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Original article

Acetylcholinesterase activity in *Lycopersicon esculentum* and its phytochrome mutants

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Abstract

Using the radiometric method, the activity of acetylcholinesterase (AChE, E.C. 3.1.1.7) was studied in seedlings of wild type (WT) and of phytochrome mutants of tomato (*Lycopersicon esculentum* Mill.). The activity of this enzyme was inhibited by an excess of substrate and by two well-known inhibitors of animal AChE, eserine and neostigmine. The activity of AChE was found in all etiolated organs as well as in light-grown seedlings. Under both conditions, the highest level of the enzyme activity was detected in cotyledons and the lowest one in root tissue. The enzyme activity was phytochrome-controlled. In WT etiolated seedlings red (R) light decreased AChE activity, whereas far red (FR) light abolished the red light effect. Furthermore, in light-grown WT seedlings the level of the enzyme activity was about twice higher than in etiolated plants. However, in the *aurea* phytochrome mutant of tomato, deficient in biosynthesis of a phytochrome chromophore, light had no effect on the AChE activity. In case of *hp*, *fri* and *tri* mutant seedlings, R and FR affected the AChE activity in a different way. Based on our results, we suggest that the type I of phytochrome is involved in the regulation of AChE activity. The type II of this photoreceptor influences the rate of the AChE synthesis de novo.

Keywords: Acetylcholine; Acetylcholinesterase; *Lycopersicon esculentum*; Phytochrome; Photomorphogenic mutants

1. Introduction

The presence of acetylcholine (ACh), an animal neurotransmitter and a member of the so-called cholinergic system, has been found in tissues of many species of plants [6,8,31,34,35,40]. In these organisms, the activity of choline acetyltransferase (ChAT, E.C. 2.3.1.6) [2,31,35] and acetylcholinesterase (AChE, E.C. 3.1.1.7) [4,14,18], enzymes that take part in the synthesis and degradation of ACh, have also been described. There is also evidence indicating a presence of ACh receptors (AChR) in plant cells, whose mechanism of action seems to be similar to that of AChR in animal cells [17,36]. It is postulated that plants possess a cholinergic system similar to that functioning in animal tissues [36].

In spite of more than 30 years of investigation, the mechanism of action of the plant cholinergic system still remains unclear [35]. The best-known phenomenon is the effect of red (R) and far red (FR) light on ACh levels in etiolated plants tissues. It has been shown that R and FR, absorbed by the photomorphogenic pigment phytochrome, increase or decrease ACh content in plants, respectively. It is postulated that phytochrome can regulate the activity of enzymes that take part in the synthesis and degradation of ACh [8,15,40]. Until now there has been only a limited number of papers concerning the properties of plant ChAT and AChE and the regulation of their activity by light [6,35]. In the present paper, we describe the properties of AChE from tomato seedlings. We also show that the activity of this enzyme is under phytochrome control. Using different phytochrome mutants, we have been able to show which type of this photoreceptor is responsible for the regulation of AChE activity.

2. Results

The influence of substrate concentration on the AChE activity is shown in Fig. 1. ACh was used at different concentrations, ranging from 0.1 to 6 mM. At lower concentrations

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; *au*, *aurea*; ChAT, choline acetyltransferase; FR, far red light; *fri*, far red light insensitive; HIR, far red light insensitive; *hp–1*, high pigment–1; LFR, low fluence response; phyA, type I of phytochrome; phyB1; B2; E; F, type II of tomato phytochromes; R, red light; *tri*, temporarily red light insensitive.

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(from 0.1 to 1 mM), the activity of AChE increased linearly. The relation between substrate and AChE activity was not linear at the higher concentrations of ACh. Moreover, at concentrations higher than 6 mM, inhibition of AChE was observed (data not shown). The K_m of tomato AChE for ACh, as determined graphically from Lineweaver– Burk equation, was 0.75 mM.

AChE was sensitive to eserine and neostigmine. The effect of these inhibitors on AChE in etiolated wild type (WT) seedlings of tomato is shown in Table 1. Addition of 0.01 mM eserine caused 18.5% inhibition of the enzyme. Ten times higher concentration of eserine inhibited AChE only a little bit stronger (reaching 31%), but the inhibitory effect of neostigmine on AChE activity was more powerful. At both concentrations studied (0.01 and 0.1 mM), neostigmine inhibited AChE activity by 90% and 93%, respectively. The I_{50} value for neostigmine was 4 μM.

Fig. 2 illustrates the distribution of the AChE activity in organs of etiolated WT tomato seedlings. The highest activity of AChE was found in whole seedlings and in cotyledons. In hypocotyls and root tissue, the enzyme activity was about 3.5 times lower than that in cotyledons. A similar distribution of AChE activity was observed in light-grown tomato seedlings of the same ages (data not shown).

The effect of continuous white light on AChE activity in the WT cotyledons and different tomato phytochrome mutants was studied (Fig. 3A–C). The level of AChE activity in light-grown WT seedlings was about 70% higher than in

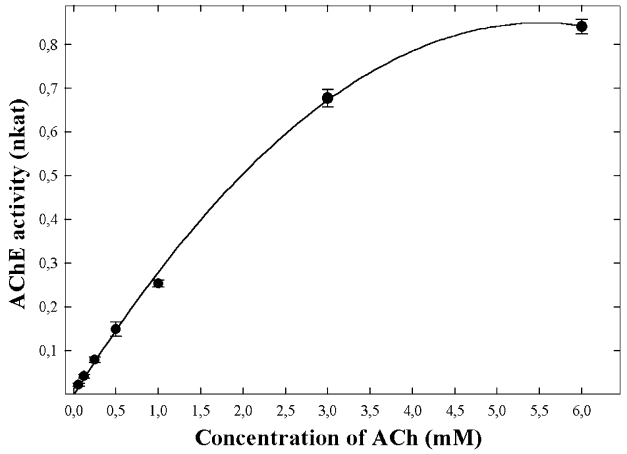

Fig. 1. Effect of ACh concentration on AChE activity in tomato seedlings.

Table 1
The effects of eserine and neostigmine on the cholinesterase activity (nkat g^{-1}FW, nanokatals g^{-1} of fresh weight)

	Activity of AChE (nkat g^{-1} FW)	% of inhibition
Control	0.3430 ± 0.0472	–
Eserine (0.01 mM)	0.2790 ± 0.0358	18.58
Eserine (0.1 mM)	0.2363 ± 0.0198	31.10
Neostigmine (0.01 mM)	0.0332 ± 0.0012	90.35
Neostigmine (0.1 mM)	0.0218 ± 0.0008	93.63

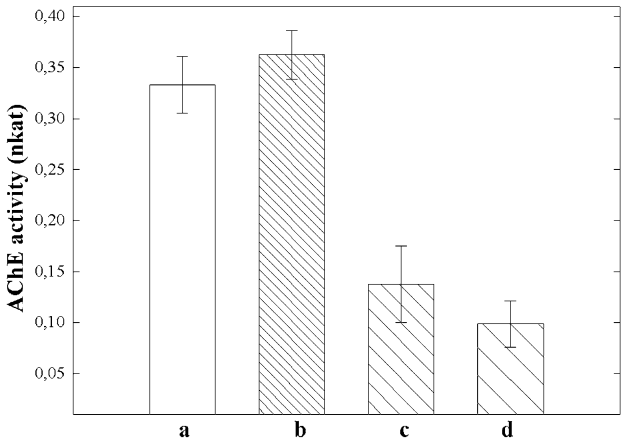

Fig. 2. AChE activity in various etiolated WT tomato organs. (a) Whole etiolated seedlings; (b) cotyledons; (c) hypocotyls; (d) roots.

etiolated ones of the same age. However, in the phytochrome-deficient *au* (*aurea*) mutant (deficient in biosynthesis of chromophore) the level of AChE activity was nearly the same in cotyledons of both etiolated and green seedlings (Fig. 3A). On the other hand, in the high pigment (*hp*) mutant seedlings, which exhibit exaggerated phytochrome responses, a higher activity of this enzyme was observed both in light-grown and etiolated plants, compared to the *au* mutant, but similarly, no significant differences were found in cotyledons of both etiolated and green seedlings (Fig. 3A).

Significant differences have been found between the activity levels of AChE in phyA–deficient far red light insensitive (*fri*) mutants and its WT seedlings grown either in light or in darkness (Fig. 3C).

The activity level of AChE was about 55% lower in the phyB1–deficient *tri* mutant than in WT seedlings grown in continuous light (Fig. 3B). The activity value in etiolated plants was about 50% higher when compared to the one determined in WT (Fig. 3B).

The effect of R and FR on AChE activity in the WT cotyledons and various tomato phytochrome mutants was also determined (Fig. 4A–D). The level of AChE activity was constant in darkness (etiolated seedlings, 10, 30, 50 min of dark treatment) whereas FR slightly stimulated the AChE activity (data not shown). It was found that in WT tomato seedlings 10 min exposure to R decreased the level of the AChE activity by about 20% (even if the material was collected after 20 min of the dark treatment) (Fig. 4A, columns a–c). However, the irradiation with FR (20 min) immediately after R abolished the inhibitory effect of the first light wavelength (Fig. 4A, column d). The same light treatment had no significant effect on the AChE activity in etiolated *au* mutant tissues (Fig. 4A, columns a'–d').

In tissues of *hp* mutant seedlings, the AChE activity level was higher in control plants (30%) compared to WT (Fig. 4B, columns a, a'). Contrary to WT plants, a 10 min exposure to R caused the increase (20%) of enzyme activity in tissues of this mutant (Fig. 4B, columns b, b'). After 20 min of treat-

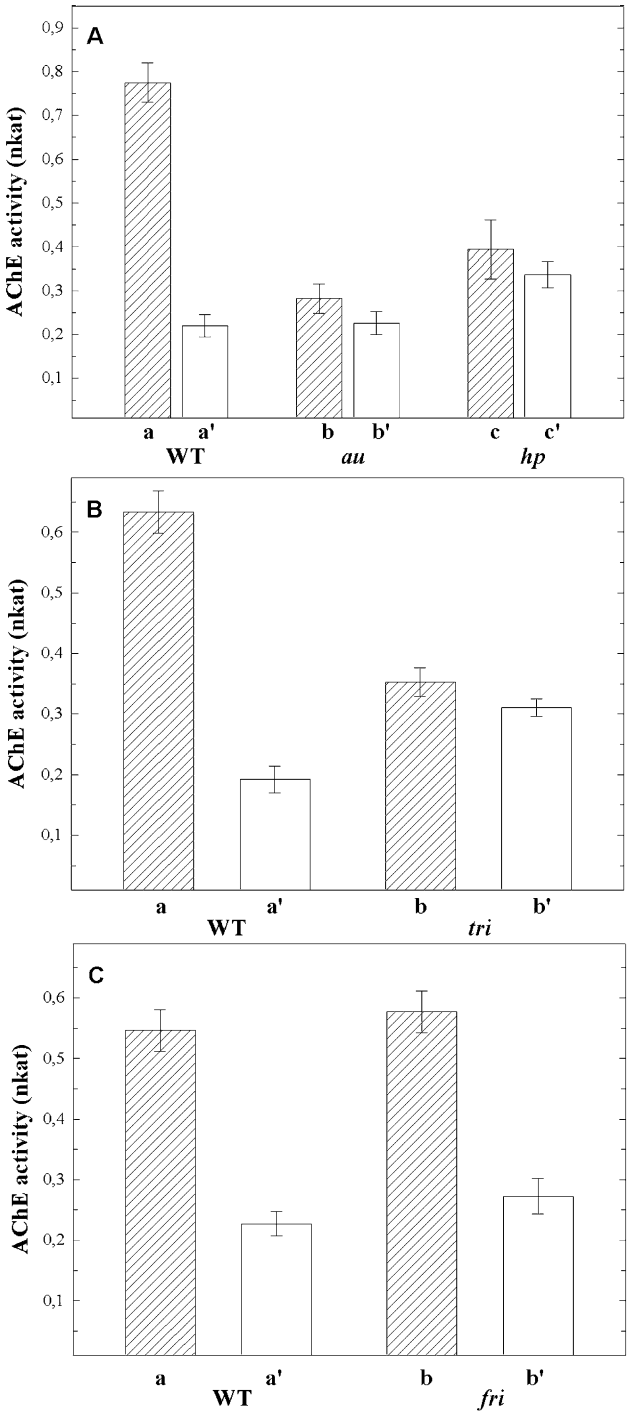

Fig. 3. AChE activity in cotyledons of phytochrome tomato mutants and their corresponding WT phenotypes cultured in continuous white light or in darkness. (A) AChE activity in tissues of *au* and *hp* mutants; (B) AChE activity in tissues of *fri* mutants; (C) AChE activity in tissues of *tri* mutants (a, b, c, d: light-grown seedlings; a', b', c', d': etiolated seedlings).

ment with R, the AChE activity decreased and was lower than that found in the control seedlings (Fig. 4B, columns c, c'). Moreover, the effect of R could not be reversed with 20 min of FR, applied directly after R (Fig. 4B, column d').

The AChE activity in the etiolated cotyledons of the phyA–deficient *fri* mutant and its corresponding WT seedlings decreased after a 10 min treatment with R, by 23% and 32%, respectively (Fig. 4C, columns a, b, a', b'). This effect was abolished only in WT seedlings by FR treatment following directly R (Fig. 4C, columns d, e), whereas in *fri* mutant seedlings no influence of FR on the AChE activity was found (Fig. 4C, columns d', e').

It was observed that, in cotyledons of the *tri* mutant (deficient of phyB1) and its isogenic WT seedlings, 10 min of R caused a decrease of the AChE activity by 47% and 27%, respectively (Fig. 4D, columns a, b, c, a', b', c'). In this mutant, the effect of R was partly reversible with FR (20 min) used directly after R (Fig. 4D, columns d', e'), while in WT seedlings the effect of R was abolished by FR (Fig. 4D, columns d, e).

The *tri* mutant is insensitive to continuous red light during the first 2 d only upon transition from darkness to this light conditions [11,13]. Thus additionally we studied, whether the activity level of AChE was changed in 5–d–old *tri* mutants and their WT tomato seedlings grown in darkness and then exposed to continuous R for 1 or 2 d. The results of this experiment are shown in Fig. 5. It was observed that in the WT seedlings the AChE activity increased on the first and second days of continuous R exposure by 50% and 64%, respectively. However, in the phyB1–deficient *tri* mutant tissues continuous R did not influence the activity of the studied enzyme in the first 2 d of deetiolation.

3. Discussion

It has been shown that the radiometric method is very sensitive and convenient for determination of AChE activity in plant tissue [19]. An additional advantage of this method is the possibility of quick and simple determination of the enzyme activity without its isolation and purification. Furthermore, the results obtained by radiometric method are similar to those obtained by using in vitro assays [19].

Using radiometric method, we found that the effect of substrate concentration on the AChE activity was similar to that described for other plant species [6,35]. At lower substrate concentrations (below 0.5 mM) a stimulation of the enzyme's activity was found. However, at higher concentrations (between 1 and 6 mM), inhibition of AChE activity was observed (Fig. 1). A similar effect of substrate concentration on the enzyme activity was also been described in different plant species by Kasturi and Vasantharajan [10], Mansfield et al. [18], Ernst and Hartmann [3], Vačkova et al. [37], Roshchina [30] and Kęsy et al. [14].

The enzyme affinity for the substrate (K_m = 0.75 mM), reported here for tomato, is higher than that described for other plant species [35]. However, most of the investigations conducted so far on the plant AChE were performed on the purified enzyme, using a calorimetric technique. Therefore, in all of these studies instead of ACh, acetylthiocholine was used as substrate.

It is well known that both animal and plant AChEs are sensitive to some ammonium compounds [35]. One of the most powerful and specific inhibitors of this enzyme is neo-

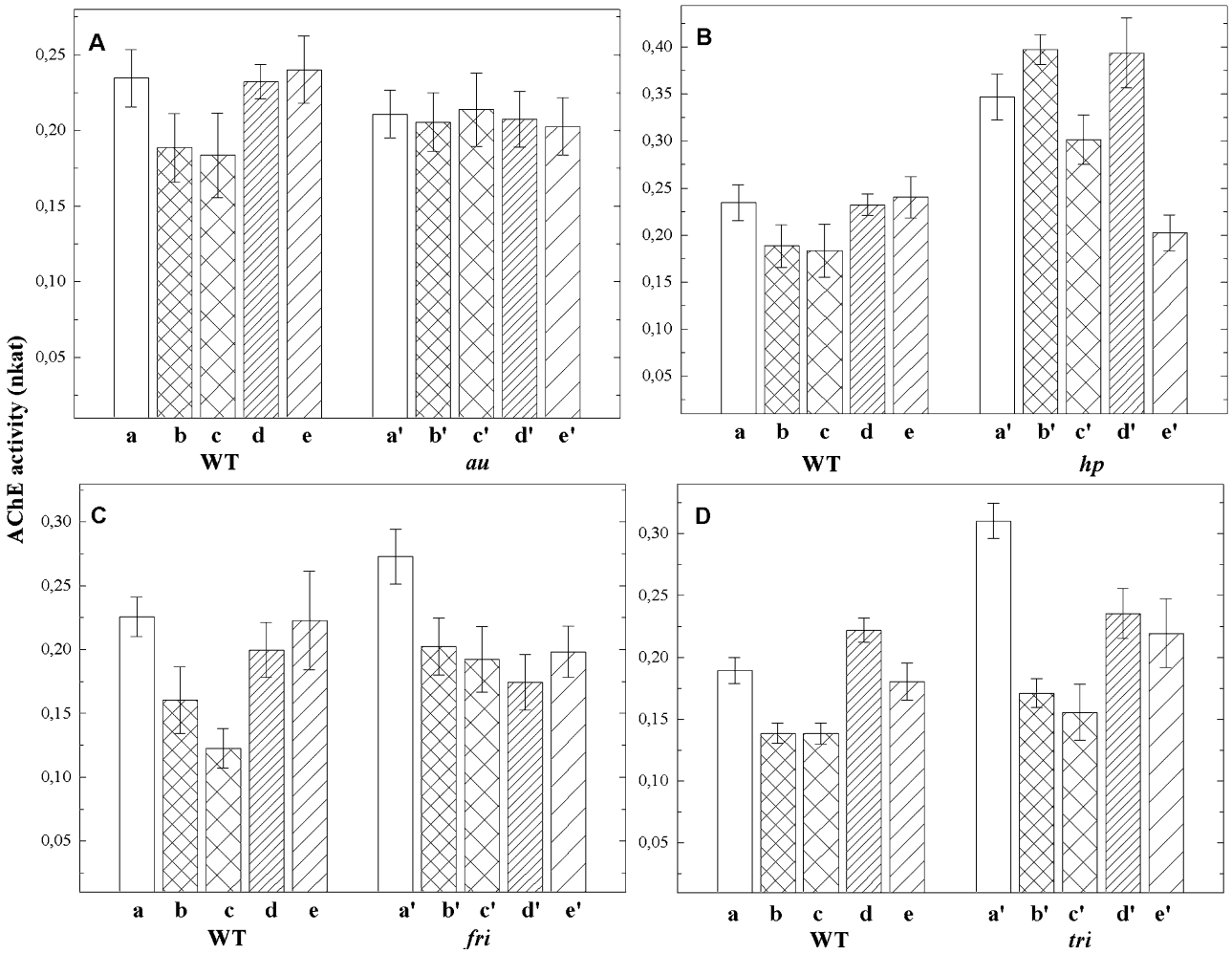

Fig. 4. AChE activity in tissue of different phytochrome tomato mutants and their isogenic WT seedlings, which were grown in dark or were irradiated with R and with FR. (A) AChE activity in tissues of *au* tomato mutants; (B) AChE activity in tissues of tomato *hp* mutants; (C) AChE activity in tissues of *fri* tomato mutants; (D) AChE activity in tissues of *tri* tomato mutants (a, a': etiolated seedlings (control); b, b': etiolated seedlings irradiated for 10 min with R (material collected immediately after irradiation); c, c': etiolated seedlings irradiated for 10 min with R (material collected 20 min after irradiation); d, d': etiolated seedlings irradiated for 10 min with R and 20 min with FR (material collected immediately after irradiation); e, e': etiolated seedlings irradiated for 10 min with R and 20 min with FR (material collected 20 min after irradiation)).

stigmine. We have shown that this compound, even at relatively low concentration (0.01 mM), inhibited AChE activity very significantly (90%) (Table 1). A similar effect of neostigmine on AChE activity was found by Riov and Jaffe [29] and Kasturi and Vasantharajan [10]. The I_{50} values for neostigmine reported here (4 μM) are of an identical magnitude as those for other plant AChEs [35]. Eserine was less active in inhibiting the AChE activity. A probable cause may be its lower affinity for the enzyme catalytic centre than in case of neostigmine [29].

The activity of AChE in tomato seedlings varied depending on tissue and conditions of plant growth. As in the case of other plant species [10,19], the AChE activity was found in all organs of dark- and light-grown seedlings (Fig. 2). The highest level of its activity was found in cotyledons, where also the highest level of ACh was detected [40]. Comparing the same organs, a higher level of AChE was found in light-grown seedlings than in etiolated ones. In 7–d–old WT green seedlings, the activity was 30.74 nM min^{-1} g^{-1} of fresh

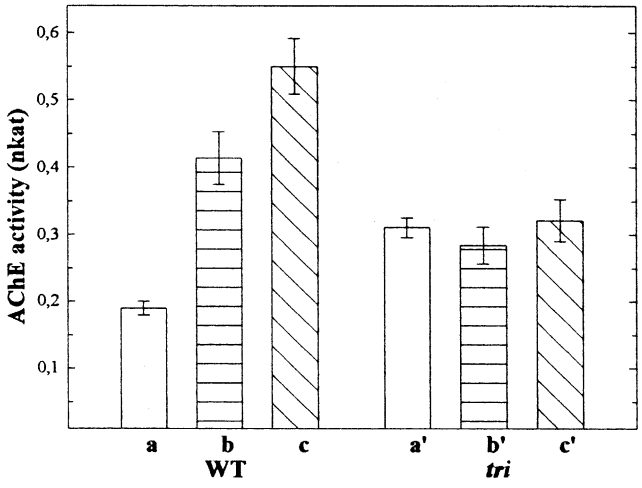

Fig. 5. AChE activity in tissues of the *tri* tomato mutant and its WT (a: cultured in dark (control); b: irradiated for 1 d with R; c: irradiated for 2 d with R).

weight but in etiolated seedlings was 12.35 nM min^{-1} g^{-1} of fresh weight. Described relation was observed in all the cultivars of both WT and phytochrome mutant (except *au*) seedlings of tomato (Fig. 3A). The level of the AChE activity found in tomato seedlings was rather high in comparison to other plant species. Using the same method Miura et al. [19] have shown that in a number of species in light-grown plants, the AChE activity varied between 37 (*Brassica oleracea*) and 0.34 nM min^{-1} g^{-1} of fresh weight (*Forsythia* sp.). Even in *Solanum melongena*, a plant species belonging to the same systematic group as tomato (*Solanaceae*), the level of AChE was five times lower (11 nM min^{-1} g^{-1} of fresh weight) when compared to light-grown tomato seedlings. These results indicate that continuous white light can influence AChE de novo synthesis. The similar effect of white light on de novo synthesis of AChE protein in pea seedlings has been described by Kasturi [9].

The influence of R and FR on the activity of the studied enzyme was found in WT tomato seedlings in all the variants tested (Fig. 4A–D). In the case of 7–d–old WT etiolated seedlings, 10–min–long R treatment inhibited, whereas FR stimulated the AChE activity. The observed phenomenon of photoreversibility is an example of a typical low-energy reaction of a phytochrome (LFR–low fluence response) [22] and confirms its participation in regulation of the AChE activity in tomato. The time during which the enzymatic reaction was studied was too short (30 min) for induction of the AChE de novo synthesis [5]. Therefore, we believe that in case of short-term experiments performed on the etiolated tomato seedlings, R and FR light are able to change such biochemical properties of the enzyme as its affinity to substrates and sensitivity to ions. The opposite effect of both R and FR on the AChE activity was described by Kasturi [9] and Kim et al. [15] in pea and oat tissues, respectively. Furthermore, Kim et al. [15] found that R and FR light might change the concentration of intracellular Ca^{2+}. On this basis they proposed that red light, acting through the phytochrome-dependent pathway, could inhibit AChE activity via increasing cytosolic Ca^{2+} concentration [9]. The inhibitory effect of calcium ions on the AChE activity in vitro was found by Kęsy et al. [14].

To test the role of the phytochrome in the regulation of the AChE activity, the experiments with *au* and *hp* mutants were performed. The *au* mutant is deficient in the phytochromobilin synthase activity that takes part in biosynthesis of a phytochrome chromophore [32,33]. Therefore, it is unable to synthesize functional phytochromes and consequently lacks both response components in the R and FR regions of the spectrum [11]. We have shown that in the case of *au*, all of the light treatments applied (white, R and FR) had no effect on both the AChE activity (see Figs. 3A, 4A) and the ACh level [40]. On this ground, we believe that phytochrome is involved in the regulation of ACh/AChE system in tomato. This hypothesis was confirmed in our further studies performed on *hp* mutant, which showed an enhanced R/FR reversible response compared with WT. However, *hp* plants exhibited a strong amplification of both the LFR and high irradiance response (HIR) response components during deetiolation [12,23–26]. Its phenotype suggests that *HP* gene encodes a negative regulator of a phytochrome signal transduction [13,21]. We have found that, contrary to WT, in *hp* tissues an increase in the AChE activity directly after the exposure to R was present (Fig. 4B). Moreover, in previous paper [40] we showed that only in tissues of *hp* an increase in the ACh level after red light treatment took place. However, after the R treatment was over, the AChE activity decreased and was lower than that observed in control seedlings. Moreover, FR used directly after R did not abolish the influence of this light wavelength on the AChE activity. The results mentioned above do not provide an answer for the question on which pool of phytochromes is involved in the regulation of AChE activity. Thus, additional experiments on *fri* (phyA–deficient mutant) and *tri* (phyB1–deficient mutant) mutants were included in the study.

The phyA–deficient mutant (*fri*) was not immunochemically and spectrophotometrically detectable PHYA [11,16,39] and lacks the LFR while retaining normal HIR [13]. We have observed that in cotyledons of *fri* mutant, red light decreased the AChE activity and FR did not reverse its influence (Fig. 4C). We believe that this effect was due to a consequence of the absence of type I phytochrome-dependent (phyA), photoreversible LFR [13]. On the other hand, we did not find any significant differences between the activity levels of AChE in *fri* mutant and its WT seedlings grown either in light or in darkness (Fig. 3C). Probably it is an effect of phyB1 on the AChE activity. We do not exclude a possible involvement of other type II phytochromes such as phyB2, phyE, phyF of tomato [1,28,32] in regulation of AChE activity. These phytochromes may be present both in dark- and light-grown seedlings [22].

Tissues of the phyB1–deficient mutant (*tri*) contain normal amounts of phyA and only one of two phyB phytochromes (phyB2), while the levels of other type II phytochromes are not changed [7,27,38]. Two *PHYB* genes are independently expressed in organ-specific manner [7]. The *tri* mutant retains the LFR, but lacks the HIR (no R/FR reversible reaction of phytochromes) [13]. We have observed that in cotyledons of the *tri* mutant a 10–min–long R treatment decreased the AChE activity, however, this effect was partly reversed with FR used directly after red light (Fig. 4D). These data suggest that phyB1 or other type II phytochromes via LFR take part in regulation of the activity level of AChE. The *tri* mutant is insensitive to continuous red light during the first 2 d only upon transition from darkness to continuous red light [11,13]. Thus additionally we investigated, whether the level of the AChE activity was changed in *tri* mutants grown under this irradiation regime. In this experiment, it was established that continuous red light did not influence the AChE activity during first 2 d after the seedlings were transferred from darkness to the red light. Moreover, it was found that the AChE activity in WT seedlings increased during subsequent days of red light treatment (Fig. 5).

Taking all our results into account, we believe that AChE from tomato can be regulated by a phytochrome in two different ways. We propose that, in light-grown tomato plants type II phytochromes dependent LFR have an effect on the rate of the de novo synthesis of this enzyme, whereas in etiolated seedlings both type I and type II phytochromes, via LFR and HIR, respectively, were involved in control of biochemical properties of the enzyme (e.g. its affinity to substrates and sensitivity to ions).

The results obtained in this study confirm the phytochrome engagement in the regulation of the AChE activity, the enzyme that is involved in regulation of the ACh degradation both in animals and in plants [35]. The presence of ACh, and the involvement of the phytochrome in regulation of the ACh concentration in tomato seedlings were described previously [40]. Results of experiments that are currently in progress suggest, that besides AChE, the activity of ChAT seems to be also phytochrome-controlled and involved in regulation of ACh turnover in tomato seedlings.

4. Methods

4.1. Plant material

All studies were performed on 7–d–old, etiolated or green, WT or mutant seedlings of tomato (*Lycopersicon esculentum* Mill.): *au*, *hp–1* (high pigment–1) cv. Ailsa Craig (AC), *fri* cv. MoneyMaker (MM), *tri* (temporarily red light insensitive) cv. GT. Tomato seeds were sterilized for 2 min with a solution of sodium perchlorate (2%,v/v), washed vigorously and soaked for 2 h in sterilized distilled water. After such a treatment, seeds were sown into plastic containers with 10 times diluted Murashige and Skoog [20] nutrient solution supplemented with 10 mM potassium nitrate and 0.8% agar. Cultivation was performed in growth chambers at 26 °C, in darkness or under white fluorescent continuous light (0.8418 μmol m^{-2} s^{-1}). A fraction of the dark-grown seedlings was treated with R and FR light. All experiments performed on etiolated seedlings were conducted under safe green light.

4.2. Light sources

Dark-grown seedlings were exposed to monochromatic irradiation with R and FR, using a specially constructed projector with a revolving holder, which contained as a light source a 250 W lamp (PHILIPS, IR–175R–PAR) with interference filters cutting out 730 nm. The fluence rates were 2.08 and 4.002 μmol m^{-2} s^{-1} for R (660 nm) and FR (730 nm) light, respectively.

4.3. Determination of AChE activity

Determination of AChE activity was carried out using the radiometric method described previously by Miura et al. [19], with our own modifications. During degradation of [^{14}C]ACh, choline and a residue of isotope-labelled acetic acid are released. This residue can be easily absorbed on and released from a column packed with ion-exchange resin. Next the radioactivity of [^{14}C]acetic acid is measured and AChE activity is determined on this basis. Tomato seedlings were cut into pieces approximately 1 × 1 mm. Twenty-five milligram of tissues were washed several times in 150 μl 0.1 M potassium phosphate buffer (pH 7.4). Plant material was preincubated for 30 min at 30 °C in the same buffer. The enzymatic reaction was started after adding into the medium 100 μl of 3 mM ACh and 50 μl of [^{14}C]ACh (Amersham Buchler, Braunschweig, Germany, 2.22 GBq/mmol). The amount of isotope was sufficient for detection of a reaction product. The reaction was carried out at 30 °C and was stopped after 60 min by adding 1 ml of dioxan (POCh, Gliwice, Poland) to the incubation medium. Eleven millilitre of a mixture consisting of dioxan/phosphate buffer (1/1, v/v) was added to the incubation tubes. The mixture was shaken and put onto the head of an ion-exchangeable column (5 ml volume) containing Amberlite IR–120 (Serva, Heidelberg, Germany) preconditioned with the same mixture. Before the experiments concerning the kinetic properties of AChE were started, the elution profile of [^{14}C]acetic acid removed from a column was determined. Thus, a small amount of KOH was added to the medium containing 100 μl of 3 mM ACh, 50 μl of [^{14}C]ACh and 150 μl of 100 mM potassium phosphate buffer (pH 7.4). After 1 min of a nonenzymatic basic hydrolysis of ACh, pH of the medium was adjusted with HCl to ca. 7.5. Afterwards, 25 ml of a mixture consisting of dioxan/potassium-phosphate buffer (1/1, v/v) were added to the solution containing degraded [^{14}C]ACh, shaken and loaded onto the column. During elution, we found that only the first 12 ml fractions contained [^{14}C]acetic acid (data not shown). Therefore, in all further experiments these fractions were collected. To 2 ml of mixture taken from each tube, a 5 ml of OptiPhase 'Hi Safe' universal scintillation cocktail (Fisons Chemicals, Loughborough Leics, UK) was added. To measure the radioactivity, the liquid scintillation counter Wallac 1409 (Wallac Oy, Turku, Finland) was used. The degree of [^{14}C]ACh degradation was strictly dependent on the relationship between the amount of tomato tissue, the hydrolysis in time of [^{14}C]ACh, and the concentration of ACh added to the incubating medium (data not shown). This dependency between the amount of tomato tissue and the degree of [^{14}C]ACh degradation was linear (data not shown). The larger amount of the tissue, the higher the rate of [^{14}C]ACh degradation observed. Since the tomato seedlings are very light, only 25 mg (fresh weight) of their tissue were used in all further experiments. The relationship between the time (5, 15, 30, 60, 90 and 120 min) of the tissue incubation in the reaction mixture and the efficiency of acetic acid residue release from radioactive ACh was also established. The reaction showed a linear character (data not shown). Based on these data, a 60–min–long incubation period for plant material in the reaction mixture was applied in the experiments. All measurements were done in three replicates and each experiment was repeated at least three times. Data are presented as means together with standard deviation (S.D.).

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