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Characterization of Chilli Pepper Hairy Roots Expressing the Parsley PAL2 cDNA

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Abstract

Transformed hairy root cultures of chilli pepper (*Capsicum frutescens* cv. cayenne) containing the CaMV 35S promoter linked to the parsley PAL-2 cDNA were generated. These transformants showed increased PAL activity at early and late stages of culture. The morphology, colour and growth rate of these transformants were quite different from the control hairy roots. Measurements of cell dry weight, content of fibre and lignin-like material, suggested that the altered characteristics of the PAL transformants might be linked to lignification. HPLC analyses of phenolic compounds in transgenic roots revealed the accumulation of several substances that were not found in the controls. In response to the addition of salicylic acid or phenylalanine, lignification of the control hairy roots increased. However, the content of lignin-like substances in the PAL transformants was not increased by these treatments. These results indicate that lignification of the PAL transformants had reached a level that could not be further increased.

Abbreviation

PAL: phenylalanine ammonia-lyase, GUS: β-glucuronidase

1. Introduction

Transgenic plants are being used, not only to improve commercial species, but also to study plant metabolism and physiology. Alterations in levels of specific enzymes have brought about changes in the quantity or quality of particular products (Hamill *et al.*, 1990, Chavadej *et al.*, 1994). Furthermore, generation of transgenic plants with differences in amount of a key enzyme of metabolism is an effective approach to analyse pathway flux.

Chilli pepper is a spice material and its pungent principle, capsaicin, is an end-product of the phenylpropanoid pathway. Capsaicin is only found in fruits and cultures of chilli pepper. The relationship between the synthesis and accumulation of capsaicin has been studied in plants and cultured cells of *C. frutescens* (Lindsey and Yeoman 1984, Lindsey 1985, Hall and Yeoman 1991, Sukrasno and Yeoman 1993). The levels of capsaicin, putative intermediates and their derivatives were measured together with chilli pepper fruit growth. The accumulation of capsaicinoids, lignin-like substances and several C6-C1 compunds occurred along with the disappearance of the three cinnamoyl glycosides and two flavonoids. This result suggests that biosynthesis of capsaicin is closely related to the flux through the phenylpropanoid pathway. In this paper, we demonstrate the increase in activity of the first enzyme of the phenylpropanoid pathway using a PAL transgene in hairy roots of chilli pepper. The increase of PAL activity resulted in increased lignin biosynthesis. The alteration of phenylpropanoid metabolism in chilli pepper hairy roots transformed with the PAL gene is also discussed.

2. Materials and Methods

2.1 Plant material

The generation and establishment of hairy root cultures of chilli pepper (*Capsicum frutescens* cv. cayenne) have been reported previously (Sekiguchi *et al.*, 1996). Hairy roots induced by *Agrobacterium*

rhizogenes strain A13 were grown in liquid Schenk and Hildebrandt medium (Schenk and Hildebrandt 1972). Transformed root cultures was produced in which either GUS gene or a PAL cDNA were introduced (Yamakawa *et al.*, 1998). Two plasmids, pBI121 and pBI12PPAL respectively, was introduced into *A. rhizogenes* strain A13. A binary vector pBI121 carried the CaMV 35S promoter-GUS gene fusion and the neomycin phosphotransferase gene as a selectable marker (Jefferson *et al.*, 1987). The chimeric plasmid pBI12PPAL was constructed with pBI121 by substituting the GUS gene with a full length parsley PAL-2 cDNA (Lois *et al.*, 1989). pBI121 transformants were prepared as a control for pBI12PPAL transformants. These transformants were also cultured in the same Schenk and Hildebrandt liquid medium (Schenk and Hildebrandt 1972) as reported previously (Yamakawa *et al.*, 1998). Three replicate flasks were used for each experiment.

2.2 Biochemical analysis

The dry weight of the roots was determined after the culture medium had been rinsed away with distilled water, and the roots were then dried to constant weight in an oven at 90°C. Soluble protein was extracted using the method described previously (Yamakawa *et al.*, 1998). Protein determinations were carried out according to the method of Bradford (1976). Free amino acid content was determined using amino acid auto analyzer (Hitachi) and the extraction procedure followed the method described in its manual. Crude fat content was measured after extraction with acetone in Soxhlet's extractor for 6 hours followed by evaporation of the solvent. Fibre residues and cell wall components, were prepared using a detergent treatment according to the method of Van Soest (1963a and b). Subsequently, the dried residues were weighed. Lignin-like substances were extracted according to the method of Sukrasno and Yeoman (1993) and the content estimated according to the method described by Johnson *et al.*, (1961) and Marth (1959). Phenolic compounds were extracted and analysed in an HPLC system (Hitachi L-6000) equipped with 3-dimensional UV detector, using a 6 mmϕ × 250 mm, C_8 column (PEGASIL, Senshu kagaku), according to the method of Hall *et al.* (1987). All analytical procedures were performed in duplicate.

3. Results

3.1 Characterization of pBI12PPAL transformants

The hairy roots of pBI12PPAL transformants grew more slowly than the control hairy roots in the liquid medium under the same culture conditions as the controls. The epidermal cells of these roots were dark brown, globular in form in contrast with the pale and cubic cells of control hairy roots (**Fig. 1**). Light microscopy revealed that cell walls of the pBI12PPAL transformants were much thicker than those of control hairy roots and accumulated dark-coloured substances. However, contents of soluble protein and total fat showed that pBI12PPAL transformants were similar to control hairy root tissue in these respects (**Table 1**). In contrast, differences in free amino acid content and dry weights were observed, indicating metabolic changes in pBI12PPAL transformants (**Table 1**). The amino acid contents of pBI12PPAL transformants were quite different to the control. Threonine, serine, glycine, alanine, valine and leucine were present in similar amounts in all hairy roots. In contrast, the proline content of the pBI12PPAL transformants was approximately 30-fold greater than the control (not shown). Cysteine and methionine were only found in the pBI12PPAL transformants. All hairy roots contained similar amounts of other amino

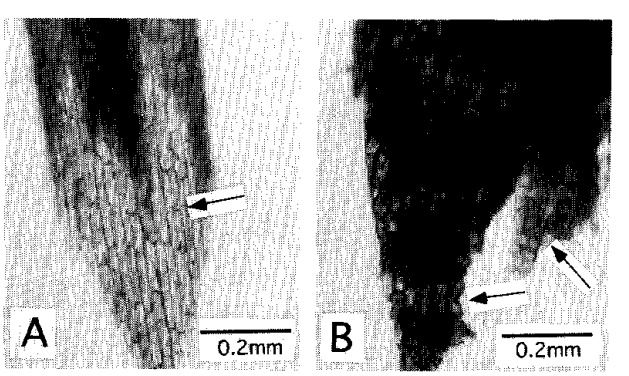

Fig. 1 Section of hairy roots.
A; control hairy roots
B; pBI12PPAL trasnsformant

Table 1. Constituents of hairy roots transformed with pBI121 and pBI12PPAL.

	Dry weight /Fresh weight	Crude Fat ($mg \cdot g^{-1}$fresh weight)	Soluble Protein ($mg \cdot g^{-1}$fresh weight)	Free Amino Acid ($\mu mol \cdot g^{-1}$fresh weight)
Control hairy root	0.071	12±0	2.3±0	3.3±0.5
pBI121 transformed hairy root I	0.078	11±1	2.4±0.2	4.1±0.5
pBI12PPAL transformed hairy root I	0.141	13±1	2.3±0	34.8±4.1
pBI12PPAL transformed hairy root II	0.162	10±1	2.1±0.2	29.8±3.5

acids including phenylalanine.

3.2 Analysis of the cell wall component of hairy roots

The pBI12PPALtransformants exhibited cell wall enlargement and greater apparent lignification. Fibre analysis of pBI12PPAL transformants suggested that some of the substances which accumulated in their cell walls were lignin-associated components (**Table 2**). A crude cell wall fractions without pectin was prepared by a neutral-detergent treatment. pBI12PPAL transformants contained more than twice the amount of this crude cell wall fraction found in the control hairy roots. The apparent accumulation of secondary metabolites in cell walls was also observed. Ligno-cellulose and insoluble materials were extracted from cell walls by acid-detergent treatment. Differences between the two cell wall fractions indicated that all hairy roots contained similar amounts of cell wall cellulose. However, the content of lignin-like substances was greater in pBI12PPAL transformants indicating that expression of the PAL transgene directly encouraged lignin synthesis in chilli pepper hairy roots (**Table 2**).

3.3 Analysis of phenolic compounds

Phenolic compounds which accumulated in hairy roots were detected by 3-D HPLC. Chromatograms of the extracts from the pBI12PPAL transformants had several characteristic peaks. Ferulic acid, a capsaicin precursor, coincided with one of these peaks. pBI12PPAL transformants accumulated about three times the amount of ferulic acid of the controls (**Table 3**). Phenylpropanoid precursors of capsaicin other than ferulic acid were not found in these HPLC analyses. These results might suggest that ferulic acid functions as a sink for the phenylpropanoid pathway in pBI12PPAL hairy roots. HPLC analysis of the phenolic compounds extracted after acid treatment of the aqueous solution showed many substances which specifically accumulated in pBI12PPAL transformants. These substances may be glucosides of phenolic compounds. These results suggest that expression of the PAL transgene affected many pathways in secondary metabolism. No phenylpropanoid precursors of capsaicin could be found in the culture medium of the three phenotypes of hairy roots.

Table 2. Contents of crude fibre and lignin-like substances in transfomed hairy roots.

	Neutral-detergent fibre ($mg \cdot g^{-1}$ fresh weight)	Acid-detergent fibre ($mg \cdot g^{-1}$ fresh weight)	Lignin-like substances ($mg \cdot g^{-1}$ fresh weight)
Control hairy root	58±2	34±0	10.3±1.4
pBI121 transformed hairy root I	57±3	26±4	9.7±1.7
pBI12PPAL transformed hairy root I	126±28	86±10	21.7±3.3
pBI12PPAL transformed hairy root II	142±16	106±4	21.6±2.8

Table 3. Ferulic acid contents in transfomed hairy roots.

	$ng \cdot g^{-1}$fresh weight
Control hairy root	0.61±0.11
pBI121 transformed hairy root I	0.77±0.20
pBI12PPAL transformed hairy root I	2.00±0.24
pBI12PPAL transformed hairy root II	2.71±0.15

3.4 The effects of salicylic acid and phenylalanine

The metabolic response was examined upon stimulation by addition of salicylic acid and phenylalanine to the liquid medium. Salicylic acid (at 0.1 and 1

Table 4. Fresh weight of transfomed hairy roots after treatment with salicylic acid and phenylalanine.

	Treatment		
	Control(g)	salicylic acid treated(g)	phenylalanine treated(g)
Control hairy root	2.9±0.3	1.2±0.0	2.6±0.3
pBI121 transformed hairy root I	3.6±0.4	1.3±0.1	3.0±0.3
pBI12PPAL transformed hairy root I	1.8±0.1	1.2±0.0	1.8±0.2
pBI12PPAL transformed hairy root II	1.7±0.0	1.2±0.1	1.6±0.1

One gram hairy root of each phenotype was grown in 200 m*l* flask.
Phenylalanine and salicylic acid were added into liquid medium
2 days after explantation and cultured for 8 days.

Table 5. Contents of lignin-like substances in the hairy roots after treatment with salicylic acid and phenylalanine. (mg lignin•g^{-1} fresh weight).

	control (mg lignin•g^{-1} fresh weight)	Salicylic acid treated (mg lignin•g^{-1} fresh weight)	Phenylalanine treated (mg lignin•g^{-1} fresh weight)
Control hairy root	10.3±1.4	17.3±3.4	13.8±2.1
pBI121 transformed hairy root I	9.7±1.7	13.0±1.1	14.8±2.4
pBI12PPAL transformed hairy root I	21.7±3.3	19.5±0.5	24.1±1.1
pBI12PPAL transformed hairy root II	21.6±2.8	21.5±0.5	18.5±1.5

One gram hairy root of each phenotype was grown in 200 ml flask.
Phenylalanine and salicylic acid were added into liquid medium
2 days after explantation and cultured for 8 days.

mM) was selected because it caused hairy roots to turn brown, whereas a range of other compounds reported to affect secondary metabolism (1 gl^{-1} and 3 gl^{-1} yeast extract, 0.1 mM, 1 mM and 10 mM glutathione, 0.02 mM, 0.1 mM and 0.5 mM $CuSO_4$, 0.05%, 0.1% and 0.2% H_2O_2, 0.5 gl^{-1} chitosan), did not cause browning (results not shown). Phenylalanine was chosen since it is the primary precursor of the phenylpropanoid pathway. Fresh weight increase of hairy roots was decreased by salicylic acid treatment (**Table 4**). Both treatments stimulated lignification for control hairy roots but not for pBI12PPAL transformants (**Table 5**). HPLC analysis showed that phenolic compounds did not accumulate in hairy roots in response to salicyclic acid treatment and little effect of phenylalanine treatment was observed (data not shown). Capsaicin biosynthesis was not observed in response to addition of any of the chemicals mentioned above which stimulate secondary metabolism.

4. Discussion

Transformation of chilli pepper hairy roots with a PAL gene altered the metabolism of aromatic compounds and also the content of amino acids. An increase in phenylalanine derivatives was observed as an accumulation of ferulic acid and a strong lignification of hairy roots. The pBI12PPAL transformants also showed some physiological changes including slow growth, greater diameter, dark brown colour and enlargement of cells. Some of these changes were related to increased lignin accumulation. These characters of the PAL gene transformants of chilli pepper have not been reported for other plants. For example, transgenic tobacco plants which exhibited reduced levels of PAL activity exhibited abnormal plant development and reduced phenylpropanoid biosynthesis (Elkind *et al.*, 1990, Maher *et al.*, 1994, Bate *et al.*, 1994). However chilli pepper hairy root cultures demonstrated a relationship between increased levels of PAL activity and phenylpropanoid biosynthesis. These data indicate that phenylpropanoid biosynthesis can be controlled by the level of PAL.

Phenylpropanoid metabolism plays an important role in plant development since lignin is one of the products that determines tissue structure. An increase in PAL activity in pBI12PPAL transformants induced slow, abnormal growth of chilli pepper hairy roots which could be a consequence of increased lignification. The selection of the pBI12PPAL transformant was not achieved on agar medium with 20 mgl^{-1} kanamycin, but succeeded in liquid medium (Yamakawa *et al.*, 1998). Only two pBI12PPAL transformants exhibiting the same increased PAL level were obtained. It may be that roots with higher PAL activities are unable to grow and are therefore not recovered. However, the difference in PAL activities between control hairy roots and pBI12PPAL transformants was relatively small (Yamakawa *et al.* 1998). It may be that the parsley PAL is not subject to the same control mechanisms as that of chilli pepper and so exhibits greater activity *in vivo*.

The reduced growth rate of pBI12PPAL transformants was apparently not related to phenylalanine deficiency, because analysis of the phenylalanine content provided evidence that the pBI12PPAL transformants have the same ability to produce phenylalanine as control hairy roots. In addition, supplementing phenylalanine to the liquid medium had no effect on the growth of the pBI12PPAL transformants. Measurements of dry weights, cell wall components and lignin-like substances demonstrated that most of the increase in end-products of the phenylpropanoid pathway were directed towards lignin-like substances. The flux through to lignin biosynthesis may be less strictly controlled than other branch pathways in root tissue. Increased lignification which occurred in pBI12PPAL transformants may limit their growth rate because the increased PAL activity may have diverted carbon from energy production into

xylogenesis. The addition of salicylic acid and phenylalanine which stimulated lignification also inhibited control hairy root growth. However, these treatments affected neither growth nor the accumulation of lignin-like substance in pBI12PPAL transformants. These data support the hypothesis that there is an upper limit of PAL activity which can be tolerated, and can be achieved either by stimulation with salicylic acid or phenylalanine, or by PAL transgene expression. Expression of the homologous PAL transgene with the CaMV 35S promoter in tobacco callus has been reported (Nagai *et al.*, 1995). The PAL activity level of the transformants increased almost 4-fold and scopoletin content increased more than 2-fold as compared to non-transformed cells. Remarkable lignification or xylogenesis was not found among these transformed clones. These results on the genetic manipulation of the PAL genes with the CaMV 35S promoter indicate that effects of the PAL transgenes may depend on the combination of the plant host species, the gene source and the tissue in which the transgene is expressed.

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