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Induction of *ent*-Kaurene Biosynthesis by Low Temperature in Dwarf Peas

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Abstract. Germinating pea (*Pisum sativum* L.) seeds of two dwarf cultivars, "Progress No. 9" and "Green Arrow," and two tall cultivars, "Alaska" and "Alderman," were treated with low temperature (3–5°C) for 14 days and then transferred to normal growing conditions (19–21°C for 16 h/14.5–16.5°C for 8 h) for an additional 10 days. Biosynthesis of [^{14}C]*ent*-kaurene from [^{14}C]2-mevalonic acid (2-MVA) was assayed in cell-free enzyme extracts prepared from shoot tips 10 days after cold treatment and was compared with activity in enzyme extracts prepared from noncold-treated, 10-day-old control plants. Shoot lengths of cold-treated plants were measured throughout a 35-day period and compared with shoot lengths of plants grown without cold treatment for 25–35 days. Low temperature induced a five- to 10-fold enhancement of *ent*-kaurene, hence potentially gibberellin (GA), biosynthesis in seedlings of the two dwarf cultivars but not in the tall cultivars. However, the lack of an increase in growth rate in the cold-treated dwarfs indicated that endogenous GA biosynthesis remained blocked at some point beyond *ent*-kaurene in the biosynthetic pathway. Since the late-flowering "Alderman" cultivar did not exhibit enhanced biosynthesis of *ent*-kaurene, it appears that if vernalization in late-flowering cultivars of peas is correlated with enhanced GA biosynthesis, it is not the early part of the biosynthetic pathway which is affected.

The many commercial cultivars of pea (*Pisum sativum* L.) are categorized as dwarf and tall and as early-flowering and late-flowering. Dwarf cultivars generally mature at shoot heights of approximately 30 cm or less, whereas tall cultivars may exceed 1 m. Late-flowering cultivars typically flower above the 15th node, respond to photoperiod as quantitative long-day plants, and are vernalizable. In contrast, early-flowering cultivars flower at the 9th or 10th node above the cotyledons, behave as day-neutral plants, and are not vernalizable (Barber 1959, Marx 1977, Moore 1964, Moore and Bonde 1962, Murfet 1977, Reid 1988, Reid and Murfet 1975).

Vernalization of late-flowering cultivars of pea, that is, treatment of germinating seeds with low temperature for one to several weeks to induce early flowering, has been investigated extensively (Barber 1959, Highkin 1956, Moore and Bonde 1958, 1962, Reid and Murfet 1975). Highkin (1956) first reported that late-flowering cultivars are vernalizable with respect to both flower formation and vegetative development. The inductive effect of cold treatment on flowering is manifested as a reduction in the number of nodes to flower and the number of days to anthesis. The inductive effect of low temperature on vegetative development is a reduction in the amount of vegetative growth accompanying flowering (i.e., height to the first flowering node). Highkin termed the latter effect "vegetative vernalization" (Highkin 1956).

The purpose of the present investigations was to test the hypothesis that vernalization of late-flowering cultivars of peas is correlated with enhanced biosynthesis of the gibberellin (GA) precursor *ent*-kaurene, hence potentially GAs. The evidence from assays of *ent*-kaurene biosynthesis from mevalonate in cell-free enzyme extracts prepared from shoot tips is that cold treatment of germinating seeds causes enhanced biosynthesis of *ent*-kaurene in dwarf seedlings, whether early- or late-flowering, but not in tall seedlings of either early- or late-flowering cultivars.

Materials and Methods

Plant Material

Four cultivars of pea (*Pisum sativum* L.) were used in all exper-

iments: "Alderman" (tall, late-flowering); "Alaska" (tall, early-flowering); "Green Arrow" (dwarf, late-flowering); and "Progress No. 9" (dwarf, early-flowering). "Alderman" peas were obtained from Harris Seed Co. (Rochester, NY, USA), and all others were obtained from Burpee Seed Co. (Riverside, CA, USA). Seeds were surface-disinfected with 0.5% NaOCl then rinsed five times with sterile-distilled water before being planted in moist, sterilized vermiculite. Cold-treated plants were germinated and grown 14 days in a dark coldroom at 3–5°C, then transferred into growth chambers under standard conditions consisting of a 16-h photoperiod at 19–21°C (1000–1200 ft-c or 1.8×10^4 to 2.2 erg $cm^{-2} s^{-1}$) and an 8-h dark period at 14.5–16.5°C for the duration of the experiment. Noncold-treated, control plants were grown exclusively in growth chambers under standard conditions. For the first 7 days in the growth chambers, the plants were irrigated with sterile-distilled water and thereafter with complete mineral nutrient solution.

Growth Experiment

Shoot growth of both control and cold-treated plants was measured at frequent intervals throughout a 25- to 35-day period for the controls and a 35-day period for the cold-treated plants. The shoot height was measured from the cotyledons to the uppermost visible node at which the stipules were still appressed.

Preparation of Enzyme Extracts

The sample unit collected as the shoot tip was that part of the shoot consisting of the oldest still-appressed stipules and enclosed structures [see Fig. 2 in Coolbaugh (1985)]. The shoot tip is an active center of *ent*-kaurene biosynthesis (Coolbaugh 1985, Coolbaugh et al. 1973, Ecklund and Moore 1974), and evidently is the site also of perception of low temperatures during vernalization (Metzger 1988). The shoot tip typically was excised above the 5th or 6th node (counting the cotyledonary node as 1) of seedlings of all four cultivars. After 10 days of growth in the growth chambers (±14 days previous cold treatment), shoot tips were harvested, counted (generally about 250), weighed (typically about 8 g fresh weight), and held in a weighing dish on ice immediately prior to preparing extracts.

General enzyme assay procedures were as described previously (Choinski and Moore 1980, Ecklund and Moore 1974). Samples of shoot tips were frozen in liquid N_2 and were ground immediately to a fine powder with a chilled mortar and pestle. As the tissue thawed it was quickly added to a slurry of insoluble polyvinylpolypyrrolidone (PVPP) (0.5 g saturated PVPP per gram fresh weight of tissue) and 0.1 M KH_2PO_4-K_2HPO_4 buffer (pH 7.1, 1.0 ml/g fresh weight of tissue) containing 3 mM dithiothreitol and 100 μM chloramphenicol and immediately homogenized. When completely thawed, the homogenate was filtered once through four layers of cheesecloth and the filtrate was centrifuged at 10,800 *g* for 10 min. The 10,800 *g* supernatant was centrifuged at 100,000 *g* for 90 min and the resulting supernatant was removed with a pipet, gently mixed, and immediately used as the enzyme extract. The mean protein content of the enzyme extracts was 8.1 mg/ml. All centrifugations were done at 4°C. These methods cause osmotic and mechanical rupture of chloroplasts, which may contain enzymes for *ent*-kaurene synthesis (Moore and Coolbaugh 1976, Railton et al. 1984).

Assay for ent-*Kaurene Biosynthesis*

The reaction mixtures contained, in a total volume of 1.0 ml: 0.7 ml enzyme extract, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 3 mM ATP, 75 mM K-phosphate (pH 7.1), and 30 μM [^{14}C]2-MVA (49 mCi/mmol, 1.836 Bq/mmol), 2.1 mM dithiothreitol, and 70 μM chloramphenicol.

Incubations were for 60 min at 30°C. The reaction was stopped by adding 1.0 ml of acetone and mixing. Each reaction mixture was extracted twice with 2 ml of benzene-acetone (3:1). Pooled organic extracts were evaporated to dryness under a stream of N_2; the residue was then redissolved in 0.2 ml acetone and spotted onto a thin-layer plate (Baker-flex Silica Gel IB, 2.5 × 7.5 cm) for chromatography. Samples of authentic *ent*-kaurene (15 mg) were chromatographed separately and the samples were visualized by spraying with 6% (wt/vol) $KMnO_4$ solution. The TLC plates were developed in n-hexane, allowing the solvent front to advance 5.0 cm from the origin. The band containing [^{14}C]*ent*-kaurene (R_f 0.75–0.95) was cut out of the chromatogram and placed in liquid scintillation mini-vials for counting. There are no interfering radioactive substances in that R_f zone (Anderson and Moore 1967, Coolbaugh et al. 1973). This was confirmed many times in the present study by cutting chromatograms into 0.5-cm bands and counting, and a few times by radioautography. Each mini-vial contained 5.0 ml of Beckman Ready Safe liquid scintillation fluid. The yield of [^{14}C]*ent*-kaurene was measured with a Beckman Liquid Scintillation Counter, Model LS1801; the counting efficiency was approximately 86%. All data presented reflect correction by deduction of activity observed in boiled-enzyme controls. Each set of assays included experimental, boiled-enzyme, and no-enzyme controls, all run in triplicate. Each experiment was repeated on three or more separate occasions.

Protein Determinations

Total protein content in each previously frozen and thawed enzyme extract was estimated by the method of Bradford (1976) using the Bio-Rad Protein Assay Kit with bovine serum albumin as a standard.

Reagents

DL-[^{14}C]2-MVA sodium salt (49 mCi/mmol, 1.836 Bq/mmol) was obtained from Research Products International Corp. (Mount Prospect, IL, USA). Dithiothreitol, chloramphenicol, ATP, and PVPP (insoluble) were obtained from Sigma Chemical Co. PVPP was prepared by boiling for 10 min in 1 N HCl and washed with 2 volumes (vol/vol) of 1 N HCl; it was then washed with distilled water until the pH was 5.0 or higher. The acid-washed PVPP was dried at 70°C for storage. Directly before use the PVPP was hydrated with 0.1 M K-phosphate buffer (pH 7.1); excess buffer was removed by suction filtration. *Ent*-kaurene was a gift from Dr. Robert K. Clark, Jr. of Abbott Laboratories (North Chicago, IL, USA).

Results and Discussion

The plants used in these investigations were physiologically younger than the corresponding cultivars

Table 1. Biosynthesis of [^{14}C]*ent*-kaurene from [^{14}C]2-MVA in cell-free enzyme extracts of shoot tips of cold-treated and noncold-treated pea seedlings.

		Yield of [^{14}C]*ent*-kaurene (pmol mg protein^{-1})					
		Exp. 1		Exp. 2		Exp. 3	
Cultivar	Phenotype	Treated	Control	Treated	Control	Treated	Control
"Progress No. 9"	Dwarf, early-flowering	5.30[a]	0.31	4.27[a]	0.37	11.01[a]	1.10
"Green Arrow"	Dwarf, late-flowering	3.04[a]	0.61	1.61[a]	0.33	2.27[a]	0.66
"Alaska"	Tall, early-flowering	0.72	0.24	0.50	0.73	0.33	0.27
"Alderman"	Tall, late-flowering	0.31	0.49	0.49	0.48	0.59	0.28

Values are the means of three separate experiments with three replicate reaction mixtures in each experiment.
[a] Significant from corresponding control at $p < 0.01$.

that were used in previous investigations in the authors' laboratory (Ecklund and Moore 1974) and those of Coolbaugh (1985) and Chung and Coolbaugh (1986). This was due to the lower temperature regimen (19–21°C/14.5–16.5°C) used in these studies compared to those used previously [i.e., 20–23°C/17–20°C (Ecklund and Moore 1974) and 23–25°C/16–18°C (Chung and Coolbaugh 1986)]. For example, the mean shoot length of 10-day-old "Alaska" seedlings in these studies was 5.0 cm, whereas in the Ecklund and Moore (1974) investigations the mean length of 10-day-old "Alaska" seedlings was 8.0 cm. It is because of the lower growing temperatures that the growth and *ent*-kaurene synthesis were lower in the present investigations than in earlier ones. The noncold-treated "Alaska" seedlings in the present study, although 10 days old, were only as tall as 7.5- to 8-day-old seedlings in the Ecklund and Moore (1974) studies. As Ecklund and Moore (1974) noted, *ent*-kaurene synthesis activity varies markedly with ontogeny. It is also the difference in stage of development that accounts for the lack of a difference in *ent*-kaurene-synthesizing activity between the control dwarf and control tall plants, which has been reported previously by the authors (Ecklund and Moore 1974) and by Chung and Coolbaugh (1986).

Assays of *ent*-kaurene biosynthesis from mevalonate in cell-free enzyme extracts prepared from shoot tips of cold-treated and noncold-treated seedlings revealed a marked induction or enhancement of *ent*-kaurene biosynthesis in the two dwarf cultivars (Table 1). A two-way analysis of variance on the *ent*-kaurene data indicated that both main effects (i.e., effects of cold treatment and cultivar) and the interaction between treatment and cultivar were significant at $p < 0.01$ in all three experiments (Table 2). Therefore, the response of the seedlings to the cold treatment depended on the particular cultivar. Treatment means for individual treated cultivars also were tested against the corresponding controls using the error mean squares from the analysis of variance (Table 2). In each experiment, treatment means for "Progress No. 9" and "Green Arrow" cultivars were significantly different from the controls ($p < 0.01$), while there were no significant differences between means associated with the "Alaska" and "Alderman" cultivars (Table 1).

Table 2. Analysis of variance table for experiments 1–3 (see Table 1).

Source of variation	Degrees of freedom	Mean square	F
Experiment 1			
Treatment	1	22.349	229.44[a]
Cultivar	3	7.992	82.05[a]
Interaction	3	8.119	83.34[a]
Error	16	0.097	
Experiment 2			
Treatment	1	9.238	280.72[a]
Cultivar	3	4.246	129.01[a]
Interaction	3	5.383	163.57[a]
Error	16	0.033	
Experiment 3			
Treatment	1	53.134	587.25[a]
Cultivar	3	44.090	487.29[a]
Interaction	3	32.746	361.91[a]
Error	16	0.090	

[a] Significant at $p < 0.01$.

Surprisingly, however, the enhancement of *ent*-kaurene biosynthesis in the dwarfs following cold treatment appears not to have been correlated with increased GA biosynthesis, since shoot growth following cold treatment was not increased in either dwarf cultivar (Fig. 1). In fact, the cold-treated plants of all four cultivars exhibited growth curves after cold treatment that were nearly identical to the controls that were grown exclusively at normal temperatures. These results are contrary to part of the original hypothesis, according to which it was ex-

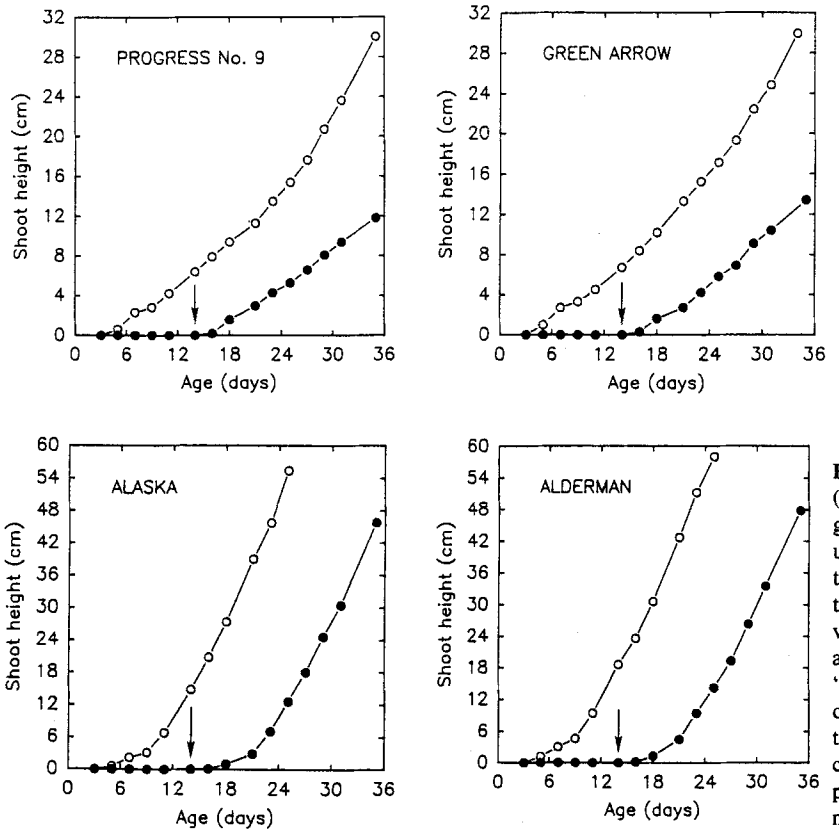

Fig. 1. Growth curves of pea seedlings with (●) and without (○) cold treatment as germinating seeds for 10 days prior to growth under normal conditions. Standard errors of the means were computed but were too small to plot. Note different calibrations on the vertical axis for the dwarf "Progress No. 9" and "Green Arrow" and tall "Alaska" and "Alderman" cultivars. The arrow denotes the day on which cold-treated seedlings were transferred to the growth chamber. The control "Progress No. 9" and "Alaska" plants had just begun to flower when measurements were discontinued.

pected that late-flowering cultivars of pea might exhibit enhanced *ent*-kaurene production in response to cold treatment. That was expected because of extensive research with numerous long-day and cold-requiring plants showing that the photoperiodic or temperature stimuli evoke increases in particular GAs (Cleland and Zeevaart 1970, Metzger and Zeevaart 1980, 1982, Paleg 1965, Rood et al. 1989, Suge 1970, Suge and Rappaport 1968, Talon and Zeevaart 1990, Zeevaart 1968, 1971, 1983). However, the results reported here do not exclude the possibility that cold treatment affects some point in the GA biosynthetic pathway beyond *ent*-kaurene in late-flowering cultivars of pea. Zeevaart and associates have found that the photoinductive effect of long days on stem growth in spinach (Metzger and Zeevaart 1980, 1982, Zeevaart 1971) and *Silene armeria* L. (Talon and Zeevaart 1990) is correlated with interconversions among certain GAs.

The effect of cold treatment on *ent*-kaurene biosynthesis in dwarf peas is particularly interesting in view of the report by Ingram et al. (1984) that the major dwarfing (recessive) gene *le* in peas blocks the 3β-hydroxylation of GA_{20} to GA_1, the latter being postulated to be the specific GA that promotes growth of peas (Ingram et al. 1983, 1986, Reid 1988, Sponsel 1985, 1986). Tall peas carry the dominant *Le* allele. Thus, it appears that enhancement of *ent*-kaurene synthesis may lead to increased amounts of GA_{20}, but that the blockage of the 3β-hydroxylation of GA_{20} to GA_1 persists in cold-treated dwarf pea plants. It should be noted, however, that seven other nonallelic internode length genes, beside *le*, have been reported for peas (Reid 1988). Three of these—*na*, *lh*, and *ls*—also are dwarfing genes and appear to influence steps in the GA biosynthetic pathway. Mutants having those genes contain little or no biologically active GAs and become phenocopies of tall types after treatment with small amounts of GA_1 (Reid 1988). Nevertheless, McComb (1977) emphasized that the main difference in internode length between familiar cultivars like the dwarf "Progress No. 9" and the tall "Alaska" can be attributed almost entirely to the *Le* locus.

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