Isolation of Differentially Expressed Genes Involved in Clubroot Disease

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Abstract

The interaction between *Plasmodiophora brassicae* and its host *Brassica rapa* is investigated by two strategies. (1) IAA-conjugate hydrolases: Root hypertrophy in club root disease is dependent on increased auxin levels and these could result from auxin-conjugate hydrolysis. So far we isolated 5 different cDNA fragments out of various tissues which revealed high identity to IAR3/ILL5, ILL2, ILL3, ILL6 and ILR1 genes from *Arabidopsis* by comparison with database entries. (2) Random priming: Using this method, we have so far obtained 26 clones from clubroot tissue, from which several sequences may be components of plant signal transduction chains, metabolic pathways and transcriptional regulation.

Keywords: *Plasmodiophora brassicae*; *Brassica rapa*; IAA-amidohydrolases; random priming

INTRODUCTION

Clubroot is a serious crucifer disease. During infection of members of the *Brassicaceae* with the obligate biotroph organism *Plasmodiophora brassicae* a strong hypertrophy of the root tissue is observed. This hypertrophy is known to be dependent on high auxin (BUTCHER *et al*. 1974) and cytokinin levels (DEKHUIJZEN & OVEREEM 1971). It was shown that the IAA (indole-3-acetic acid) content in infected plants of *Brassica rapa* was approximately 60–70% higher than that of the non-infected controls (LUDWIG-MÜLLER *et al*. 1993). Considering our present knowledge about auxin homeostasis, one possible origin of the hormonally active free IAA is via auxin-conjugate hydrolysis. In *Arabidopsis thaliana* ILR1, IAR3, ILL1 and ILL2 encode IAA-amino acid hydrolases and three additional amidohydrolase-like genes (ILL3, ILL5, ILL6) have been isolated (BARTEL & FINK 1995; DAVIES *et al*. 1999; LE CLERE *et al*. 2002). Therefore we used degenerated oligonucleotides homologous to conserved domains of these amidohydrolases to isolate corresponding fragments of infected and healthy tissues of *Brassica rapa* via RT-PCR.

In a second approach we are looking for genes differentially expressed under infection conditions. One method to determine and isolate these fragments is via random priming. Based on preliminary studies we used the degenerated primers mentioned above also in a random priming experiment with cDNA of infected roots as template.

MATERIALS AND METHODS

To amplify amidohydrolase-like genes by RT-PCR the following tissues of *Brassica rapa* were used: uninfected seedlings (5 days), leaves and stems of uninfected plants (24 days) and roots of infected seedlings (11, 14, 21 days after infection). After 10 days of growth, infection of the plants was performed as described earlier (RAUSCH *et al*. 1983). Total RNA was isolated according to NAGY *et al*. (1988) and for reverse transcription the M-MLV-reverse transcriptase from Gibco BRL and oligo (dT) priming was used. PCR was performed according to standard procedures with the degenerated primers described by DAVIES *et al*. (1999), using the following program: initial denaturation at 95°C for 5 min, 40 cycles of 95°C for 45 s, 55°C for 60 s, 72°C for 90 s and final extension at 72°C for 10 min. After separation of the PCR products on an agarose gel, the DNA fragments were cleaned using the Stratagene gel slice kit and cloned into the pGEM-T Easy vector (Promega). Sequencing was performed by Seqlab GmbH (Göttingen). Homology was determined using the NCBI-BlastN.

For RT-PCR based random-priming infected roots 14 and 21 days after infection were used. Methods were the same as described above.

RESULT AND DISCUSSION

IAA-conjugate hydrolases. Using the degenerated oligonucleotides resembling conserved domains of the published IAA-amino acid hydrolases and amidohydrolase-like genes in *Arabidopsis* we isolated several fragments (~354 bp) with high homology to these genes via RT-PCR. Homologies were found to the IAA-amino acid hydrolases ILR1, IAR3 and ILL2 and to the amidohydrolase-like genes ILL3 and ILL6 (Table 1). To assign our isolated fragments to the described genes in the database, we simply mark them with the abbreviation "Br-" (for *Brassica rapa*) followed by the abbreviation of the gene with which the highest homology was found using BlastN. However, we are aware that our available sequences are too short for exactly classifying our fragments and that only the full-length clones can give more information concerning their identity.

Since the classification of one isolated fragment is not clear, we denoted it as IAR3/ILL5. In *Arabidopsis thaliana* ILL5 is the closest homolog of IAR3 and it is discussed to be a pseudogene (DAVIES *et al*. 1999).

The highest homologies are observed for the fragments Br-ILR1, Br-ILL6 and Br-IAR3/ILL5. In Table 2A the percent identity of the isolated *Brassica rapa* cDNA fragments with homology to IAA-conjugate hydrolases is shown, demonstrating high similarity between the sequences and protein alignment revealed the presence of highly conserved regions (data not shown). To support the classification of fragments a dendrogram was generated (Table 2B) and in comparison with the corresponding genes in *Arabidopsis* (LE CLERE *et al*. 2002) our fragments can be classified in the same three distinct branches: IAR3/ILL5/ILL2; ILL6 and ILL3/ILR1.

In Table 3 the isolated fragments with homology to IAA-amidohydrolases from *Arabidopsis* are listed according to the different tissues of *Brassica rapa* which were used as template for RT-PCR. In *Arabidopsis thaliana* expression of the IAR3 gene has been reported for different tissues, including stems

Table 1. Homology of the isolated fragments with database entries according to NCBI BlastN. A. Fragments isolated under specific PCR amplification conditions to identify possible amidohydrolase-like genes. The abbreviation "Br" denotes *Brassica rapa*, and the following abbreviations used, as for example "ILR1", demonstrate the highest homology of the isolated fragment with the corresponding database entries for IAA-amino acid hydrolases and amidohydrolase-like genes in *Arabidopsis*. B. Fragments isolated using the degenerated primer combination in a random priming approach. Results are presented using the E [x] (Expect value) and (%) identity

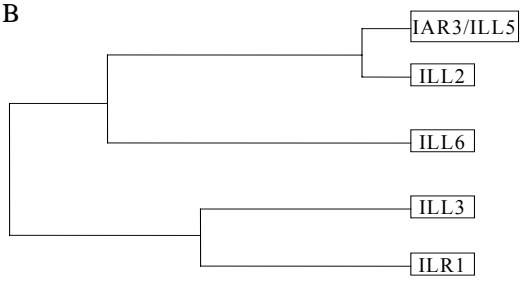
	Homology BlastN	Expect value E [x]	Identity (%)
A.	Br-IAR3/ILL5	–95	91
	Br-ILL2	–46	84
	Br-ILL6	–99	87
	Br-ILL3	–45	83
	Br-ILR1	–96	91
B.	n-calpain-1 large subunit, putative	–96	93
	putative GTP-binding protein	–74	89
	putative calcium-binding protein	–76	89
	anion channel protein	–23	86
	NADH-ubiquinone oxidoreductase	–100	92
	3-deoxy-D-arabino-heptulosonate 7-phosphate, putative	–72	94
	pectinacetylesterase precursor	–77	88
	putative hydroxymethyltransferase	–10	90
	splicing factor-like protein	–70	90
	putative exportin, tRNA (nuclear export receptor for tRNAs)	–64	86
	putative transcriptional regulator	–51	87
	putative zinc finger	–12	83

Table 2. Percent identity of *Brassica rapa* cDNA fragments (249 bp) with homology to IAA-conjugate hydrolases based on the predicted amino acid sequence. B. Dendrogram of the same sequences. The PCGENE program was used to determine the percentage of identity and to generate the dendrogram

A

	Br-IAR3	Br-ILL2	Br-ILL3	Br-ILL6
Br-ILR1	44	53	54	46
Br-IAR3		64	49	54
Br-ILL2			53	50
Br-ILL3				48

B



and shoots, comparable with our results (DAVIES *et al*. 1999). Considering the results for the infected roots it is interesting to notice, that during the course of infection the number of isolated fragments with homology to different IAA-amino acid hydrolase genes and amidohydrolase-like genes increased from 11 days past infection (dpi) over 14 dpi to 21 dpi. It is tempting to speculate that there could be a correlation between the increased auxin content during infection and the expression of different genes with possible auxin-conjugate hydrolase activity.

Random priming. For this approach we used cDNA of roots 14 and 21 days after infection. So far we have obtained 26 clones from these tissues, whereas most fragments were derived from the early infection stage (24 clones). In Table 1B only the fragments with homology to genes with known or putative functions are listed. Several of these sequences may be components of plant signal transduction chains, metabolic pathways and transcriptional regulation.

Table 3. Origin of the isolated fragments with homology to IAA-amidohydrolases from *Arabidopsis* amplified out of cDNA from the different tissues of *Brassica rapa*

Source (*Brassica rapa*)	Isolated fragments with homology to IAA-aminohydrolases from *Arabidopsis*
Uninfected seedlings (5 days)	ILL6
Leafs of uninfected plants (24 days)	ILL3, IAR3/ILL5
Stems of uninfected plants (24 days)	IAR3/ILL5
Roots infected with *Plasmodiophora brassicae* 11 dpi	IAR3/ILL5
Roots infected with *Plasmodiophora brassicae* 14 dpi	ILL2, IAR3/ILL5
Roots infected with *Plasmodiophora brassicae* 21 dpi	IAR3/ILL5, ILL3, ILR1

dpi = days past infection

Calpains for example are cytosolic proteases that are believed to function in stimulating Ca^{2+} signaling on cell activation, resulting in cell differentiation, proliferation and death (SATO & KAWASHIMA 2001). Although these genes were initially described for animal systems, calpains are also present in the plant kingdom and seem to be important in plant signal transduction (LID *et al*. 2002).

For certain enzymes of the prechorismate pathway like 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHP, EC 4.1.2.15) it could be shown, that a distinct accumulation of transcript was observed during wounding and pathogen attack in *Arabidopsis thaliana* (KEITH *et al*. 1991), implying a function during clubroot development.

To further determine the function of these isolated fragments in the course of clubroot infection, expression studies are under way.

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