Secondary wall deposition in tracheary elements of cucumber grown *in vitro*

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Abstract

It is a matter of controversy whether secondary wall deposition is dependent on lignification during the development of tracheary elements. To understand this, tracheary element differentiation was studied in the homogeneous calli obtained from the cotyledonary explants of *Cucumis sativus* subsequent to treatment with plant growth regulators, such as naphthalene acetic acid (NAA) and benzylamino purine (BAP), which are necessary for the induction of tracheary elements, along with metabolic blockers such as 2-aminoindan-2-phosphonic acid (AIP), 2,3,5-triiodobenzoic acid (TIBA) and nifedipine. Calli treated with AIP, a potential inhibitor of L-phenylalanine ammonia-lyase (PAL), have no PAL activity at any time during the culture period. There was a complete inhibition of lignification although secondary wall deposition was unaltered. Similar results were obtained using TIBA, an inhibitor of auxin transport, and nifedipine, a known calcium channel blocker. Thus the present study suggests that secondary wall deposition in the course of tracheary element differentiation need not to be dependent on lignification.

Additional key words: AIP, *Cucumis sativus*, lignification, PAL, TIBA, xylogenesis.

Introduction

Lignification is an important aspect of secondary wall formation in differentiating tracheary elements (Hepler *et al.* 1970, Wardrop 1981, Savidge 1996). Lignification and tracheary element (TE) differentiation under *in vitro* conditions have been well

Received 19 November 1997, *accepted* 20 September 1998.
Acknowledgements: This work forms a part of the Ph.D. thesis submitted by the senior author to the Bharathidasan University, Tiruchirappalli. This thesis was critically evaluated by Dr. Jack B. Fisher, Fairchild Tropical Garden, Miami, United States, which had helped in the refinement of the matter contained in this paper. The authors are thankful to him for his suggestions. The senior author is also thankful to the Department of Science & Technology, India for the financial assistance.
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studied and have provided much information regarding factors controlling vascular differentiation (Bengochea *et al.* 1983, Fukuda and Komamine 1985, Phillips 1980, Roberts 1976, Sugiyama and Komamine 1990, Torrey *et al.* 1971, Church 1993 and Savidge 1996). During differentiation of tracheary elements, secondary wall formation and lignification are considered to be very closely interrelated. It has been stated that lignin deposition appears to require the presence of a cellulosic matrix (Siegel 1956) and further that in *Zinnia* cultures, only cells with secondary walls become lignified (Fukuda and Komamine 1981, Ingold *et al.* 1988, Lin and Northcote 1990, and Northcote 1995). Some workers have claimed that lignification is not necessary during secondary wall deposition (Ingold *et al.* 1988, Smart and Amrhein 1985). This has lead to the question whether lignification is absolutely necessary during secondary wall deposition in such elements. The present work, therefore, has been undertaken to settle this controversy.

Materials and methods

Cotyledonary explants of aseptically grown seedlings of *Cucumis sativus* L. produced homogeneous calli (Bolwell 1987) through repeated subculturing in a Murashige and Skoog's (1962; MS) basal medium supplemented with 1 mg dm^{-3} 2,4-dichlorophenoxy acetic acid (2,4-D), 3 % (m/v) sucrose, and 8 % (m/v) agar. Further experiments involving the treatments of homogeneous calli using various metabolic blockers, *e.g.*, 2-amino indan-2-phosphonic acid (AIP), 2,3,5-triiodobenzoic acid (TIBA) and nifedipine were carried out either in a culture medium with 8 % agar or in a liquid medium.

AIP, an excellent inhibitor of phenylalanine ammonia-lyase (PAL) both *in vitro* and *in vivo* and superior to both 1-α-aminooxy-β-phenyl propionic acid (AOPP) and 1-amino-2-phenyl ethyl phosphonic acid (APEP) (Zon' and Amrhein 1992) was used to inhibit L-phenylalanine ammonia-lyase (PAL) activity in the prospective tracheary elements. This was added at a concentration of 4×10^{-4} M to the xylogenic medium [containing 0.5 mg dm^{-3} naphthalene acetic acid (NAA) and 1.0 mg dm^{-3} benzylaminopurine (BAP)] at the time of implanting the 2,4-D grown calli and these calli were analysed cytochemically for its effect on lignification at frequent intervals, ranging from 0 upto 28 d.

The activity of PAL (E.C. 4.3.1.5) was assayed in calli grown in absence of plant growth regulators (PGRs) (control), in calli grown with PGRs + sucrose and in calli grown with PGRs + sucrose + AIP using the method of Bolwell (1987). The activity of PAL was assayed by taking 0.05 cm^3 of the extract separately with *a*) 0.95 cm^3 of 10 mM L-phenylalanine and *b*) 0.95 cm^3 of 10 mM D-phenylalanine, both *a*) and *b*) were prepared in 0.1 M Tris-HCl, pH 8.8. Using the tube *b*) with D-phenylalanine as a blank, absorbance was read at 290 nm; in the L-phenylalanine tube 5 min after starting the reaction.

In another set of experiments, the homogeneous calli were treated with TIBA (an inhibitor of auxin transport). It was added as a solution in sterile-filtered water to the xylogenic medium, to give a final concentration of 0.25, 0.5, 1.0, 2.0, 3.0, 4.0 or

5.0 mg dm^{-3}. Observations were made at 1, 3, 7, 11 and 15 d after treatment. As controls, the callus tissues were cultured on a medium containing only the added PGRs for similar periods of time.

A role for Ca^{2+} in the induction of tracheary elements in callus cultures was indirectly examined using nifedipine, which is a prominent representative of the 4-aryl dihydropyridines, an important class of organic Ca^{2+} channel blocker. The effect of nifedipine was estimated by taking 30 cm^3 of the cell suspension culture, previously maintained in liquid MS medium containing 0.1 mg dm^{-3} NAA and 0.001 mg dm^{-3} BAP (corresponding to 1 g fresh mass of cells). To this 0.5 mg dm^{-3} NAA, 0.25 mg dm^{-3} BAP and nifedipine (*Sigma*, Hyderabad), dissolved in dimethyl sulfoxide (DMSO) at various concentrations ranging from 10 to 200 μM were added. As control, the cell suspension culture in the absence of nifedipine was used. Observations were made at 1, 3, 7, 11 and 15 d after treatment.

The tracheary elements were detected under a light microscope based on the presence of secondary wall thickening. This was done after hydrolysing the callus tissue in 45 % acetic acid at 60 °C for 1 min (Gahan *et al.* 1994) and stained with 0.5 % aqueous safranin or with toluidine blue O which stained the lignified walls red or blue (Krishnamurthy 1988).

Results

The homogeneous parenchymatous callus tissue, when subjected to treatment with xylogenic medium comprising 0.5 mg dm^{-3} NAA and 1.0 mg dm^{-3} BAP, tracheary element differentiation commenced on the 3rd day of culture and fully differentiated elements were seen on the 7th day. Peak appearance of tracheary elements was noticed on the 14th day of culture. PAL activity was found during the entire period of callus cultures. The peak PAL activity coincided with the peak xylogenesis on the 14th day subsequent to which there was a decline (Table 1). The content of this enzyme was very low in calli grown in the absence of PGRs.

Table 1. Phenylalanine ammonia-lyase (PAL) activity in callus cultures of *Cucumis sativus* grown for 28 d in MS salt medium without (control) or with the added plant growth regulators (0.5 mg dm^{-3} NAA and 1.0 mg dm^{-3} BAP). Mean of 3 replicate determinations ± SE.

	PAL [μg g^{-1}(f.m.)]					
	0 d	3 d	7 d	14 d	21 d	28 d
Control	1.2 ± 0.11	2.1 ± 0.05	2.3 ± 0.11	1.9 ± 0.08	2.1 ± 0.05	1.8 ± 0.05
PGR	2.4 ± 0.05	4.8 ± 0.05	7.2 ± 0.11	9.8 ± 0.17	6.3 ± 0.08	1.7 ± 0.11

When the homogeneous callus tissue was subjected to treatment with xylogenic medium consisting of PGRs, along with AIP, PAL activity could not be detected any time during the period of observation. None of the prospective tracheary elements

underwent lignification although they showed secondary walls including the reticulate-banded thickenings (Fig. 1).

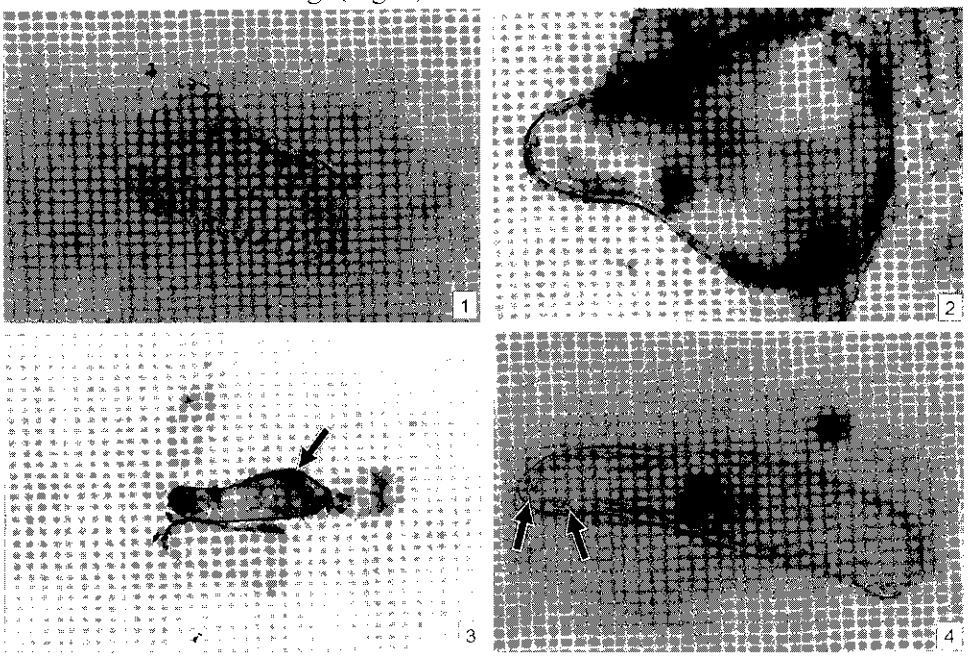

Fig. 1. A single "mature" tracheary element stained with toluidine blue O showing complete lack of lignification due to AIP treatment of the callus. Note the secondary wall including the thickening bands have been fully developed but not lignified as there is the absence of colour (× 850).
Fig. 2. A single prospective tracheary element, treated with TIBA, showing one pole bloats into a balloon like structure while the other pole remains very narrow (× 850).
Fig. 3. Prospective tracheary elements, treated with nifedipine, stained with phloroglucinol showing complete lignification (*arrow*) (× 225).
Fig. 4. A prospective tracheary element, treated with nifedipine, stained with phloroglucinol showing total absence of lignification. Note the wall of the cell is not lignified although it is secondary in nature (*arrows*) (× 850).

TIBA induces very characteristic morphological changes in the prospective tracheary cells. One pole of the affected cell bloats into a balloon like structure (Fig. 2), while the other pole remains very narrow. Interestingly, such changes were not observed in ordinary callus cells or in prospective phloem cells. In a number of prospective tracheary elements, secondary wall deposition commenced but not completed with lignification. The lower concentrations of TIBA, ranging from 0.25 to 3.0 mg dm^{-3}, affected only a decline in the number of tracheary elements by 30 % (data not shown) over control, when the observation was made on the 7^{th} day. On the other hand, in the higher concentrations of TIBA, 4.0 and 5.0 mg dm^{-3}, there was almost a total absence of fully differentiated tracheary elements even on the 7^{th} day.

After addition of nifedipine in all the concentrations used no tracheary elements upto 7 d of culture were observed. But after 11 d, a few mature tracheary elements were noticed. When such elements were stained for lignin, some showed complete

lignification (Fig. 3), while others showed the total absence of lignification (Fig. 4). In the partially lignified and totally unlignified tracheary elements, secondary wall deposition, however, proceeded normally, although to unequal thickness, at different loci in the non-lignified regions.

Discussion

The relationship between PAL activity and lignification during tracheary element differentiation has been a matter of dispute. In Jerusalem artichoke calli little correlation was found between changes in PAL and lignification (Minocha and Halperin 1976). However, the activity of PAL has been reported to be one of the earliest markers associated with prospective tracheary elements (Fukuda and Komamine 1982, Ingold *et al.* 1988). This enzyme has been shown to be in peak during the period of maximum xylogensis in calli and that greater PAL content was present in tracheid differentiating calli than in tracheid free calli (Rubery and Fosket 1969, Roberts 1988). It was also shown that this enzyme was directly involved in the first step in lignification, that is, the deamination of phenylalanine into transcinnamic acid (Jones 1984, Northcote 1985, Kavi Kishor 1989, Church 1993); although it may also be involved in active protein synthesis (Rubery and Fosket 1969). Tritiated tyrosine, phenylalanine and methionine application resulted in the labelling of xylem thickenings in *in vitro* systems (Picket-Heaps 1968). Inhibition of PAL activity arrested lignification in the secondary walls of differentiating tracheary elements. The present study also categorically demonstrates the importance of PAL in lignification. This was proved by using AIP, a potent inhibitor of PAL. Thus, not only peak activity of PAL coincided with peak xylogenesis on the 14^{th} day but also complete inhibition of lignification by AIP was recorded although secondary wall deposition is unaltered. The result obtained by us is in agreement with the observations made by Smart and Amrhein (1985) using 1-α-aminooxy-β-phenyl propionic acid (AOPP), another inhibitor of PAL activity, in mungbean. Thus the data reported in the present study and the experiments of other workers, suggest that PAL is one of the earliest markers of tracheary element differentiation, through its influence on lignification.

One area that needs critical discussion and further investigation relates to the roles of calcium in vascular differentiation (Roberts *et al.* 1988). Calcium is reported to regulate many processes in plant development, and in xylogenesis it may control responses of cells to growth regulators, auxin transport, microtubule organisation, cellulose synthesis and deposition, and lignification (Dedman *et al.* 1979, Sabnis and Hart 1982, Delmer *et al.* 1984, Hepler and Wayne 1985, Roberts and Baba 1987, Roberts *et al.* 1988, 1992, Fukuda and Kobayashi 1989, Roberts and Haigler 1989, 1990, Church 1993). The basis for these conclusions were the application of calcium deprivation and calcium channel blockers, both of which were reported to inhibit the differentiation of tracheary elements.

In the present investigation, nifedipine, a known calcium channel blocker (Lee and Tsien 1983, Hepler and Wayne 1985, Kauss 1987), was added to the medium that promoted xylogenesis. Upto 7 d none of the xylem elements underwent lignification

but showed secondary wall deposition. After 7 d the majority of elements did not undergo lignification. The present study, therefore, clearly shows that calcium affects only lignification and not secondary wall deposition, including cellulose synthesis, as has been claimed by some investigators like Delmer *et al.* (1984) and Roberts and Haigler (1990). Calcium perhaps blocks lignification by releasing wall bound peroxidase as has been stated by Wardrop (1976). Our conclusions are also supported by the other result obtained by us, when calli were treated with 2,3,5-TIBA, a substance known to block movement of calcium (Roberts 1976, Burgess and Linstead 1984, De Guzman and Dela Fuente 1984) as well as auxins, the latter probably through the first. TIBA-treated prospective tracheary elements underwent secondary wall deposition but not lignification. These cells were invariably bloated at one pole of the cell, possibly indicating the failure of auxin transport since calcium channels were blocked by TIBA, thus proving the other role contemplated for calcium, that is, involvement in auxin transport.

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