



Cloning and characterisation of cytochrome P450 genes from barley (*Hordeum vulgare*)

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Introduction

Plant P450s belong to the cytochrome group that are membrane-bound enzymes, usually found in plant endoplasmic reticulum. This gene family is diverse in structure and function, which enables these enzymes to participate in numerous biosynthetic and degradative pathways. In plants, P450s are known to play important roles in production of hormones, pigments, oils, and defensive compounds. P450s are also involved in herbicide detoxification in cereal crops, including barley. In addition, the heme group of P450s is responsible for several catalytic reactivities of cytochrome P450 in plants.

Purification of functional P450 enzymes has proven to be difficult due to their low abundance and lability (Chaple, 1998). However, in the last decade, molecular cloning techniques have been successfully used to isolate a large number of P450 genes from many species. Recently, sequencing of the complete Arabidopsis genome identified a total of 224 cytochrome P450 genes. However, the function of most of these genes is still unknown.

In this paper, we describe our strategies to apply molecular cloning techniques to isolate P450 genes from barley. Two approaches have been utilised. The first approach is the application of Polymerase Chain Reaction based methods to clone gene fragments from genomic DNA. The second approach is the use of the International Triticeae EST Cooperative (ITEC) database (<http://wheat.pw.usda.gov/genome/index.html>) to search for P450 gene sequences. Expression techniques are being employed to investigate gene expression patterns of isolated barley P450 clones.

Materials and Methods

Approach 1: Molecular cloning of barley P450 genes by Polymerase Chain Reaction based methods

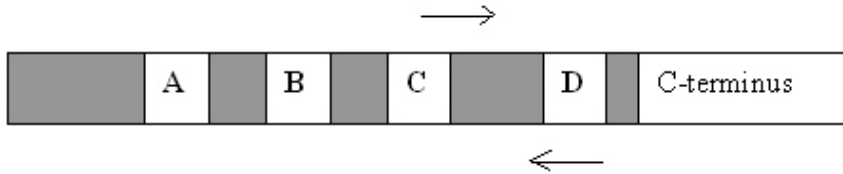


Figure 1. Strategy to amplify amino acid sequence around the haem-binding by PCR primers. The four regions of sequence conservation are found in most cytochrome P450 sequence including plant P450s. These regions, also called domains, are named A, B, C, and D. Each domain has its own pattern of amino acid sequence (Kalb and Loper, 1988). Among these domains, D is located near the C terminal end and contains a highly conserved FxxGxxxCxG that corresponds to the haem-binding group of most P450 plants (Chaple, 1998).

Template DNA was extracted from barley seedlings (cv. Chebec and Harrington), using DNeasy plant mini kit (QIAGEN) according to the manufacturer's protocol. Two forward and four reverse primers were designed based on consensus amino acid sequence occurring between domain C and the haem-binding of most cytochrome P450 sequences. Degenerate primers were employed to amplify nucleotide sequences around the haem-binding region. PCR products were cloned into TA cloning vector (Promega). Inserts of positive clones were sequenced by ABI BigDye Terminator. Cloned sequences were analysed using MacVector (version 6.5.3, Oxford Molecular Ltd.). Translated sequences (with or without primer sequences) were compared to the Genbank database using BLASTP program. Multiple sequence alignment was performed with Clustal W program (MacVector version 6.5.3, Oxford Molecular Ltd.).

Approach 2: Gene identification from Expressed Sequence Tags.

Using FASTA program, approximately 24000 wheat and barley sequences from the ITEC database were screened for P450 genes. Full-length sequences from the 9 representative clans of the P450 superfamily were searched against the ITEC database. Using BLAST searches, ESTs that showed high similarity to P450 clan representatives were compared to sequences in Genbank to confirm their classification.

Results

Molecular cloning of barley P450 genes by Polymerase Chain reaction based methods

Table 1. List of P450 gene fragments isolated by PCR based methods

Clone ID	Translated sequence ^T	Amino acid sequence identity between isolated clones and best matched P450 genes/species	
LN PCR 1	FMECGSAAAMDYKGTDFSYLP	9/13 (69%)	Rice
LN PCR 2 ^a	FMECGSAAAMDYKGDDFSYLP	19/33 (57%)	Maize
LN PCR 3 ^a	FMECGSAAAMDYKGNDFSHLP	17/31 (54%)	Petunia x hybrida
LN PCR 4	FMECGSAAAMDYKGNDFSYLP	9/11 (81%)	Arabidopsis
LN PCR 5 ^a	FMECGSAAVMDNKGNDFFSYLP	18/33 (54%)	Petunia x hybrida
LN PCR 6	FMECGSAAIMDYKGNDFSYLP	9/11 (81%)	Arabidopsis
LN PCR 7 ^a	FMECGSAATMDYKGSDFSYLP	14/30 (46%)	Petunia x hybrida
LN PCR 8	FMECGSAATMDYKGNDFSYLP	9/11 (81%)	Arabidopsis
LN PCR 9	FMKCGSAATMDYKGNDFSYLP	9/11 (81%)	Arabidopsis
LN PCR 10 ^a	FSQRVCYIQIRVHFA YIYVLP	12/34 (35%)	Maize
LN PCR 11 ^a	FSQRVCYIQIRVHFA YIHVLP	12/34 (35%)	Maize
LN PCR 12	FQNKNINYMGAYSEFTP	10/17 (58%)	Wheat
LN PCR 13	FQNKNINYKGAYREFTP	11/17 (64%)	Wheat
LN PCR 14	FQNKNINYKGAYSEFTP	11/17 (64%)	Wheat
LN PCR 15	FEDKDVDVFNGAHFELLP	13/17 (76%)	Rice
LN PCR 16	FEDKGVDFNGAHFELLP	12/17 (70%)	Rice
LN PCR 17	FEDTTVDYNGTQFEYLP	10/17 (58%)	Prunus dulcis
LN PCR 18	FEDTTVDYNGTQFECLP	10/17 (58%)	Lolium rigidum
LN PCR 19	FEDTTEDYNGTQFEYLP	10/17 (58%)	Prunus dulcis
LN PCR 20	FESGMVDFKGTNFEYIP	11/16 (68%)	Brassica napus
LN PCR 21	FENDSTNYGGTYFEFIP	13/17 (76%)	Wheat
LN PCR 22	FEKNTINFNGTYFEFLP	11/17 (64%)	Wheat
LN PCR 23	FENNNVDYNGTSFEFTP	13/17 (76%)	Wheat
LN PCR 24	FENNNMDYNVTYFEFIP	13/17 (76%)	Wheat

LN PCR 25	FEDNNVDYNGTSFEFTP	12/17 (70%)	Wheat
LN PCR 26	FVGSATDFRGNSEFIF	11/17 (64%)	Soybean
LN PCR 27	FLGSTIDFRGVDFELLP	11/17 (64%)	Mentha piperia
LN PCR 28	FKPSFEVVTKNNYFP	7/19 (37%)	Barley
LN PCR 29	FKPSFEVVSKNNYFP	7/19 (37%)	Barley
LN PCR 30 ^a	FGVVEVHSPMGISVVFSWELCLYP	14/37 (37%)	Soybean

^T The first and last amino acid residues of each sequence (F, P) are derived from sequences of degenerate primers.

^a Translated sequence including primer sequences were used to search against the Genbank database to confirm their classification of P450.

We have cloned 30 barley P450 gene fragments using PCR strategy (Table 1). Out of these clones, both LN PCR 28 and 29 have 37 % amino acid sequence identity with CYP73A34 from barley (Nelson, D.R. 2001). LN PCR 3,5,7 have matched P450 sequences of Flavonoid 3'-Hydroxylase (petunia x hybrida) but this matching was only found when translated sequence containing the primer sequences were used. Clone 26 showed 64% identity to CYP71D10 in soybean but the function of this gene is still unknown. Among isolated clones which were homologous to cytochrome P450 in rice, clone 15 and 16 showed a high level of identity (76% and 70% respectively) to the same sequence (gi|12583813|gb|AAG59665.1|AC084319_23). Eight out of 30 isolated clones showed similarity to cytochrome P450s in wheat.

P450 gene identification by Expressed Sequence Tags

Table 2. List of selected P450 clones identified from ITEC database

Barley EST Clones	Best match species	Function of matching genes	Amino acid sequence identity between EST clones and matched genes
ITEC EST 1	Barley	Allene oxide synthase	83/85 (97%)
ITEC EST 2	Barley	Allene oxide synthase	83/83 (100%)
ITEC EST 3	Madagascar periwinkle	Cinnamate 4-Hydroxylase	101/134 (75%)
ITEC EST 4	Populus	Cinnamate 4-Hydroxylase	175/243 (72%)

Sixteen barley EST sequences were identified in the ITEC database by this approach. Among these EST sequences, four cloned ESTs showed homology to known function

P450 genes (Table 2). While the rest of barley EST sequences identified by this strategy matched most closely to P450 genes of unknown function.

Discussion

Several methodologies such as protein purification, differential screening, transposon & T-DNA tagging have been used to isolate P450 genes in plants, but only PCR and EST techniques can target specifically P450 genes. Moreover, these techniques have shown a potential to isolate a large number of P450 genes. In the past, PCR approach was applied successfully to isolate numerous P450 genes in other species for example petunia (Holton and Lester, 1996) and Arabidopsis (Mizutani *et al.*, 1998).

It is interesting to note that the Flavonoid 3'-Hydroxylase and the Cinnamate 4-Hydroxylase gene fragments isolated from barley were most homologous to genes in the bioflavonoid pigment synthesis pathways of fruit and flower development.

As mentioned above, both approaches have yielded more than 30 barley P450 gene fragments. Expression patterns of these isolated clones are being investigated by northern blot and microarray analysis. In this way perhaps the function of pigmentation genes in barley may be resolved.

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