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. cayemne) 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 compounds 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. cayemne) 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 Mark (1959). Phenolic compounds were extracted and analysed in an HPLC system (Hitachi L-6000) equipped with 3-dimensional UV detector, using a 6mm.φ × 250mm, C4 column (PEGASIL, Sen-

| Table 1. Constituents of hairy roots transformed with pBI121 and pBI12PPAL. |
|-----------------|-----------|-----------------|-----------------|-----------------|
|                  | Dry weight | Crude Fat       | Soluble Protein | Free Amino Acid |
|                  | /Fresh weight | (mg·g⁻¹fresh weight) | (mg·g⁻¹fresh weight) | (µmol·g⁻¹fresh weight) |
| Control hairy root | 0.071 | 12±1 | 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 |

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 acids.
3.2 Analysis of the cell wall component of hairy roots

The pBI12PPAL transformants 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 fraction 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 capsicin 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 capsicin could be found in the culture medium of the three phenotypes of hairy roots.

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

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<tr>
<th>Table 2. Contents of crude fibre and lignin-like substances in transformed hairy roots.</th>
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<tr>
<td>Control hairy root</td>
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<tr>
<td>pBI21 transformed hairy root I</td>
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<tr>
<td>pBI12PPAL transformed hairy root I</td>
</tr>
<tr>
<td>pBI12PPAL transformed hairy root II</td>
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<td>pBI12PPAL transformed hairy root II</td>
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<th>Table 3. Ferulic acid contents in transformed hairy roots.</th>
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<td>Control hairy root</td>
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</tr>
<tr>
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<td>pBI12PPAL transformed hairy root II</td>
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<th>Table 4. Fresh weight of transformed hairy roots after treatment with salicylic acid and phenylalanine.</th>
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<td>Treatment</td>
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<tr>
<td>Control hairy root</td>
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<tr>
<td>pBI12PPAL transformed hairy root II</td>
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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 g l⁻¹ and 3
g l⁻¹ yeast extract, 0.1 mM, 1 mM and 10 mM glutathione,
0.02 mM, 0.1 mM and 0.5 mM CuSO₄, 0.05%, 0.1%
and 0.2% H₂O₂, 0.5 g l⁻¹ chitosan), did not cause
browning (results not shown). Phenylalanine was
chosen since it is the primary precursor of the phenyl-
propanoid 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 trans-
formants (Table 5). HPLC analysis showed that
phenolic compounds did not accumulate in hairy roots
in response to salicylic 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 metab-
olism.

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 trans-
formants 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 accumula-
tion. These characters of the PAL gene transform-
nants 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 rela-
tionship 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 trans-
formant was not achieved on agar medium with 20
mg l⁻¹ kanamycin, but succeeded in liquid medium
(Yamakawa et al., 1998). Only two pBI12PPAL trans-
formants 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 activ-
ities 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 transform-
nants was apparently not related to phenylalanine
deficiency, because analysis of the phenylalanine con-
tent provided evidence that the pBI12PPAL transform-
nants 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. Mea-
measurements of dry weights, cell wall components and
lignin-like substances demonstrated that most of the
increase in end-products of the phenylpropanoid path-
way 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

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<tr>
<th>Control hairy root (mg lignin·g⁻¹ fresh weight)</th>
<th>Salicylic acid treated (mg lignin·g⁻¹ fresh weight)</th>
<th>Phenylalanine treated (mg lignin·g⁻¹ fresh weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control hairy root</td>
<td>10.3±1.4</td>
<td>17.3±3.4</td>
</tr>
<tr>
<td>pBI121 transformed hairy root I</td>
<td>9.7±1.7</td>
<td>13.0±1.1</td>
</tr>
<tr>
<td>pBI12PPAL transformed hairy root I</td>
<td>21.7±3.3</td>
<td>19.5±0.5</td>
</tr>
<tr>
<td>pBI12PPAL transformed hairy root II</td>
<td>21.6±2.8</td>
<td>21.5±0.5</td>
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</tbody>
</table>

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.
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 pBI122 PAL trans- 
mants. 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. Remark-
able 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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