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Gibberellic Acid-Promoted Lignification and Phenylalanine Ammonia-lyase Activity in a Dwarf Pea (Pisum sativum)[1]

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Abstract. **The effects of gibberellic acid on lignification in seedlings of a dwarf and a tall cultivar of pea (*Pisum sativum*) grown under red or white light or in the darkness, were studied. Gibberellic acid (10^{-6}–10^{-4} M) promoted stem elongation in both light and dark and increased the percentage of lignin in the stems of the light-grown dwarf pea. The gibberellin had no effect on the lignin content of the tall pea although high concentrations (10^{-4} M) promoted growth of the tall plants. Time course studies indicated that the enhanced lignification in the gibberellin-treated dwarf plants occurred only after a lag period of several days. It was concluded that gibberellic acid-enhanced lignification had no direct relation to gibberellic acid-promoted growth. The activity of phenylalanine ammonia-lyase (E.C. 4.3.1.5) was higher in gibberellin-treated dwarf plants grown under white or red light than in untreated dwarf plants. Gibberellic acid had no detectable effect on the activity of this enzyme when the plants were grown in darkness, just as it had no effect on lignification under dark conditions. The data suggest that in gibberellin-deficient peas the activity of phenylalanine ammonia-lyase is one of the limiting factors in lignification.**

Lignins occur in almost all nonaquatic higher plants (3, 23) as integral components of the differentiated cell wall (27). It is generally considered that it is lignin which gives the stem its strength and rigidity. The intermediates of the biosynthetic pathway leading to the formation of the lignins are for the most part now known (3). However, with the exception of phenylalanine ammonia-lyase (E.C. 4.3.1.5), which has been extensively purified (7) and studied (2, 4, 8, 18, 19, 22, 26, 31, 32), and is implicated as one controlling agent in lignification (10, 29, 30), there is relatively little information regarding most of the enzymatic reactions involved (3, 23, 24). Consequently, the factors which control lignification are poorly understood but appear to vary with environmental conditions (21), developmental stage (11, 23, 25) and the species of the plant (23, 24).

Light may be one factor which regulates lignification. Stems are stiffer and stem elongation is retarded under illumination (9). While there are relatively few studies on the effects of light on lignification, presumably the stiffening of the stem upon exposure to light results in part, at least, from an enhanced deposition of lignin. Phillips (21) reported that the formation of lignin in the stems of ash required exposure of the leaves to light. Ishikawa and Takaichi (12) found that dark-grown bean plants had less than one half the lignin content of plants grown in white light. These authors also noted a wave-length effect with yellowish-green light promoting lignification the most. On the other hand, light had no effect on lignin accumulation in excised internodes of *Phleum* (23) or sorghum (24). Kratzl (15) also reported lignin in etiolated potato sprouts and concluded that light was not required for lignification.

Gibberellic acid (GA) promotes stem elongation and increases cell wall plasticity (17) and thus might be expected to influence lignification. Studies (13, 20, 23, 28) of the effects of GA on lignification, however, are limited. GA was reported to promote lignification in cultures of carrot tissue (13). Parups (20) also noted that in the hypocotyls of dark-grown peas 10^{-4} M GA slightly promoted formation of eugenol-lignin whereas 10^{-2} M GA was inhibitory. Indoleacetic acid in combination with GA promoted the formation of lignified vessels in several woody species (28) whereas in white pine GA alone stimulated differentiation of new sieve cells (5). GA does not invariably stimulate lignification; Stafford (23), working with excised internodes of *Phleum*, found no detectable effect of GA on lignification except a slight inhibition at high (2.9×10^{-4} M) concentrations.

The opposing effects of light and GA on stem elongation and the apparent light-enhanced lignification in some tissues (12, 21) prompted us to study the effects of GA on lignification. Preliminary experiments with a dwarf pea indicated that GA

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promoted lignification as well as stem elongation. This observation raised some interesting questions. First, how does GA promote lignin accumulation? Second, since lignified cells are not considered readily extensible, what is the relationship of GA-promoted lignification to GA-promoted growth, if any? The purpose of the experiments reported here was to study the possible effects of light and GA on stem elongation and lignification.

Materials and Methods

Growth of Plant Material. Two cultivars of peas (*Pisum sativum*), a tall variety (Tall Telephone) and a dwarf variety (Improved Laxton's Progress), were grown in a growth chamber in vermiculite in dark, or under red or white light. The temperature was maintained at 26 ± 1° for seed germination and 20 ± 1° for seedling growth. White-light grown peas were irradiated for 14 hr a day by four 25 w incandescent bulbs and 8 cool-white 40 w fluorescent lamps which gave a light intensity of 1000 ft-c at the level of the seedlings as measured by a Weston model 756 illumination meter. Red light was furnished for 14 hr a day by one 40 w fluorescent lamp wrapped in red cellophane. All manipulations with the dark-grown plants were done under a green, 10 w incandescent bulb.

Lignin Analysis. Different methods for lignin determination all give slightly different results. Nevertheless, it is possible to obtain reproducible "lignin" fractions which presumably give an estimate of the lignin content of the tissue. In the experiments reported here, the debladed whole stem was used for lignin analysis by the method of Armitage *et al.* (1). This method consisted of extracting 1 g finely ground oven dried material in 40 ml of boiling ethanol-benzene (1:2, v/v) for 2.5 hr. The residue was refluxed with 100 ml of 5 % (w/v) HCl for 1 hr, filtered, and then incubated at 38° for 18 hr in 100 ml 0.25 % (w/v) Na_2CO_3 containing 100 mg trypsin. After the tryptic digestion the residue was again collected by filtration and dissolved in 10 ml of 72 % (w/v) H_2SO_4. The acid solution was held for 2 hr at 15° to 20°, then refluxed with an additional 240 ml of water for 2 hr, and finally filtered through a tared Gooch crucible. The "lignin" fraction thus collected was washed with hot water, dried, and weighed. All results for lignin are expressed on a percentage dry weight basis or on a per stem basis.

Extraction and Assay of Phenylalanine Ammonia-lyase. Acetone powders prepared from debladed whole stems were extracted with 0.1 M borate (Na^+) buffer, pH 8.7. The soluble protein was precipitated by 70 % (w/v) saturation with $(NH_4)_2SO_4$ and resuspended in 0.2 M borate (Na^+) buffer, pH 8.7. Enzymic activity was determined spectrophotometrically at 290 mμ essentially as reported previously (18). The reaction mixture (3.0 ml) contained L-phenylalanine (20 μmoles), pH 8.7 borate (Na^+) buffer (100 μmoles) and enzyme. One unit (U) of enzyme is defined as the amount of enzyme catalyzing the formation of 1 μmole of cinnamate per min at 30°. Enzymic activities are reported here in terms of milliunits (mU) per stem or per mg protein. Protein was determined by the Biuret method.

Results and Discussion

Influence of GA on Lignification in a Tall and a Dwarf Pea Cultivar. It is well established that differences in the ability to form lignin exist between species (23, 24). The data presented in table I indicate that variations in lignification also occur within a species. The percentage of lignin in the untreated plants of the tall variety was significantly higher than that in the dwarf variety. The differential effect of GA on lignification in the 2 cultivars illustrates even more strikingly the varietal differences. Lignin accumulation in the tall variety was not affected by the GA treatments although the highest GA concentration (10^{-4} M) promoted growth of the tall plants by about 50 %. In contrast, in the dwarf cultivar GA in a concentration range between 10^{-6} and 10^{-4} M promoted both growth and lignification. However, the enhanced accumulation of lignin was not proportional to the GA-promoted growth. Lignification in the dwarf was approximately doubled by 10^{-5} M GA and apparently saturated by this amount of GA whereas stem elongation was still promoted by 10^{-4} M GA. The simplest interpretation of these observations is that GA promoted the activity of one or more rate-limiting steps in lignin biosynthesis in the dwarf variety. In the tall variety either these rate-limiting reactions were not responsive to GA, a possibility we consider unlikely, or the tall plants contained sufficient endogenous GA that exogenous GA could elicit no further response.

Time Course of Lignin Accumulation. The dwarf plants exhibited an enhanced growth rate

Table I. *The Lignin Content of the Stems of Dwarf and Tall Peas Treated With Gibberellic Acid*

White-light grown, 10-day old seedlings were treated with GA by pouring 250 ml of an aqueous solution directly into 8 inch pots each of which contained 10 plants. After a further 9 days growth under white light the plants were harvested for lignin determination. At time of treatment, the dwarf and tall plants were 1.8 cm and 2.6 cm, respectively. Each treatment was duplicated. The standard deviation is given.

	Tall		Dwarf	
Gibberellin treatment	Increase in height	Lignin	Increase in height	Lignin
	cm	*%*	*cm*	*%*
None	24.8±0.4	4.8±0.37	9.8±0.07	3.1±0.07
10^{-6} M	24.2±1.5	4.2±0.24	12.6±0.04	4.5±0.25
10^{-5} M	26.8±1.3	4.2±0.74	26.9±0.60	7.7±0.44
10^{-4} M	36.0±0.6	4.2±0.55	44.3±1.07	6.8±0.60

within 1 day after treatment with GA. In order to determine the relationship between GA-promoted growth and the enhanced lignification in the tissue treated with GA, a time course study of lignin accumulation was conducted. Dwarf seedlings grown under white light for 8 days were treated with GA (3×10^{-4} M) and harvested for lignin determination at intervals over the following 10 days. The lignin content of the GA-treated plants was higher than that of the controls at all sampling dates (fig 1) but the percentage of lignin was not significantly higher in the treated plants until 4 days after treatment. This lag of 4 days before there was a significant accumulation of lignin and the lack of proportionality between GA-enhanced growth and lignification in both the dwarf and tall cultivars (table I) indicate that the enhanced accumulation of lignin, *per se*, in the GA-treated tissue has no direct relation to the GA-promoted growth.

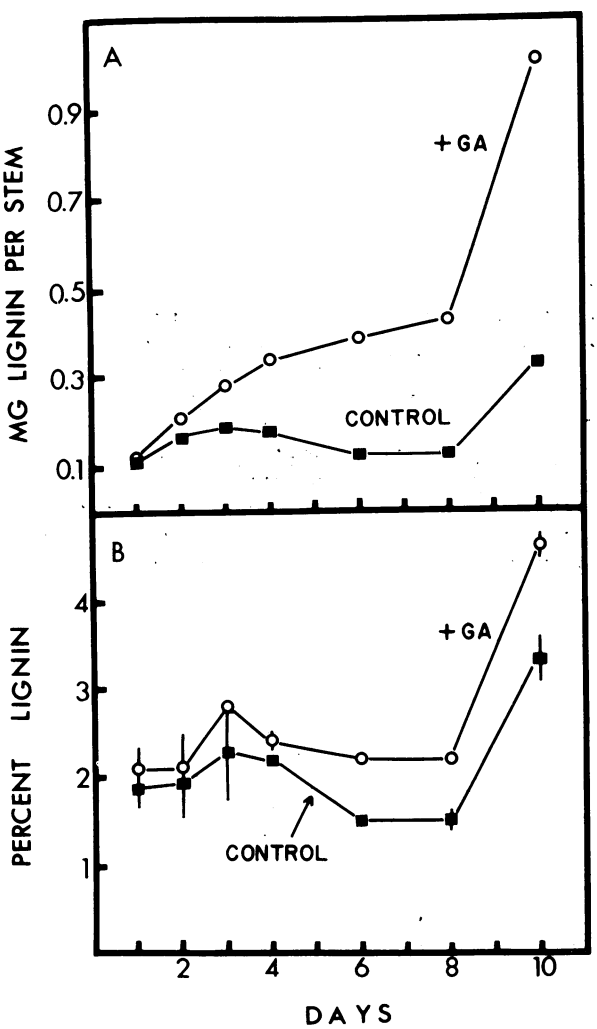

FIG. 1. Time course of lignin accumulation in the stems of dwarf peas grown under 1000 ft-c white light. Eight-day old seedlings were sprayed once with 50 ml gibberellic acid (3×10^{-4} M). Triplicate samples, comprised of 20 to 30 stems each, were taken for lignin analysis over the following 10 days. The total lignin content per stem (A) and the percentage lignin (B) are illustrated. The standard deviation for the percentage lignin is shown by the vertical bars.

The percentage of lignin was relatively constant (approximately 2%) for the first 8 days of the time-course experiment (fig 1) but then during the period which corresponded to 16 to 18 days after germination there was a sudden and marked increase in the lignin content in both the controls and the GA-treated tissue, analogous to that observed in "heading" wheat (25). Whether the increased lignin content observed here was due to a quantitative or qualitative change in lignin formation is not known. However, it appears that at least several weeks prior to blossoming a marked change in lignin metabolism occurs in this dwarf pea, a phenomenon which may prove of value in future studies of factors regulating lignification.

Effects of Light and GA on Lignification. The effects of light, darkness and GA on stem elongation are well known (16). Experiments were conducted to determine the effect of these factors on lignification in the dwarf pea stem. The growth retardant 2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidenecarboxylate methyl chloride (AMO-1618) is reported to be an inhibitor of GA biosynthesis (6). This inhibitor had no effect on the growth of the dwarf pea under white light (data not reported) but did retard stem elongation of dwarf plants grown in the dark (table II). AMO-1618 was included in the dark and red-light experiments to determine the effects of a reduced endogenous level of GA on lignification under these conditions. GA and AMO-1618 markedly promoted and retarded, respectively, stem elongation under dark or red-light conditions (table II). However, with 1 exception, these growth regulators had a negligible effect on the percentage lignin in the stems. Plants grown under red light did have a slightly higher percentage of lignin when treated with GA than did the untreated plants or the plants treated with AMO-1618.

In accord with an earlier report (12) the percentage of lignin was 2-fold higher in the stems of plants grown in white light than it was in those grown in the dark. However, the total amount of lignin was 2- to 3-fold higher in the stems of untreated plants grown in the dark than it was in corresponding tissue grown under white light. The greater amount of lignin in the stems of plants grown in the absence of light may be in part a function of the greater stem length. Stafford (23) has postulated that the number of sites available for lignin deposition may be a factor in lignification. The results obtained with AMO-1618 (Expt. 2, table II) suggest, however, that neither the percentage nor the total amount of lignin is strictly a function of stem length. In this experiment the growth retardant reduced stem length by 40% but had a negligible effect on both total and percentage lignin.

Table II. *The Lignin Content and Phenylalanine Ammonia Lyase Activity of the Stems of Dwarf Peas Grown Under Different Light Regimes and Treated With Gibberellic Acid or AMO-1618*

Eight-day old seedlings were sprayed once with 50 ml of GA (3×10^{-4} M) or AMO-1618 (1.4×10^{-4} M) and harvested 10 days later.

Expt. No.	Treatment	Increase in height	Lignin[1]		Phenylalanine ammonia lyase[2]	
		cm	*mg/stem*	*%*	*munits/stem*	*munits/mg protein*
			Darkness			
1	None	15.7	0.9±0.03	1.5±0.07	0.13	0.08
	GA	33.9	1.4±0.10	1.7±0.13	0.13	0.08
2	None	20.8	0.7±0.04	1.7±0.14	0.12	0.07
	AMO-1618	12.3	0.6±0.03	1.8±0.19	0.15	0.08
			Red Light			
	None	7.4	0.4±0.01	1.7±0.03	0.08	0.15
3	GA	31.3	1.0±0.01	2.0±0.07	0.28	0.25
	AMO-1618	6.7	0.3±0.01	1.6±0.04	0.07	0.10
			White Light			
4	None	3.7	0.3±0.05	3.3±0.26	0.10	0.18
	GA	10.4	1.1±0.01	4.6±0.13	0.43	0.35

[1] Each treatment was replicated 4 times with 30 plants per replication. The standard deviations are given.
[2] Each assay was run at least twice on duplicate samples comprised of 20 plants each.

Phenylalanine Ammonia-Lyase. The reported correlation between the activity of phenylalanine ammonia-lyase and the synthesis of phenylpropanoid compounds (19, 22, 31) and lignification (10, 24, 29, 30) led us to check the effect of GA on this enzyme. The measurable activity of phenylalanine ammonia-lyase was very low (0.10 mUnits) in the stems of the dwarf pea grown under white light as compared to normal corn (17 mUnits) (18). Treatment with GA increased phenylalanine ammonia-lyase activity in the pea stem by 60% within 1 day. Although the total activity of the ammonia-lyase enzyme remained on the average 3-fold higher in GA-treated tissue than in the control for the course of a 10-day experiment, which paralleled the time course study of lignification (fig 1), there was no correlation between enzyme activity and the sudden increase in lignification observed 16 to 18 days after germination.

The data for phenylalanine ammonia-lyase activity presented in table II do suggest, however, a correlation between the GA-enhanced lignification and GA-promoted activity of the enzyme. The percentage lignin and the specific activity of the enzyme were both about twice as high in the untreated plants grown under white light as they were in plants grown in the dark. Under dark conditions GA had no effect on either lignification or phenylalanine ammonia-lyase activity. It was only in light, both red and white, that GA had an effect; under these conditions both lignification and enzymatic activity were promoted by GA.

Increased phenylalanine ammonia-lyase activity and a concomitant enhanced production of phenylpropanoid compounds following exposure of tissue to light is well documented in a number of plants (2, 4, 22, 31, 32) although the light effect may not be universal (18, 26). Studies (22, 26, 31, 32) employing inhibitors of protein and nucleic acid synthesis indicated that the increase in phenylalanine ammonia-lyase activity is a result of enzyme induction. Attridge and Smith (2) have reported that in Alaska pea, a tall cultivar, activation of phenylalanine ammonia-lyase in the terminal bud is mediated by phytochrome. We assume that in our work the illumination provided was sufficient to convert at least part of the phytochrome to the far-red form. The promotion of phenylalanine ammonia-lyase activity by GA in the dwarf plants grown under light but not in dark suggests that GA and a photoreaction, perhaps the phytochrome reaction, may both be required for induced protein synthesis. An alternative possibility is that GA either inhibits the action or prevents the synthesis of the recently reported (26, 32) phenylalanine ammonia-lyase inactivating protein.

The data reported here suggest that in the gibberellin-deficient pea tissue the reaction catalyzed by phenylalanine ammonia-lyase is 1 rate-limiting step in lignin biosynthesis. We have no direct evidence of the other reactions which must be limiting in the present case. However, since phenylalanine is a protein amino acid, its supply normally cannot be limiting else exogenous phenylalanine could be expected to stimulate protein synthesis and growth. The observation (14) that gibberellic acid stimulated a 3-fold increase in the concentration of 3 phenolic precursors of lignin in peas suggests that the limiting reaction(s) may be at the polymerization stage.

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