Effect of a Cytokinin Antagonist on Cytokinin and Light-Dependent Amaranthin Synthesis in *Amaranthus Tricolor* Seedlings

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Abstract. In *Amaranthus tricolor* seedlings, amaranthin synthesis can be induced under the effect either of a cytokinin or of white light. The 3-methyl-7-(n-pentylamino)pyrazolo(4,3-d)pyrimidine (PAMPP), a cytokinin analog that strongly inhibits the growth of tobacco callus, antagonizes the stimulating effect of cytokinin as well as stimulation by light. In dose-response terms, the inhibitory effect of PAMPP was described as competitive with respect to N^6-benzyladenine (b^6Ade) or light. The inhibition by PAMPP of the b^6Ade amaranthin test response or the inhibition by this cytokinin analog of the light amaranthin test response were both reversed by either subsequent light or b^6Ade treatment.

The use of cytokinin antagonists is of considerable interest in clarifying the mechanism of action of the cytokinins. Hecht et al. (1971) reported that several substituted 3-methylpyrazolo(4,3-d)pyrimidine analogs are highly potent inhibitors of tobacco callus growth. The first compound described was the 3-methyl-7-(3-methylbutylamino)pyrazolo(4,3-d)pyrimidine. The most potent in a series of 7-substituted 3-methylpyrazolo(4,3-d)pyrimidines was the pentyl derivative (Skoog et al. 1973). This compound also inhibited the growth of tobacco cell suspensions (Gregorini and Laloue 1980, Laloue 1980).

Synthesis of other analogs in the substituted-pyrrolo (2,3-d)pyrimidine series was first reported by Skoog et al. (1975). These compounds were tested as cytokinins and anticytokinins in the tobacco bioassay. Little is known about the activity of analogs in plant systems other than cultured callus or suspension cultured cells. Iwamura et al. (1979) reported the effect of sixteen 4-substituted-2-methylpyrrolo(2,3-d)pyrimidines in the *Amaranthus* bioassay. Only the 4-hydroxyethyl derivative exhibited inhibitory activity. The effect of 1 μM i[6]Ade was partly reversed by this analog at concentrations higher than 10 μM. These observations led us to consider the effect on amaranthin synthesis of PAMPP, a cytokinin analog recognized as being particularly active on the growth of tobacco callus (Skoog et al. 1973).

Amaranthus tricolor seedlings, which synthesize only one betacyanin, amaranthin, were used for this study (Colomas 1976). Large quantities of pigment are produced under the effect of a cytokinin or under white light (Colomas 1975). The object of the present study was to determine whether PAMPP was able to antagonize the stimulant effect of b[6]Ade or of light. A reciprocal antagonism between PAMPP and each of the two inductive factors and the reversal of the effect of the analog were investigated.

Materials and Methods

Amaranthus tricolor L. var. *bicolor ruber* Hort. seeds were placed at 26°C in the dark on filter paper soaked with distilled water. After 48 h, the seed coats were removed under a green safety light (Withrow and Price 1957). Fifty whole seedlings were transferred to a Petri dish containing 5 ml of a solution of b[6]Ade and/or the analog. Seedlings were maintained in these conditions for 16 or 24 h, either in darkness or under white light.

For reversion studies, two stepwise treatments of the seedlings were applied. Seedlings 48 h old were first grown for 16 h either on b[6]Ade and PAMPP solutions or exposed to light in the presence of PAMPP. They were then transferred and maintained for 16 h either on b[6]Ade solutions in darkness or on buffer solution under light conditions. Control experiments were performed by omitting PAMPP during the first part of the treatment.

Solutions of b[6]Ade or analog were usually prepared in a phosphate buffer (Na_2HPO_4-KH_2PO_4, pH 6.8) using the optimum Na^+/K^+ ratio of 10 meq/l, Na^+; 5 meq/l, K^+ (Elliott 1979). However, a series of experiments was carried out in the absence of K^+ ions. Seedlings were illuminated by fluorescent tubes (Mazdafluor, blanc super, 65 W), exhibiting an important emission band between 520 and 640 nm. The intensity of the light at the seedling level was 10, 20, or 40 $W.m^{-2}$, as stated for each experiment. Amaranthin was extracted by the procedure of Piattelli et al. (1969). Its absorbance was measured at 538 nm, and the concentration was calculated using a molar extinction coefficient of 5.66×10^4. In some experiments, the results were expressed as % inhibition relative to control amaranthin synthesis. All the experiments were repeated at least five times. Statements on significance are based on Student test at the 95% confidence level.
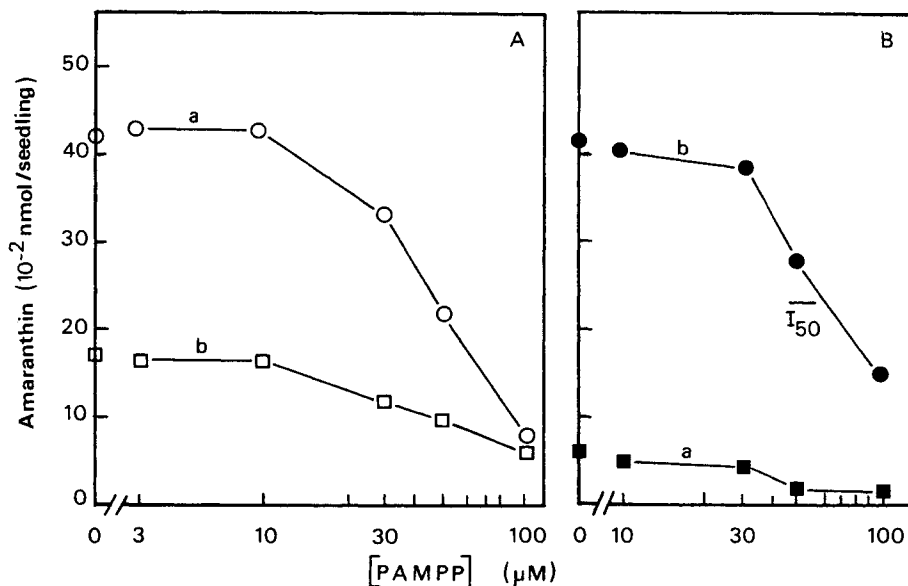
Fig. 1. Panel A: Effect of PAMPP on amaranthin synthesis induced under light treatment (10 $W.m^{-2}$ for 24 h) in the presence (curve a) or in the absence (curve b) of K^+ ions. Panel B: Effect of PAMPP on amaranthin synthesis induced in darkness in the presence of K^+ ions, without (curve a) or with (curve b) 5 μM b^6Ade. For each experiment controls were performed omitting PAMPP. I_{50} corresponds to concentrations of PAMPP that inhibit 50% of control amaranthin synthesis.

Results

Inhibitory Effect of PAMPP

The effect of the cytokinin analog was studied when the inducing factor was either light or b^6Ade. The quantity of amaranthin induced by light (10 $W.m^{-2}$ for 24 h) was enhanced 2.3-fold in the presence of K^+ ions (Fig. 1A, controls). The addition of PAMPP strongly inhibited light-dependent amaranthin synthesis at concentrations higher than 10 μM. It can be noted that inhibition was greater in the presence of K^+ ions (Fig. 1A, curve a), then 100 μM PAMPP induced 80% inhibition instead of 60% in the absence of K^+ ions (Fig. 1A, curve b). Because of these results, all the experiments were conducted in the presence of K^+ ions. In the dark, the low synthesis of amaranthin due to K^+ ions alone was strongly enhanced in the presence of 5 μM b^6Ade (Fig. 1B, controls). As illustrated in Fig. 1B, addition of PAMPP at concentrations higher than 30 μM induced an inhibition of amaranthin production. This inhibitory effect, which was barely detectable under conditions allowing only a low level of synthesis (Fig. 1B, curve a), became obvious in the presence of b^6Ade (Fig. 1B, curve b). Thus, for concentrations higher than 30 μM, amaranthin synthesis strongly decreased. The inhibition reached about 50% at 60 μM.
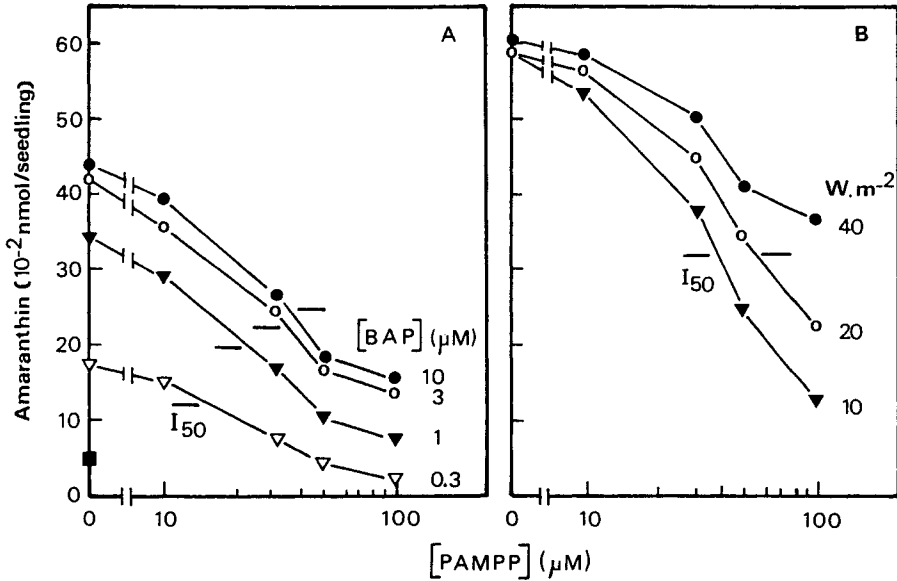
Fig. 2. Panel A: Reciprocal antagonism between PAMPP and b[6]Ade in darkness, in the presence of K^{+} ions. Seedlings 48 h old were grown on b[6]Ade and PAMPP solutions for 24 h. The symbol (■) corresponds to the quantity of amaranthin induced in darkness under the effect of K^{+} ions alone. Panel B: Reciprocal antagonism between PAMPP and light in the presence of K^{+} ions. Seedlings 48 h old transferred onto b[6]Ade (5 μM) and PAMPP solutions were exposed to light for 24 h. I_{50} corresponds to concentrations of PAMPP that inhibit 50% of control amaranthin synthesis.

Reciprocal Antagonism of PAMPP–b[6]Ade and PAMPP–Light

When 48-h-old seedlings were grown on b[6]Ade and PAMPP solutions at different concentrations for 24 h, we observed that the inhibitory effect of PAMPP was antagonized by the increase of b[6]Ade concentrations (Fig. 2A). Thus, 100 μM PAMPP completely inhibited amaranthin production in the presence of 0.3 μM b[6]Ade, but only 70% inhibition could be observed with 10 μM b[6]Ade. In order to obtain the same rate of inhibition (50%), the concentration of PAMPP had to be increased with increasing b[6]Ade concentrations.

To study the reciprocal antagonism between PAMPP and light, assays were performed under conditions allowing a high level of amaranthin synthesis, i.e. in the presence of 5 μM b[6]Ade. Whatever the light intensity (10, 20, or 40 $W.m^{-2}$), the same quantity of pigment was obtained ($60 \cdot 10^{-2}$ nmol per seedling). The analog inhibitory effect was observed for concentrations higher than 10 μM and decreased with respect to light intensity (Fig. 2B). Thus, a concentration as high as 100 μM did not induce 50% inhibition when the light intensity was 40 $W.m^{-2}$. Similar results were obtained when experiments were conducted in the absence of b[6]Ade, except that quantities of amaranthin synthesized were lower under these conditions.
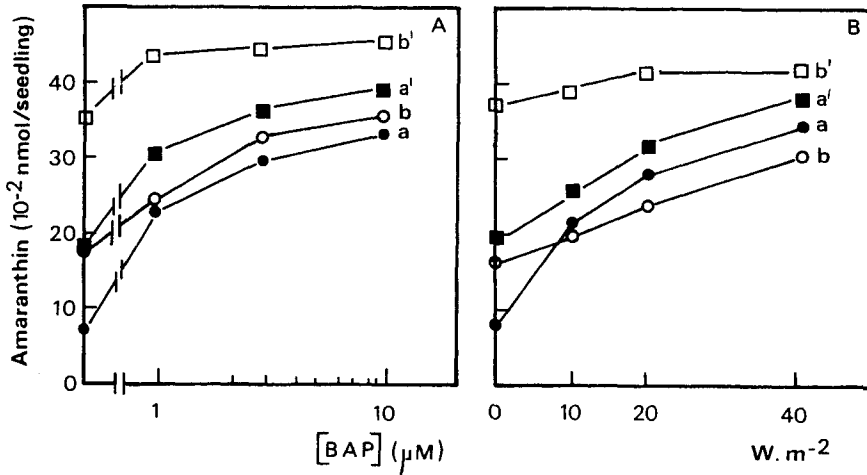
Fig. 3. Panel A: Reversal by b⁶Ade of the inhibitory effect of PAMPP on amaranthin synthesis induced by b⁶Ade or by light. Seedlings 48 h old were grown for 16 h either on 0.3 μM b⁶Ade and 30 μM PAMPP (curve a) or exposed to light (10 W.m⁻² for 16 h) in the presence of 50 μM PAMPP (curve b). In both cases, seedlings were transferred and maintained for 16 h either on b⁶Ade solutions or on buffer solution. Panel B: Reversal by light of the inhibitory effect of PAMPP on amaranthin synthesis induced by b⁶Ade or by light. Seedlings 48 h old were grown for 16 h either on 0.3 μM b⁶Ade and 30 μM PAMPP (curve a) or exposed to light (10 W.m⁻² for 16 h) with exogenous supply of 50 μM PAMPP (curve b). Then the seedlings were transferred onto buffer solution and exposed for 16 h to light treatment. Curves a' and b' illustrate the respective control experiments performed in omitting PAMPP during the first step of treatment.

Reversal of the Inhibitory Effect of PAMPP on Amaranthin Synthesis Induced by b⁶Ade or by Light

Reversal of the effect of PAMPP by an exogenous supply of b⁶Ade or by a light treatment was attempted. Seedlings 48 h old were grown in the dark on 0.3 μM b⁶Ade and 30 μM PAMPP solutions. Under these conditions, the amaranthin production induced by b⁶Ade alone was inhibited 75% by PAMPP (Fig. 2A). Reversal of this inhibition was obtained by transferring the seedlings onto b⁶Ade solutions of increasing concentrations (Fig. 3A, curve a). Stimulation of the amaranthin synthesis was already observed for 1 μM b⁶Ade and increased up to 10 μM. In contrast, when the seedlings were transferred onto buffer solutions, no increase of amaranthin production occurred ($8 \cdot 10^{-2}$ nmol per seedling). When induced by light (10 W.m⁻² for 16 h), amaranthin synthesis was inhibited 50% by 50 μM PAMPP ($18 \cdot 10^{-2}$ nmol per seedling, instead of $37 \cdot 10^{-2}$). This inhibition could also be reversed by the application of b⁶Ade, and this reversal was more efficient when the concentration of b⁶Ade increased (Fig. 3A, curve b). When the seedlings were transferred onto buffer solutions, no increase in amaranthin production ($7.7 \cdot 10^{-2}$ nmol per seedling) was noted.

As illustrated in Fig. 3B (curve a), the inhibitory effect of 30 μM PAMPP on

amaranthin synthesis induced by 0.3 μM b[6]Ade was also antagonized by light. We observed that a light treatment at the lowest intensity (10 W.m^{-2} for 16 h) was sufficient to restore the synthesis obtained when PAMPP was omitted (21 $\cdot$ 10^{-2} nmol per seedling, instead of 18 $\cdot$ 10^{-2}). The production of amaranthin increased with respect to light intensity. As shown in Fig. 3B (curve b), the inhibitory effect of 50 μM PAMPP on light-dependent amaranthin synthesis (10 W.m^{-2} for 16 h) was slightly antagonized by a light treatment. Amaranthin production increased slowly and linearly in direct relation to light intensity. The comparison of curves a and b (Fig. 3B) showed that the reversal was more efficient when the inducing factor was b[6]Ade.

Discussion

The results obtained demonstrate clearly the inhibitory effect of PAMPP on amaranthin synthesis induced in darkness in the presence of b[6]Ade. At a concentration of 60 μM, PAMPP causes a 50% reduction in synthesis induced by 5 μM b[6]Ade. Therefore this substance seems to be more active than the derivatives of the series of 4-substituted-2-methylpyrrolo(2,3-d)pyrimidines tested by Iwamura et al. (1979) on *Amaranthus caudatus*. The most active derivative of this series had to be applied at a concentration of 10^3 μM in order to cause 50% inhibition when the cytokinin used was i[6]Ade at 1 μM. One of the essential observations of this study was the effect of the analog on light-induced amaranthin synthesis. At concentrations of 50 and 100 μM, it causes a considerable reduction in synthesis induced by light.

Furthermore, it proved to be possible to reverse the inhibition produced by the analog, either by an exogenous supply of b[6]Ade or by light treatment; the degree of reversal was a function of b[6]Ade concentration or light intensity. This study shows that a reciprocal antagonism exists between PAMPP and b[6]Ade on one hand, and PAMPP and light on the other. Thus, there is considerable similarity between the effect of light and b[6]Ade on amaranthin synthesis. Köhler et al. (1980) suggested that light acted on amaranthin synthesis by modifying the endogenous cytokinin contents. Our results agree with this hypothesis. The only definite conclusion possible is that light and cytokinins both possess, in whole or in part, a mechanism sensitive to cytokinin analogs. Direct proof of Köhler's hypothesis would require demonstration of the existence of a pool of endogenous cytokinins in the presence of light, and of the effective action of these cytokinins on the same molecular mechanism, which itself has yet to be characterized.

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