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Polyamine Sparing May Be Involved in the Prolongation of Cell Division Due to Inhibition of Phenylpropanoid Synthesis in Cytokinin-Starved Soybean Cells

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Abstract. Effects on growth, mostly of an inhibitory nature, have been attributed to phenolic compounds in vivo and in vitro. This suggests that L-α-aminooxy-β-phenylpropionic acid (L-AOPP), a competitive inhibitor of phenylalanine ammonia-lyase (PAL), the enzyme controlling the first step in phenylpropanoid synthesis, might stimulate growth in soybean suspension cultures (*Glycine max,* cv. Acme). The promotive effect of L-AOPP, measured as an increase in cell number, was more clearly detected in the growth-limiting condition of cytokinin starvation. At least one more cell division cycle was completed in the presence of L-AOPP before growth by division ceased and growth continued by expansion only. Phenolic acids are known to conjugate with polyamines, modulating the free levels of these plant growth substances. Thus, the effect of L-AOPP on the titers of free and conjugated polyamines (putrescine, spermidine, and spermine) was investigated by high performance liquid chromatography in the course of cytokinin starvation. An increased level of free putrescine was detected in the presence of L-AOPP relative to controls, especially in the initial period before growth became restricted to cell expansion. The decrease in free putrescine associated with the cessation of cell division was temporarily delayed, suggesting that an interaction between phenolic acids and polyamines is involved in the mechanism of growth promotion by L-AOPP.

Key Words. *Glycine max*—Polyamines—Phenolic compounds—L-AOPP—Cell division—Cell elongation—Cytokinin starvation

Abbreviations: IAA, indole-3-acetic acid; ABA, abscisic acid; PAL, phenylalanine ammonia-lyase; L-AOPP, L-α-aminooxy-β-phenylpropionic acid; PA(s), polyamine(s); HPLC, high performance liquid chromatography; PUT, putrescine; SPD, spermidine; SPM, spermine.

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Growth and accumulation of phenolic compounds are generally inversely related. By altering mineral uptake, water relations, photosynthesis, carbon flow, and phytohormone activity (Einhellig 1995) phenolics inhibit the growth of the plant itself, of pathogens as phytoalexins, and of other plants as allelochemicals. Apart from this prominent function as growth inhibitors there are conditions in which certain phenolics can promote growth in low concentrations. Elevated levels of phenolics correlate with improved growth of some galls (Abrahamson et al. 1991). Applied phenolics increased growth rates in radish seedlings (Ray 1986), promoted hypocotyl elongation in lettuce seedlings (Li et al. 1993), and callus growth in a concentration-dependent manner related to the supply of auxin (Lavee and Avidan 1982). Phenolics may prolong auxin action by inhibiting the oxidation of IAA (Krylov et al., 1994, Pilet 1964, Tomaszewski and Thimann 1966) and antagonize some ABA effects (Li et al. 1993).

As the enzyme controlling the first step in phenylpropanoid synthesis, phenylalanine ammonia-lyase (PAL) is the metabolic link between primary and secondary metabolism. PAL activity (Moreno et al. 1994) and subsequent accumulation of phenylpropanoids (Cline et al. 1989, Cvikrová et al. 1991, 1994, Miyamoto et al. 1994) correlate with growth retardation. Consistent with that, inhibitors of PAL are able to stimulate growth (Ingold et al. 1990, Kudakasseril and Minocha 1986). In soybean suspension cultures the PAL inhibitor L-α-aminooxy-β-phenylpropionic acid (L-AOPP) stimulated cell division and delayed the effect of cytokinin starvation and nutrient depletion (Mader and Hanke 1996).

The present study investigates the possibility of participation by polyamines (PAs) in the mechanism of growth stimulation by L-AOPP. The prominent part played by PAs in the control of growth and development is now established. Regulation is achieved by balancing the levels of free and conjugated PAs in development.

High levels of free PAs correlate with growth by cell division, low levels with cell expansion (Egea-Cortines and Mizrahi 1991, Davidonis 1995). Since the phenylpropanoid pathway provides conjugation partners for PAs, its activity may contribute to the regulatory machinery of plant growth. To obtain evidence for this, the effect of PAL inhibition by L-AOPP on levels of free and conjugated PAs was measured in batches of cells in which growth was limited by cytokinin starvation for comparison with cultures in which growth was slowed gradually by nutrient depletion.

Materials and Methods

Suspension Culture of Soybean (*Glycine max* (*L.*) *Merr., cv. Acme*)

The cell line used was derived from callus initiated on cotyledon segments and has maintained a cytokinin requirement for growth over 20 years of subculturing at weekly intervals to approximately sixfold dilution with a ratio of volume suspension to volume culture flask of 1:4. Cultures were kept on an orbital shaker (100 rpm) in the dark at 25°C. The medium described by Miller (1967) was used with omission of inositol and addition of 0.5 mg/liter (2.3 μM) kinetin and 2 mg/liter (10.7 μM) α-naphthaleneacetic acid.

For experiments, cultures in the exponential phase of growth were used 3 days after subculturing. In experiments on kinetin starvation extracellular kinetin was removed by three washes with kinetin-free medium by centrifugation (1,500 ×*g*, 5 min) and resuspension. L-AOPP was added by sterile filtration (22-μm pore size) to kinetin-starved cultures and non-kinetin-starved controls to a final concentration of 10^{-7} M. Determination of cell number was carried out according to Brown and Rickless (1948) by hemocytometer (modified Fuchs Rosenthal, depth 0.2 mm). Sterilin plastic pots (50 mL, 10-mL suspension) were used in the experiments.

Analysis of Polyamines by HPLC

The acid-soluble fraction of PAs was extracted, benzoylated, and analyzed for free and conjugated PAs as described previously by Pfosser et al. (1990) with minor modifications. The benzoylated PAs were extracted in diethyl ether only once but in a larger volume (i.e. in 5 mL instead of 2 times 2 mL).

The experiments were carried out four times. The characteristics of the cell line used changed over time, judged by the changes in absolute levels of PAs. However, the same relative effects were found repeatedly, leading to the conclusions illustrated here by one set of representative results.

Results and Discussion

Effects of L-AOPP on Growth by Cell Division vs Growth by Cell Expansion

Generally, L-AOPP stimulated cell division and delayed the effect of the limiting factors, cytokinin starvation or nutrient depletion in the presence of cytokinin (Fig. 1).

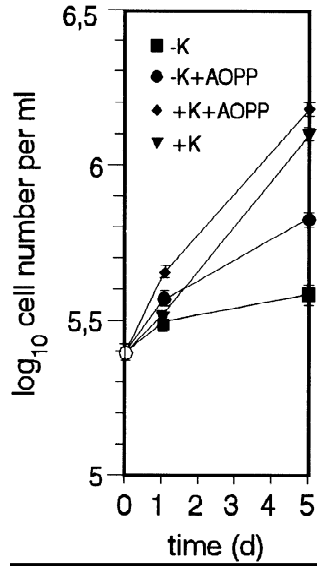

Fig. 1. Effect of L-AOPP on cell number: response in a 3-day-old suspension culture after transfer to media supplemented with combinations of L-AOPP (10^{-7} M) and kinetin (2.3 μM). ◆, kinetin and L-AOPP; ▼, kinetin only, ●, L-AOPP only; ■, without either. A representative experiment consisting of three replicates is shown. *Bars* indicate the highest and lowest values.

The effect of cytokinin starvation, a prompt cessation of cell division and the onset of increase in cell size, was postponed. Cells starved of kinetin in the presence of L-AOPP completed at least one more division cycle than cells starved of kinetin without L-AOPP. Comparable findings were obtained with cultures supplied with kinetin, growing normally. The increases in cell density with time (Fig. 1) indicate that cell division activity increased in the presence relative to the absence of L-AOPP. Despite the acceleration of nutrient depletion, the phase of active cell division appeared to be extended, postponing the onset of increase in cell size. Cell size seemed promoted following treatment with L-AOPP.

Since cell elongation involves increased wall extensibility and turgor, the action of L-AOPP may facilitate it both by reduction of phenolic cross-linking in the wall responsible for lowering its plasticity (Beimen et al. 1992, Miyamoto et al. 1994, Tan et al. 1991) and by prevention of phenolic-related effects on water relations. Phenolic acids reduce the water needed for cell expansion and uptake of ions (Booker et al. 1992 and references therein) and thereby affect growth.

Further, bearing in mind the promotion of expansion growth as a result of cell wall loosening by increased acidity (Rayle and Cleland 1970, 1977, 1992, Kutschera 1994 and references therein), the changes in the pH of the medium associated with ongoing elongation were investigated. In kinetin-starved cultures with elongated cells the pH fell to about 4.5 compared with 5.5 in ki-

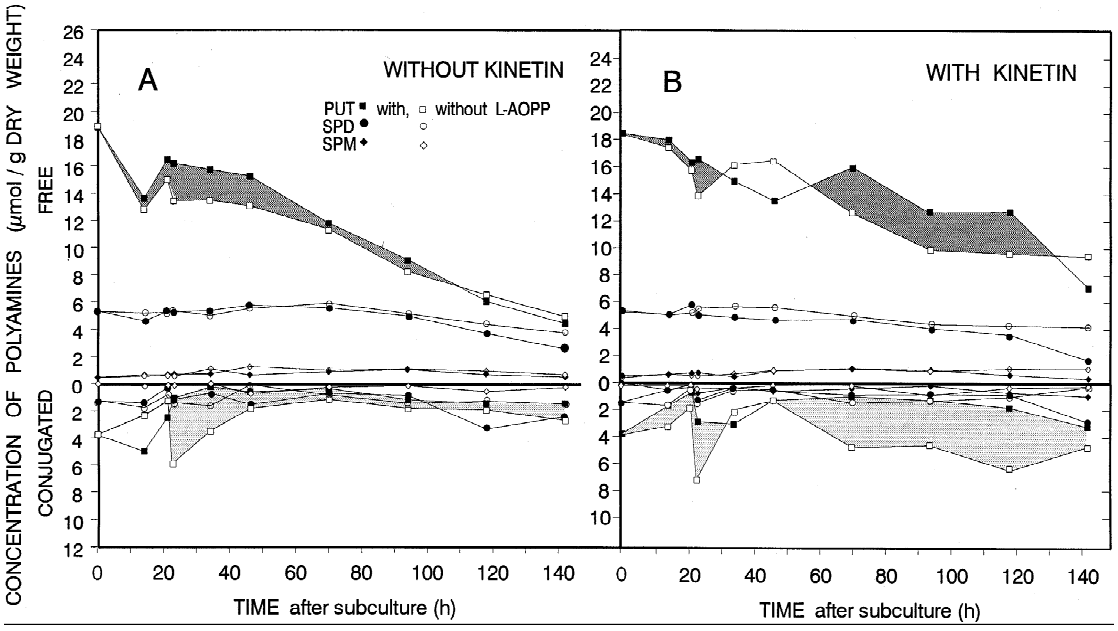

Fig. 2. Free and conjugated PAs in cytokinin-dependent suspension cultures of *G. max:* the effect of treatment with L-AOPP on the level of free and conjugated PUT (■), SPD (●), and SPM (◆ in kinetin-starved (*A*) and kinetin-supplied cells (*B*). The *shaded areas* represent the increase in free PUT (■) and the decrease in phenolic acid conjugated PUT (■) in the presence of L-AOPP. Data of a representative experiment are shown.

netin-supplied cultures. This matches the optimum pH predicted by the acid growth hypothesis and is consistent with a mechanism of elongation with an optimum pH lower than 5, as shown for *Avena* coleoptiles (Cleland et al. 1991, Cleland 1992). Possible effects of L-AOPP on medium pH would require more detailed investigation, but they are smaller than those brought about by cytokinin starvation.

Effect of L-AOPP on the Distribution of Free and Conjugated PAs

Cytokinin treatment is known to result in an increase in PA synthesis in some tissues (Rastogi and Davies 1991) and, consistent with this, levels of free putrescine (PUT) decreased under kinetin-starved conditions. Free spermidine (SPD) and spermine (SPM) remained more or less unchanged (Fig. 2*A*). Since SPD and SPM are derived from PUT by successive incorporation of aminopropyl moieties, this result suggests that the stimulatory effect of cytokinin treatment is confined to earlier steps, e.g. in the synthesis of PUT. The decline in free PUT was counteracted by L-AOPP, especially in the initial period of kinetin starvation (Fig. 2*A*), paralleling the delay in cessation of cell division. The effectiveness of L-AOPP at increasing PUT levels declined in the later phase of kinetin starvation possibly because of the loss of cytokinin-maintained biosynthetic capacity. By contrast, in kinetin-supplied cultures the elevation of free PUT levels in response to L-AOPP (stretched over the whole period of observation) was most noticeable later in the time course (*dark shaded* area in Fig. 2*B*) but before nutrient limitations. Here, the decreased level of conjugated PUT (*light shaded* area in Fig. 2*B*) clearly identified the mechanism of the L-AOPP effect as sparing newly formed PUT from conjugation by reduced availability of phenolic conjugation partners. In kinetin-starved cultures, however, the effect was not as clearly seen as the cells were characterized by already low levels of conjugated PUT. Clearly, in these cultures, growth of new tissue is the stronger sink for the limited free PUT than conjugation. Only kinetin-supplied cultures with uninhibited phenylpropanoid synthesis were able to accumulate conjugated PUT. Following from these results it will be of interest to investigate to what extent exogenous PAs can substitute for cytokinins in cytokinin-starved cultures.

Whether in kinetin-starved cells release of free PUT from conjugated PUT may compensate for reduced PA biosynthesis cannot be determined from these results. The results presented here are consistent with reports that the state of intensive cell division is characterized by high levels of free PAs (Egea-Cortines and Mizrahi 1991) and low levels of phenolic acids (Cvikrová et al. 1991) which inhibit cell division (Vaughan and Ord 1988). In addition, the correlation between cell elongation and decreasing levels of free PUT is in agreement with the induction of cell expansion by inhibitors of PA

synthesis (Altamura et al. 1993, Berlin and Forche 1980, Fallon and Phillips 1988, Mengoli et al. 1987).

The peaks and troughs in the levels of free and conjugated PAs observed, especially over the initial period, are likely to result from a combination of causes. Induction of PAL activity is a stress response elicitable by light and in suspension cultures on subculturing (Barnes and Jones 1984, Berlin et al. 1991, Jones 1984). Thus, changing the liquid medium and, in the process, subjecting dark-grown cultures to light will induce the phenylpropanoid pathway, resulting in an increase in conjugated PAs. An additional explanation, in view of cell cycle-dependent changes in PA metabolism (Pfosser et al. 1990, Pfosser 1993, reviewed by Serafinin-Fracassini 1991) may be partial synchronization of the culture caused by transfer to fresh medium. In this case, the peaks and troughs, ebbing away with the loss of putative synchrony, may reflect cell cycle phases. It can be noted that in suspension cultures of *Nicotiana tabacum* and *Medicago varia* the peak and trough characteristics of PA distribution in a time course after synchronization (Pfosser et al. 1990, Pfosser 1993) were quite similar to those found in this study.

Conclusion

Growth and development involve mutual coordination of cell division and cell expansion, processes with inverse requirements for free PAs. This study has discovered evidence that phenylpropanoid-derived compounds are involved in the regulation of development by reducing growth by cell division. One component of the mechanism appears to be conjugation with the free PAs required for cell division.

In conclusion, PAL is involved in the control of the switch from primary metabolism, characterized by growth by cell division, to secondary metabolism, both of which utilize the same resources. What has been indicated here is that the induction of PAL is not simply a control point switching on secondary metabolism, but the enzyme also contributes toward switching off the preceding phase of primary growth.

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