

# Evidence that Auxin-induced Xylogenesis in *Lactuca* Explants Requires Calmodulin

Sango Baba

(PROFESSOR OF BIOTECHNOLOGY)

The calmodulin-binding drugs trifluoperazine and chlorpromazine completely inhibited auxin-induced xylogenesis in cultured pith parenchyma explants of *Lactuca*.

Callus formation was only slightly suppressed under the same cultural conditions. Chlorpromazine sulfoxide, a non-antagonistic CP analog, did not inhibit xylogenesis. Addition of the calcium ionophore A23187 ( $\text{Ca}^{2+}$  salt) to a TFP-containing medium was ineffective in reversing TFP inhibition. Verapamil at low concentrations (20–50  $\mu\text{M}$ ) had no appreciable effect, but stimulated xylogenesis at 100  $\mu\text{M}$ . Lanthanum consistently stimulated xylogenesis with low variation in TE cell counts (50–100  $\mu\text{M}$ ). The possible significance of the web patterns of secondary wall thickening is discussed in terms of the Falconer and Seagull hypothesis.

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## INTRODUCTION

A recent hypothesis regarding the regulation of physiological events by auxin suggests that the auxin-receptor complex acts via a transducer on a  $\text{Ca}^{2+}$  gate which, in turn, regulates intracellular calcium and calmodulin-controlled enzyme systems.<sup>1)</sup> This hypothesis is supported by the finding that calmodulin-binding compounds are highly effective in blocking auxin-dependent wheat (*Triticum aestivum*) coleoptile growth<sup>7)</sup> and auxin-induced elongation of oat (*Avena sativa*) coleoptile segments.<sup>16)</sup> Since TE differentiation in cultured explants is also an auxin-induced phenomenon,<sup>17)</sup> it was of interest to determine the possible role of calmodulin in this process.

A specific requirement for calcium in xylogenesis has never been clearly established. Davis<sup>5)</sup> found a general decrease in vascular

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differentiation in shoot meristems of *Pinus taeda* during periods of calcium deficiency. In this same study the root apical meristems showed precocious formation of tracheids near the apex following calcium deficiency. Further details on the anatomical and cytological effects of calcium deficiency has been reviewed by Hewitt.<sup>11)</sup>

The present study describes the results of some experiments on auxin-induced xylogenesis in cultured explants of pith parenchyma of lettuce in the presence of calmodulin antagonists. Further experiments were conducted on antagonists of calcium flux and apoplastic calcium.

## MATERIALS AND METHODS

Tissue explants were prepared as previously described<sup>4,6)</sup> from the core excised from heads of Romaine lettuce (*Lactuca sativa* cv Romaine) obtained from commercial sources. The trimmed core was then surface sterilized in a 10% (v/v) aqueous solution of sodium hypochlorite for 10 min, and subsequently rinsed four times with sterile double-distilled water. Cylinders of pith parenchyma, removed with a 5 mm inside diameter sterile cork borer, were sliced into 2 mm thick explants. The explants were rinsed twice in sterile doubledistilled water, blotted lightly on Whatman No. 1 filter paper, and transferred singly to glass scintillation vials containing 10 cm<sup>3</sup> culture medium.

The medium consisted of a Murashige and Skoog<sup>14)</sup> basal medium supplemented with *myo*-inositol (100 mg 1<sup>-1</sup>), thiamine-HCl (0.1 mg 1<sup>-1</sup>), glycine (2.0 mg 1<sup>-1</sup>), nicotinic acid (0.5 mg 1<sup>-1</sup>), pyridoxine-HCl (0.5 mg 1<sup>-1</sup>), indole-3-acetic acid (10 mg 1<sup>-1</sup>), kinetin (0.2 mg 1<sup>-1</sup>), sucrose (2% w/v), and Bacto-Agar (1% w/v). The culture tubes, capped with aluminum foil, were placed in glass culture jars (7 vials

per jar). The jars, wrapped with aluminum foil, were dark incubated at 25°C for 7 days.

At the termination of each experiment the explants were weighed and TE cell counts were determined by a modification of the Brown and Rickless maceration procedure.<sup>2,6)</sup> Samples were placed singly in 1.0 cm<sup>3</sup> of a chromic acid-HCl maceration fluid for 24 hr at room temperature. The maceration fluid was removed and replaced with 0.5 cm<sup>3</sup> distilled water. The disrupted sample was drawn repeatedly into a hypodermic syringe equipped with a 22 gauge needle. The cell suspension was brought to a final volume of 2 cm<sup>3</sup>, and 1.0 cm<sup>3</sup> of the suspension was transferred to a Sedgwick-Rafter plankton counting chamber. The differentiated TE were counted in 10 optical fields with the assistance of a Whipple eyepiece micrometer (X100). The total number of TE in each explant was calculated.<sup>6)</sup> TE were also examined in explants that had been cleared and stained with safranin O.<sup>6)</sup>

Calmodulin-binding compounds employed in this study included trifluoperazine (10  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M) and chlorpromazine (10  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M). Chlorpromazine sulfoxide, an inactive analog of CP, was tested at concentrations of 10  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M. An attempt was made to reverse the inhibitory effect of TFP (10  $\mu$ M) by the addition of the Ca<sup>2+</sup> salt of the ionophore A 23187 (64  $\mu$ M) to the xylogenic medium containing TFP. Experiments were also conducted with verapamil (20  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M) and lanthanum chloride (LaCl<sub>3</sub>; 50  $\mu$ M, 100  $\mu$ M). A sample of CPS was provided gratis from Smith, Kline, and French. All other calcium antimetabolites were obtained from the Sigma Chemical Co. Stock solutions of all antimetabolites were freshly prepared prior to each experiment, and the reagents were

initially taken into solution with a few drops of dimethylsulfoxide. All components of the media were sterilized by autoclave.

## RESULTS AND DISCUSSION

The calmodulin antagonists TFP and CP completely inhibited xylogenesis at concentrations of 50  $\mu\text{M}$  and 100  $\mu\text{M}$ , respectively (Table 1). Callus formation was only slightly suppressed at these same concentrations. CPS, an analog of CP that is ineffective in blocking calmodulin,<sup>16)</sup> had relatively little effect on xylogenesis and callus in the range of 50–100  $\mu\text{M}$  (Table 1). The data provides evidence that the physiological effects of CP are specific for calmodulin. An unsuccessful attempt was made to reverse TFP inhibition of xylogenesis by incorporating the  $\text{Ca}^{2+}$  ionophore A23187 ( $\text{Ca}^{2+}$  salt) in the xylogenic medium containing TFP. Because of the wide variation in the TE cell counts, it can be assumed that the ionophore is incapable of consistently reversing the blocking effect of TFP (Table 2).

Two other calcium antagonists are verapamil and lanthanum. Veramil blocks movement of  $\text{Ca}^{2+}$  through voltage-dependent channels in the plasmalemma, and it is less effective in blocking receptor operated channels not involving membrane potential differences.<sup>18,20)</sup> Verapamil had no noticeable effect on cell division. At low concentrations it had no consistent effect on xylogenesis, although verapamil stimulated this process at 100  $\mu\text{M}$  (Table 3). Verapamil has been reported to block  $\text{Ca}^{2+}$  transport from the apoplast into the cell thereby preventing the budding of *Funaria*.<sup>18)</sup> Assuming verapamil blocks voltage-dependent  $\text{Ca}^{2+}$  influx, one must assume that xylogenesis is not dependent on a supply of apoplastic calcium.

Although the movement of lanthanum is restricted to the apoplast,<sup>3,15,19)</sup> this rare earth metal is a competitive antagonist of calcium in the cell wall.<sup>19)</sup> The addition of  $\text{LaCl}_3$  (50  $\mu\text{M}$ , 100  $\mu\text{M}$ ) to the xylogenic medium was definitely stimulatory to auxin-induced xylogenesis (Table 3). Although the reason for this stimulation is not known, it might result from a sparing effect on apoplastic calcium. The latter essential element, released from cell wall binding sites, would be available for uptake via plasmalemma channels. Other workers have taken an opposite view on the assumption that verapamil and lanthanum block  $\text{Ca}^{2+}$  transport from the wall into the cytoplasm.<sup>18)</sup> Further experiments should be undertaken in order to establish the effect(s) of verapamil and lanthanum on intracellular levels of calcium.

Two distinct patterns of secondary wall thickening were evident in populations of TE differentiated in the presence of low concentrations of TFP and CP. Falconer and Seagull<sup>9)</sup> have given the terms "bands" (scalariform) and "webs" (reticulate) to these secondary wall patterns. Random arrays of cortical MT were associated with web patterns in isodiametric cells, whereas parallel arrays of MT were found in elongated cells with band patterns.<sup>9)</sup> An experiment was devised to test the hypothesis that calmodulin antagonists are capable of influencing the developing wall pattern. Pith parenchyma explants were cultured for 72 hr on a xylogenic medium as previously described. Secondary wall deposition is initiated after approximately 72 hr of culture.<sup>10)</sup> The explants were subsequently transferred to a similar xylogenic medium containing TFP (100  $\mu\text{M}$ ). Many of the TE differentiated under these conditions had abnormal secondary walls with unique patterns (Fig. 1a,b). In some cases web and band patterns appeared within the same TE in unique configurations (Fig. 1b). This is the first evidence

that calmodulin may play some role in the establishment of the secondary wall pattern in developing TE. The hypothesis can be made that localized disturbances of MT arrays by the calmodulin antagonists have resulted in localized web patterns. The close association between MT orientation and wall pattern in developing TE was clearly shown by Falconer and Seagull.<sup>8)</sup>

Immunohistochemical techniques revealed significant amounts of calmodulin localized in differentiating metaxylem elements in root apices.<sup>12)</sup> Additional work is needed in order to demonstrate whether or not calmodulin is preferentially associated with cortical MT prior to the establishment of the secondary wall pattern in differentiating TE. The techniques developed by Wick and her colleagues<sup>21)</sup> might resolve this question.

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Table 1. Effect of trifluoperazine, chlorpromazine and chloromazine sulfoxide on xylogenesis and callus formation in *Lactuca* explants. Explants dark cultured 7 days (23°C). Initial fresh weight approximately 85 mg. Fresh weight expressed in mg/explant + SE. TE cell counts + SE given per explant based on a mean of 10 samples.

Treatment	Fresh Weight	TE Cell Count	Effect
Control (TFP)	173±9	32,800±3,600	—

TFP, 10 $\mu$ M	169 $\pm$ 15	14,500 $\pm$ 5,900	-55.8%
TFP, 50 $\mu$ M	132 $\pm$ 13	—	-100%
TFP, 100 $\mu$ M	132 $\pm$ 10	—	-100%
Control (CP, CPS)	178 $\pm$ 33	35,000 $\pm$ 4,000	—
CP, 10 $\mu$ M	171 $\pm$ 20	29,000 $\pm$ 9,000	-17%
CP, 50 $\mu$ M	182 $\pm$ 15	8,000 $\pm$ 4,000	-77%
CP, 100 $\mu$ M	147 $\pm$ 14	trace	-99%
CPS, 10 $\mu$ M	168 $\pm$ 8	33,000 $\pm$ 3,000	-5%
CPS, 50 $\mu$ M	162 $\pm$ 12	31,000 $\pm$ 9,000	-11%
CPS, 100 $\mu$ M	179 $\pm$ 10	38,000 $\pm$ 6,000	+9%

Table 2. Effect of ionophore A 23187 ( $\text{Ca}^{2+}$  salt) on reversing tri-fluoperazine inhibition of xylogenesis (Table 1 for further details).

Treatment	Fresh Weight	TE Cell Count	Effect
Control	149 $\pm$ 12	41,000 $\pm$ 5,200	—
TFP, 10 $\mu$ M	145 $\pm$ 7	18,600 $\pm$ 9,000	-55%
TFP, 10 $\mu$ M+			
A23187, 64 $\mu$ M	148 $\pm$ 6	21,000 $\pm$ 12,000	-49%

Table 3. Effect of verapamil and lanthanum chloride on xylogenesis and callus formation in *Lactuca* explants. (see Table 1 for further details).

Treatment	Fresh Weight	TE Cell Count	Effect
Control (VP)	162 $\pm$ 9	42,000 $\pm$ 5,000	—
VP, 20 $\mu$ M	161 $\pm$ 9	39,600 $\pm$ 12,000	-7%



VP, 50 $\mu$ M	165 $\pm$ 14	41,100 $\pm$ 16,900	- 2 %
VP, 100 $\mu$ M	163 $\pm$ 10	56,400 $\pm$ 6,100	+25.5%
Control (LaCl <sub>3</sub> )	150 $\pm$ 9	11,800 $\pm$ 2,900	—
LaCl <sub>3</sub> , 50 $\mu$ M	156 $\pm$ 18	15,500 $\pm$ 2,300	+31%
LaCl <sub>3</sub> , 100 $\mu$ M	154 $\pm$ 11	13,600 $\pm$ 3,800	+15%

## LEGEND TO FIGURE 1

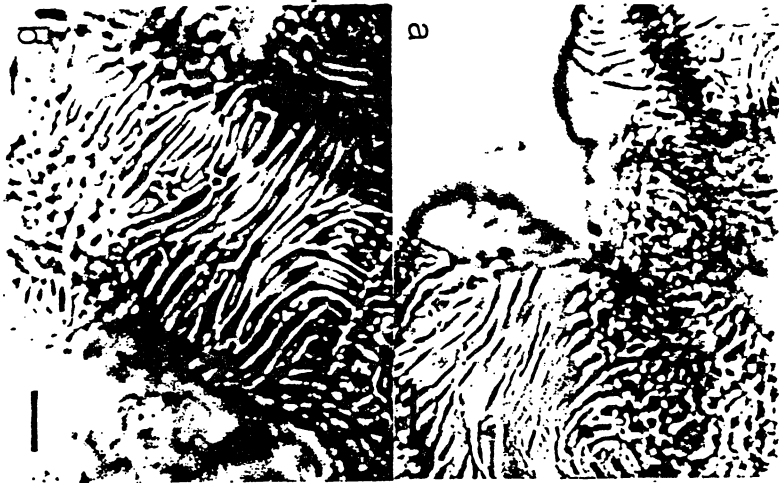


Fig.1. Micrographs of aberrant tracheary elements differentiated in pith parenchyma explants of *Lactuca* after 72 hr culture on a xylogenic medium followed by 48 hr of culture on a similar medium containing trifluoperazine (100  $\mu$ M). (a) Clump of TE showing web and band patterns of secondary wall thickening (upper left). The extreme web pattern seen in the largest cell in the micrograph (lower right) is uncharacteristic of TE differentiated in plant tissue cultures. (b) TE showing both web and band patterns within the same cell. Scale bars represent 25  $\mu$ M in (a) and 22  $\mu$ M in (b).

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