in 50 % Clorox (about 2 % Cl_2), and washed three times for 5 min in sterile distilled water. Seeds were then aseptically placed into glass jars and sown on 0.8 % agar medium containing Murashige and Skoog (1962) basal salt mixture, MS (Sigma-Aldrich, Deisenhofen, Germany). Seedlings were grown at 25 °C for 7 days either in darkness or under continuous white light (WL).

Tissue culture

Hypocotyls and cotyledons were excised from 7-day-old light- and dark-grown seedlings. The excised organs were cut into 5-mm-long segments. Two explants (5 mm long) were excised from the central region of hypocotyls. Single cotyledonary explants (5 mm long) were obtained from the middle of the cotyledon.

Hypocotyl and cotyledonary explants were transferred to Petri dishes containing regeneration media composed of MS supplemented with *myo*-inositol (100 mg L^{-1}), thiamine (10 mg L^{-1}), pyridoxine (1 mg L^{-1}), nicotinic acid (1 mg L^{-1}), 3 % (w/v) sucrose and 0.8 % (w/v) agar, pH 5.7.

Both shoot- (SIM) and root-inducing media (RIM) were used. In order to induce shoot formation 10 µmol BAP and 1 µmol IAA were added to 1,000 mL of supplemented Murashige-Skoog basal salt mixture (see above). For root formation, the same medium containing 1 µmol L^{-1} IAA was used. Explants were cultured 28 days on SIM and 10 days on RIM.

Irradiation

White light was obtained from Osram 30 W/11-860 «Daylight» fluorescent tubes (Osram, Berlin, Germany). In all experiments standard irradiation with white light was 431 µmol m^{-2} s^{-1} (PAR). Continuous RL was obtained by growing explants in polystyrene culture vessels (Phytatray II, Sigma-Aldrich, Deisenhofen, Germany), which transmit light of above 590 nm with a transmission peak at 660 nm. RL intensity at the level of explants was 6 µmol s^{-1} m^{-2}.

For experiments with 5- and 10-min-long irradiations with RL and far-red light (FRL), red light was obtained by passing white light from a Xenon lamp (2500 W) through a narrow band filter (transmission maximum at 660 nm, half band widh 9 nm). Light from the same lamp was used to obtain FRL. The filter used gave maximal transmittance at 750 nm (half band widh 7 nm). Light intensity at the level of explants was 1.0 and 0.02 µmol s^{-1} m^{-2} for red and far-red light, respectively.

Presentation of results

Each experiment was repeated three times, with at least 20 explants in each experiment. Mean and standard error were calculated. Error bars shown in all figures represent standard errors calculated from all repetitions of each experiment.

Results

The effect of darkness and continuous irradiation with white and red light

In the first experiment cotyledon and hypocotyl explants isolated from green WT and mutant seedlings were cultured on SIM containing 10 µmol L^{-1} BAP and 1 µmol L^{-1} IAA. Cultures were grown under continuous white light (WL), red light (RL) or were kept in darkness (Fig. 1). In darkness shoot regeneration from both hypocotyl and cotyledon explants was absent or very rare in all genotypes used (Fig. 1 A).

The number of shoots formed by hypocotyl explants isolated from light-oversensitive high pigment (*hp*) mutant was the highest under all light conditions tested. Light-insensitive mutant *aurea* (*au*) (Fig. 2 B) formed no shoots or formed them only occasionally under both white and red light. Explants derived from WT plants regenerated an intermediate number of shoots (Fig. 2 A). That white light stimulated shoot formation more efficiently then red light was especially visible in *hp* hypocotyl explants (Fig. 1 B, C).

The organogenetic competence of cotyledonary explants was not affected by photomorphogenic mutations as strongly as that of hypocotyl explants. Under WL, *hp* explants formed

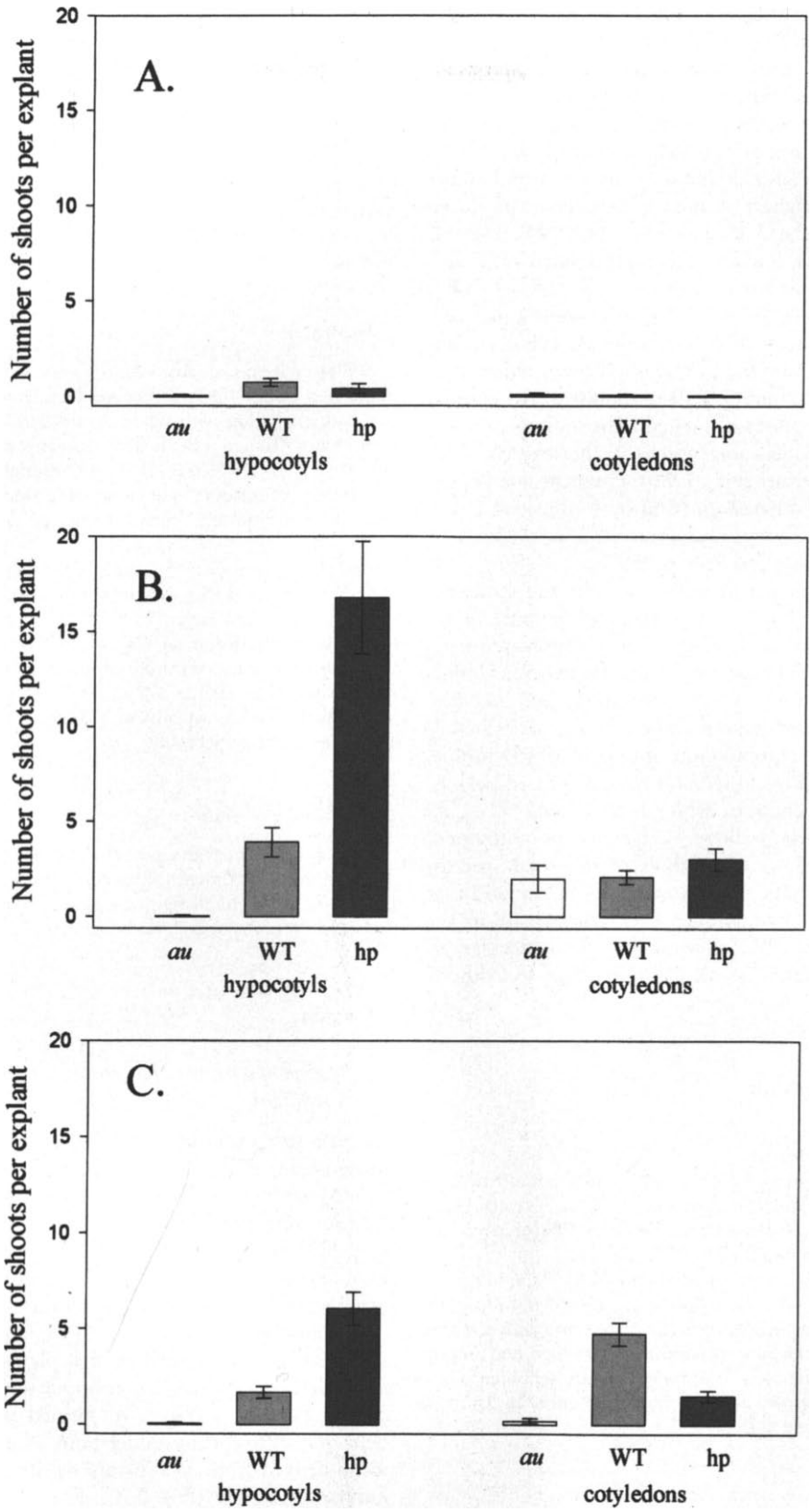

Fig. 1: Shoot formation on explants isolated from hypocotyls and cotyledons of light-grown WT, *au* and *hp* seedlings cultured in darkness (A) or under continuous white (B) and red (C) light. Regenerants were counted after 28 days of culture on SIM (1 μmol L^{-1} IAA, 10 μmol L^{-1} BAP).

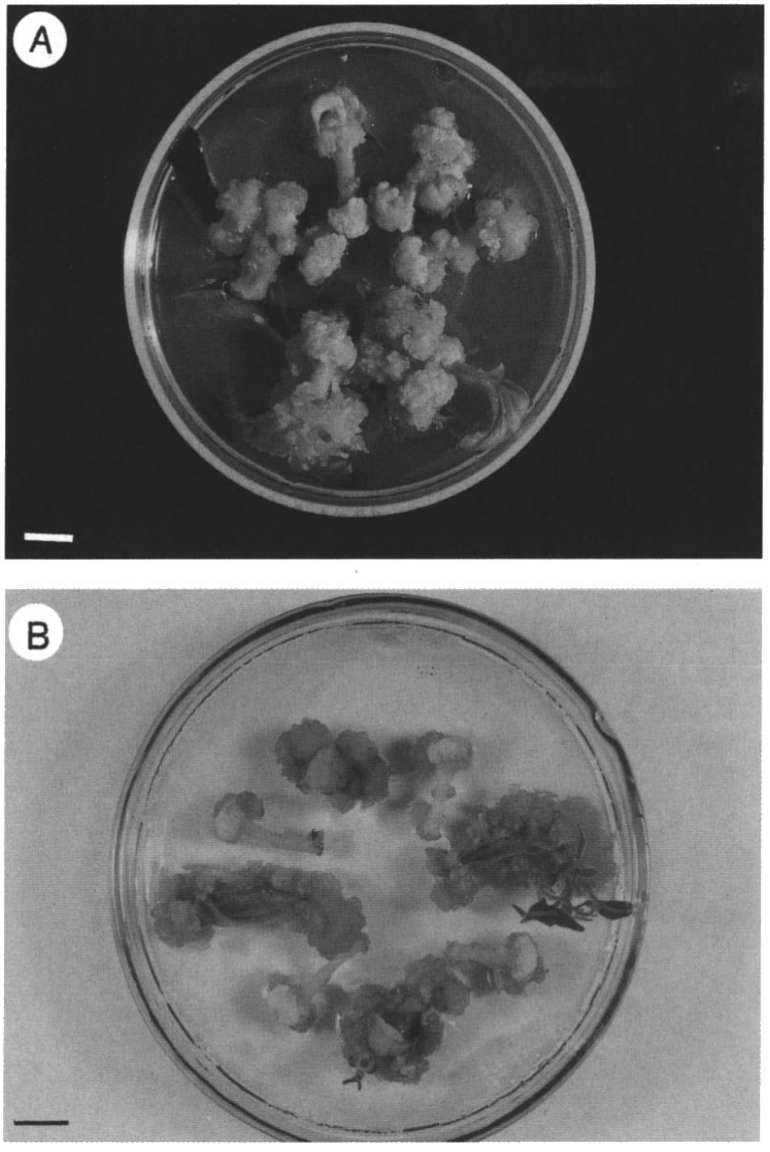

Fig. 2: Shoot-bud formation on explants isolated from hypocotyls of light-grown seedlings of WT (A) and hypocotyls and cotyledons of *aurea* (B) cultured for 28 days on SIM (1 µmol L^{-1} IAA, 10 µmol L^{-1} BAP) under continuous irradiation with white light. Note the lack of regeneration from hypocotyl explants of *aurea*. Bar = 1 cm.

only slightly more shoots than WT and *aurea*. Under red light the largest number of shoots was formed by WT plants.

Organogenetic response of etiolated hypocotyl explants

In the next experiment, explants isolated from hypocotyls of etiolated seedlings were used. Like green explants, etiolated ones also do not produce shoots if they are cultured in darkness. In contrast to explants isolated from green seedlings, however, they undergo root formation, which was not observed if explants were derived from green seedlings. Segments of etiolated hypocotyls regenerate roots in spite of being cultured on SIM (10 µmol L^{-1} BAP, 1 µmol L^{-1} IAA) in the presence of a high cytokinin concentration, which is usually known to inhibit adventitious root formation (Eriksen, 1974; Fabijan et al., 1981; Bollmark and Eliasson, 1986). Additionally, it was found that root formation from etiolated hypocotyls cultured on SIM was stimulated by 5-min-long pulses of red light applied daily during the culture period. Red light stimulated root regeneration from *hp* explants most effectively, less effectively from WT explants, and there was no stimulatory effect on *au* explants. The stimulatory effect of red light was effectively reversed by subsequent 10-min-long irradiation with far-red light (Fig. 3). Roots differentiated *via* indirect organogenesis from callus tissue, which developed on the edges of explants (Fig. 4).

Short red light irradiations stimulated root formation but were not able to induce shoot regeneration from etiolated hypocotyl explants (Fig. 4). Minimal shoot formation was induced when 2-h-long daily irradiations with white or red light were applied. The intensity of shoot formation induced by 2-h-long pulses of WL was dependent on the sensitivity of plants to light. The highest and the lowest shoot regeneration was obtained from *hp* and *au* explants, respectively, with an intermediate response from WT. If RL was used, a different situation occurred with the highest regeneration coming from WT explants. Shoot formation was accompanied by root regeneration. In contrast to shoot formation, rhizogenesis was

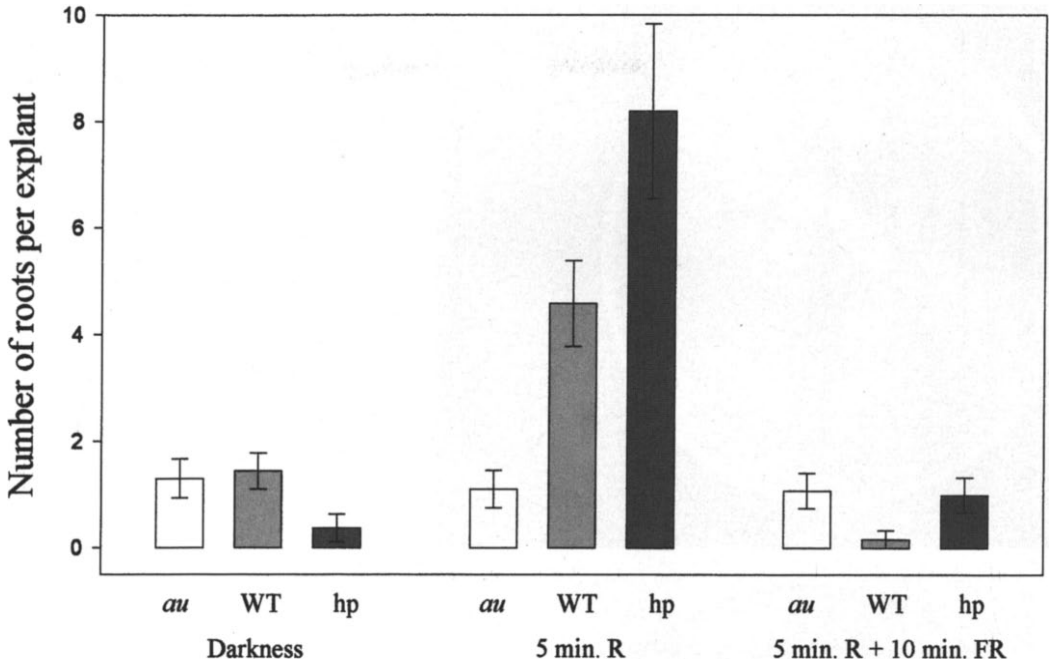

Fig. 3: The regulatory effect of red (R) and far-red light (FR) on root formation on hypocotyl explants isolated from etiolated WT, *au* and *hp* seedlings. Explants were cultured on SIM (10 µmol/L BAP and 1 µmol/L IAA). They were grown in darkness or were irradiated either with 5-min-long red (R), 5-min-long R followed by 10-min-long far-red (FR).

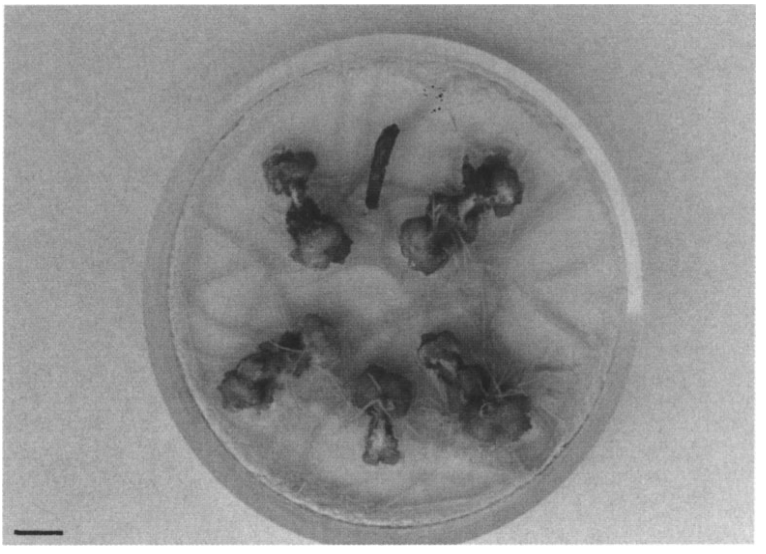

Fig. 4: Root formation on explants isolated from hypocotyls of etiolated seedlings of WT plants cultured in darkness on SIM (1 µmol L^{-1} IAA, 10 µmol L^{-1} BAP). Explants were given 5-min-long daily irradiation with red light. Bar = 1 cm.

reversely coupled with light sensitivity. The highest number of roots regenerated from *aurea* explants. Fewer roots were formed on WT and *hp* explants respectively (Fig. 5).

The effect of red and far-red light irradiation on root formation on etiolated hypocotyl segments cultured on root inducing medium

As noted above, explants excised from etiolated hypocotyls of tomato seedlings form roots when grown in darkness on SIM (in the presence of 10 µmol L^{-1} BAP and 1 µmol L^{-1} IAA), which favours shoot formation if explants are grown under irradiation. Moreover, it was found that rooting is stimulated with 5-min-long daily pulses of R, the effect of which is reversed by subsequent irradiation with FR (Fig. 3). We attempted to check whether this kind of photoreversible control is also present if etiolated explants are cultured on a medium that favours root formation (RIM) or is specific if rooting occurs in the presence of cytokinin. It was observed that both WT and mutant explants cultured on a medium supplemented with 1 µmol L^{-1} IAA as the only growth regulator form abundant roots irrespective of genotype. No signifi-

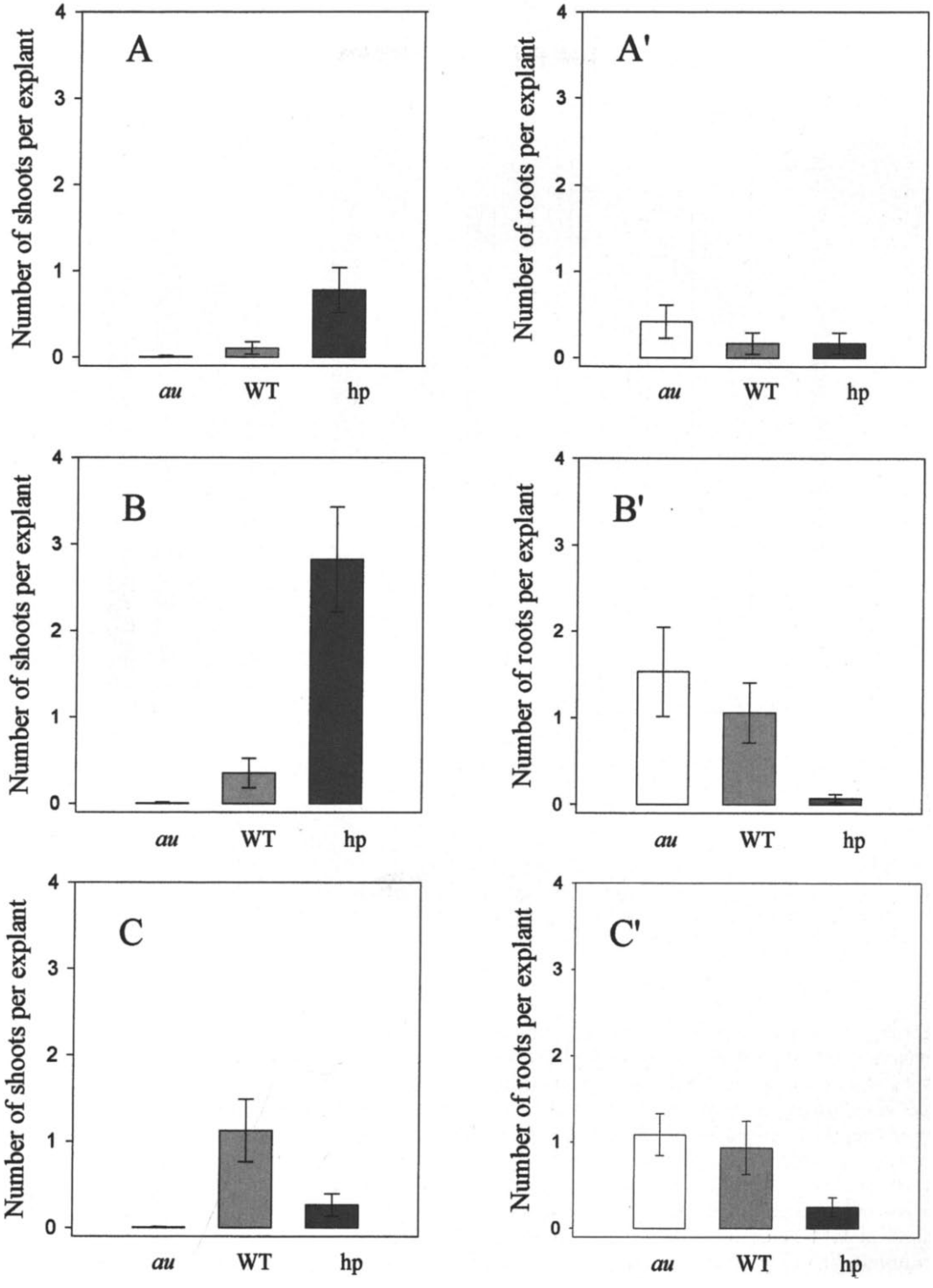

Fig. 5: Shoot and root organogenesis on explants isolated from etiolated WT, *au* and *hp* seedlings cultured on SIM (10 µmol/L BAP and 1 µmol/L IAA). Explants were grown in darkness (A) or were irradiated daily with 2-h-long pulses of white (WL) (B,B′) and red (R) (C,C′) light.

cant differences in the number of roots formed were found between explants that obtained daily R, R+FR and FR pulses, except WT, where slight red-light inhibition and far-red light stimulation of rooting was observed (Fig. 6).

Discussion

We have shown that efficient shoot formation on tomato seedling explants occurs only in light conditions. We have

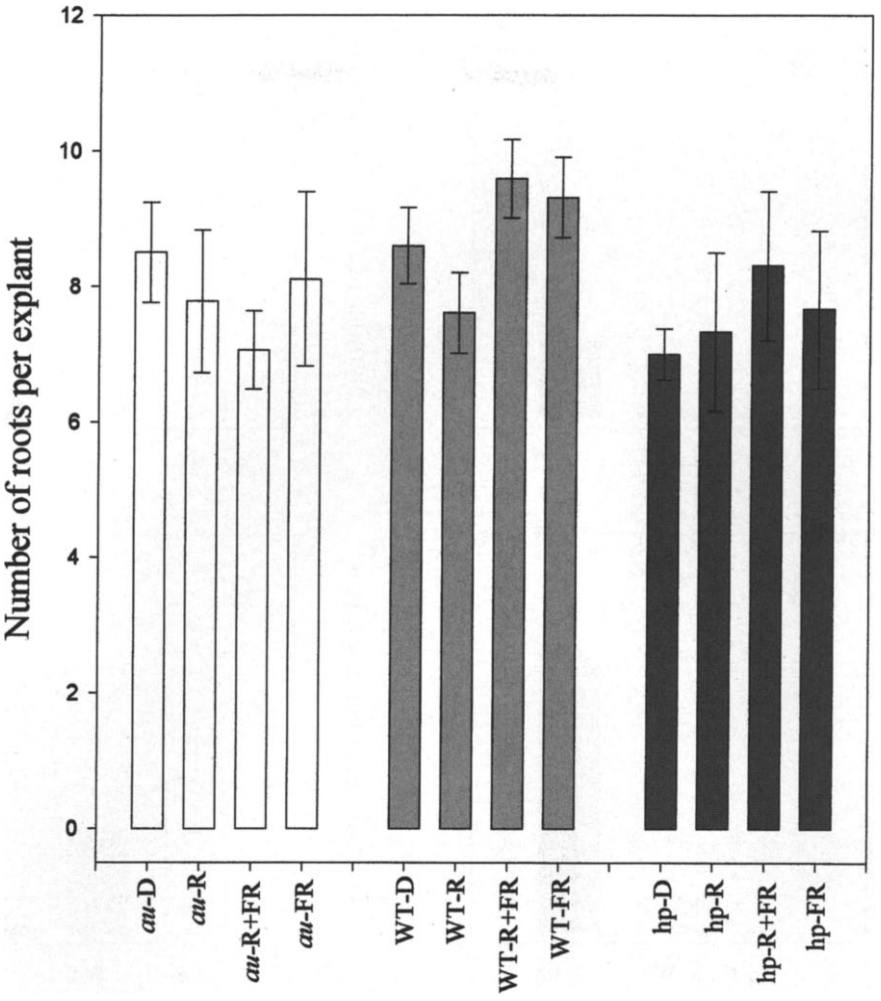

Fig. 6: Root organogenesis on explants isolated from etiolated WT, *au* and *hp* seedlings cultured on RIM. Explants were grown in darkness (D), or were irradiated either with 5-min-long red (R), 5-min-long R followed by 10-min-long far-red (FR) or 5-min-long FR pulses. Explants were cultured on RIM containing 1 μmol L^{-1} IAA.

also found that photomorphogenic mutations significantly affect shoot formation. Hypocotyl explants of the light-insensitive mutant *aurea* exhibit an extremely reduced shoot-forming capacity. The *high pigment* mutant, on the other hand, shows increased regeneration potential when compared with wild-type plants. This observation suggests that light perception is necessary for shoot-formation in tomato.

Explants isolated from green (deetiolated) seedlings of WT and *hp* placed on SIM underwent shoot organogenesis only in light conditions. Explants excised from hypocotyls of *aurea* (which preserves the etiolated phenotype when grown in light) do not form shoots either in darkness or in light. Therefore, the regeneration of shoots requires both a deetiolated phenotype of seedlings before explant dissection and light conditions during explant culture.

In contrast to our results, Kraepiel et al. (1995) have found that hypocotyl segments of wild-type and two photomorphogenic mutants of tobacco, *pew1* (chromophore mutant, deficient in all phytochrome types) and *pew2* (specifically deficient in phytochromes expressed in darkness), developed calli and shoots both in light and in darkness when cultured in the presence of exogenously applied auxin and cytokinin. Under white light conditions double mutant *pew1/pew2* developed etiolated shoots as did the wild-type in darkness (Kraepiel et al., 1995).

We have found that etiolated hypocotyl explants cultured in darkness on shoot inducing medium (SIM) form roots, and that root regeneration is under photoreversible control of red and far-red light. Therefore, we suggest that the phytochrome system is involved in the control of rooting in this experimental system. However, red/far-red light effects seem to be obligatory only if etiolated explants are cultured on SIM. Root regeneration from etiolated hypocotyl segments cultured on a medium supplemented with auxin as the only growth regulator (which favours rooting) is not significantly affected by irradiations analogous to those that effectively modulated rooting on SIM. Thus, we suggest that the etiolated/deetiolated phenotype affects competence of seedling explants for shoot or root differentiation. While shoot regeneration was obtained only from deetiolated explants, etiolated

tissues seem to be predetermined for root formation. A preformed pattern of competence is stimulated by short red light pulses that can be replaced by root promoting auxin treatment.

The phytochrome effect on adventitious root formation was reported by Pfaff and Schopfer (1974), who found that rooting of mustard (*Sinapis alba*) seedlings is stimulated by the Pfr form of phytochrome. It was suggested that phytochrome is necessary for the production of a hormonal rooting factor in cotyledons (Pfaff and Schopfer, 1974).

The results of experiments where pulses of WL or RL were applied to etiolated hypocotyl fragments reveal that 2 h of light daily is the minimum time of irradiation for shoot formation. Production of both roots and shoots was observed under this light treatment. However, the root/shoot ratio was dependent on the plant genotype used. It was highest in the *aurea* mutant that produced only roots, intermediate in WT and the lowest in *hp* mutant. Therefore, we conclude that photosensitivity of plants may be an important factor regulating both the quantitative effect of organogenesis (the number of organs per explant) and the pattern of differentiation (the kind of organs produced by explants). Root formation observed on SIM seems to be affected by light in a dual way. It is stimulated by short daily irradiations with RL and is inhibited by long (minimal 2-h-long) irradiations with WL or RL.

Acknowledgements

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