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CELL-WALL MUTANTS FROM HIGHER PLANTS: A NEW METHOD USING CELL-WALL ENZYMES

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Abstract—Several enzymes were located in the cell wall of *Datura innoxia*. Rapid screening for released cell-wall enzymes, which are not usually secreted into the growth medium, was used to isolate a series of variants in some of these enzymes.

Cell-wall enzymes are very important for wall deposition and tissue differentiation. Numerous enzymes have been shown to be located in the cell wall both by histochemical and fractionation techniques (see [1, 2]). Oxidases (phenol oxidase, ascorbic acid oxidase and peroxidases), hydrolases (phosphatases, pectin methyl esterases and glycosidases) and dehydrogenases have all been found. Varying levels of many of these enzymes are found at different stages of differentiation and growth, suggesting a central role in these processes.

Dissection of metabolic pathways by isolation of mutants has had limited success [3–7] due to the lack of good screening procedures. The present approach has been to utilize haploid suspension cultures of *Datura innoxia* and to screen mutagenized cells for aberrant cell-wall enzyme activity and then find the effect of the mutation on the regeneration and development capabilities. This first report deals with a rapid screening method for the recognition of variants in cell-wall enzymes.

Growth medium, cells and cell walls were screened for enzyme activity (Table 1). Alkaline phosphatase, neutral β-galactosidase and β-glucosidase have fairly high activities in the cell wall but are virtually undetectable in the growth medium. These enzymes were then assayed in the screening process. Acid phosphatase is present in all fractions and this proved to be very useful in the development of the assay technique. If filter paper was used to draw off the surface moisture on the cultures, only areas immediately surrounding each microcallus gave a

Table 1. Enzyme assays of the cell fractions from *D. innoxia*

Substrate	pH	Cell fraction		
		Medium	Cells	Cell walls
4-Methylumbelliferone phosphate	5	++++	++++	++
	7	++	++	+
	9.5	−	+++	+++
4-Methylumbelliferone galactoside	5	++	++	(+)
	7	−	+	+
	9.5	−	−	−
4-Methylumbelliferone glucoside	5	+	+++	+
	7	−	++	++
	9.5	−	−	+
Guaiacol	6.8	+	++	++

Centrifuged growth medium (1 ml), equivalent numbers of cells (usually 10^6) and cell-wall material from the same number of cells were used for the assays.

−, no detectable change; (+), slight fluorescence; +, easily discernible fluorescence; ++, good fluorescence; +++, strong fluorescence; ++++, very strong fluorescence.

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positive result for the acid phosphatase. No other enzymes were detected in this way before mutagenesis and so this assay was used to screen for secretor variants of the other enzymes.

It is noteworthy that alkaline phosphatase has only been found in plants growing in phosphate-deficient medium. In *Chlamydomonas* they are induced and appear to be localized at the cell periphery [8]. In *Lemna* [9] the induced phosphatase was not localized and in *Spirodela* [10] about half was found in the growth medium. Supplementing the growth medium used in this study with phosphate (5 mM instead of 1 mM) decreased the alkaline phosphatase levels. A similar alkaline phosphatase to the *Datura* was found in the cell walls of sycamore, carrot, soybean and tomato suspension cultures, by incubating cell-wall fractions with 4-methyl umbelliferone phosphate in alkaline buffer. It appears to be a *bona fide* wall enzyme and is non-specific, being able to hydrolyse *p*-nitrophenol phosphate, 4-methyl umbelliferone phosphate, ATP, glycerol phosphate and glucose-6-phosphate. It is also inhibited by sodium fluoride (100 mM) although not completely.

Using the filter paper assay large numbers of microcalli could be screened in a short time. Several screening attempts for secreted alkaline phosphatase, β-glucosidase and peroxidase, using *ca* 1000 microcalli for each enzyme, gave four presumptive mutants of alkaline phosphatase, 20 for β-glucosidase and none for peroxidase. Since peroxidase is a very important enzyme in growth and differentiation (e.g. lignin synthesis [11]) the assay could be made more sensitive to detect an over-producer.

The presumptive mutants were then transferred to fresh medium and retested after another 4 weeks of growth. One of the alkaline phosphatase secretors and at least one of the β-glucosidase secretors were found to be stable. These are being cultured further with the aim of regenerating half of the callus and making suspension cultures from the other half. One problem encountered so far is the slow growth rate. It seems likely that the variations arise in the cell wall itself (albeit the enzyme) and by expanding the techniques described here (such as using fluorescent lectins to detect carbohydrates or glycoproteins not normally secreted by the cell) additional variants could be isolated. These along with those already found could prove very useful in the elucidation of cell-wall morphogenesis and function.

EXPERIMENTAL

Haploid *D. innoxia* plantlets (gift from J. Ranch, Iowa State University) were maintained on M and S medium [12] supplemented with 1 μM benzyladenine. Small plantlets were transferred to medium without hormones for rooting and then grown in a greenhouse. Callus was initiated from petiole explants and suspension cultures made from placing 1–3 g of callus into M and S medium supplemented with 10 μM 2,4-D and shaking (120 rpm). Single cell suspension cultures were mutagenized by addition of NaN_2 (to 10 μM, for 24-hr) which had a negligible effect on cell viability (measured by trypan blue [13]).

4-Methylumbelliferone-β-galactoside, β-glucoside and phosphate were used at 4 mM in three buffers: 100 mM acetate (pH 5), imidazole–HCl (pH 7) and glycine–NaOH (pH 9.5). The release of 4-methyl umbelliferone was assurred qualitatively by using a long-wave UV source. Peroxidase was detected by using 5 mM guaiacol [14] in 100 mM Pi buffer (pH 6.8) containing 5 mM H_2O_2.

Cells for assay, were separated from the growth medium by filtration (polypropylene 120-μm mesh). Cell walls were isolated by sonication (Braunsonic 1510, maximum setting) followed by filtration and extensive washing, first in 1 M NaCl then H_2O [1].

Mutagenized cells were plated on agar and grown for 3–4 weeks until the microcalli were about 1 mm in diameter. A dry, sterile filter paper (Whatman No. 3, 7 cm dia) was carefully lowered over the exposed colonies and agar. The surface moisture was absorbed in 3–5 sec. The filter paper was then removed and tested with the appropriate substrate (by dipping into a solution of the substrate in buffer) either immediately or after drying. Only one substrate could be tested for on any one culture.

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