Characterization of *Fragaria vesca* – *Rhizoctonia fragariae* Interaction by Gene Expression Analysis

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Abstract

The present work was carried out to characterize the *Fragaria vesca* – *Rhizoctonia fragariae* interaction by analysis of differentially expressed DNA. Four *Fragaria vesca* genotypes obtained from a specific breeding programme were utilized. Two of them were shown to be resistant against *Rhizoctonia* (*Rhizoctonia* – resistant); the others were susceptible to *Rhizoctonia* disease. For each genotype as a whole 45 plants were utilized. In particular 25 of them were artificially inoculated with *Rhizoctonia* mycelium, the others were used as testers (non-inoculated plants). Five sampling times were applied; 15, 30, 45, 60 and 75 days after inoculation. For each sampling time 5 inoculated and 4 tester plants were collected. Plant roots, crown regions and leaves were separately collected and stored in liquid nitrogen. RNA was purified separately from roots, crowns and leaves. In order to isolate differentially expressed genome fractions, cDNA was obtained. Specific and random primers were applied to isolate differentially expressed sequences among susceptible and resistant genotypes. All the differentially expressed fragments obtained were isolated and sequenced. All sequences were compared to a database to evaluate similarity with genes already isolated. All fragments isolated were used to produce a specific cDNA library. A large percentage of the fragments sequenced showed homologies with ESTs. Considering the fragments showing homologies with genes encoding proteins with known activity, particular attention was directed to the expressed fractions related to beta-galactosidase, chitinase and glucanase genes. The fragments encoding for beta-galactosidase synthesis were isolated only in the resistant genotypes, starting from the first sampling time. Considering the enzymatic activity of this gene, it is possible to assume an indirect control of the pathogen.

INTRODUCTION

The understanding of molecular mechanisms underlying host-pathogen interactions has a major role in devising strategies to control diseases.

Differential display (DD) was first reported by Liang and Pardee (1992). This method can be used to amplify low abundance transcripts by polymerase chain reaction (PCR).

DD has been used to isolate plant genes which are involved in physiological events (Yoshida et al., 2001), signal transduction, stress response (Yi and Hwang, 1998) and secondary metabolism. With DD, scientists have been able to isolate genes encoding membrane proteins and transcription factors (Park et al., 2001); these genes occur in small amounts and they are typically difficult to identify. The experimental design can play an important role to reduce mistakes and improve the investigation capability of this technique.

In the cultivated strawberry the study of genetic control and gene expression could be complicated by the high ploidy level (8x) and for this reason the possibility to study this at the lowest ploidy level was considered. Cytological (Hancock, 1999) and phylogenetic evidence suggests that *F. vesca* and *F.* × *ananassa* have a common ancestor and because genetic analysis of polyploid species is very difficult, it has been suggested

that *F. vesca* can be used as a model for genome investigation. The evidence suggests that transcriptomic inferences obtained analysing diploid genomes could be related to related species at higher ploidy level. The present study was directed to investigate and characterize the gene expression patterns involved in the *Fragaria vesca – Rhizoctonia fragariae* interaction.

MATERIALS AND METHODS

Plant Material

Four *Fragaria vesca* genotypes obtained from a specific breeding programme were utilized. Two of them (PZ99C101 and PZ99C102) showed evidence of resistance against *Rhizoctonia* (*Rhizoctonia* – resistant); the other two (PZ99SP7.1 and PZ99SP8.1) were classified as susceptible to *Rhizoctonia* infection. For each genotype 45 plants were used; 25 of them were artificially inoculated with *Rhizoctonia* mycelium, the other 20 were used as controls (non-inoculated plants). Five sampling times were applied, respectively 15, 30, 45, 60 and 75 days after the inoculation. For each sampling time and for each genotype 5 inoculated and 4 tester plants were collected. From each plant we separately collected roots, crown regions and leaves and stored them in liquid nitrogen.

Mycelium Multiplication and Plant Inoculation

Rhizoctonia mycelium was multiplied on PDA medium starting from a fungus strain previously isolated from an infected plant. A mixture of mycelium and talcum powder was produced. The infected plants were obtained by applying the mixture to the roots. After that the plants were placed in the pot. The same procedure was applied to the non-infected plants using only talcum powder without mycelium. To confirm the infection of each plant a root fragment was collected and placed on PDA medium at each sampling time.

RNA Extraction and cDNA Synthesis

The sampled tissues were used for the RNA extraction. Total RNA was extracted starting from 2 g of tissue usingan extraction buffer: 0.2 M Tris HCl pH 8.5; 0.35 M NaCl; 7 M Urea; 0.2 M EDTA pH 8; 2% (w/v) SDS. Particular attention was paid during the cleaning step to obtain highly purified RNA; cDNA synthesis was carried out following the protocol described in Bachem et al. (1996).

Analysis Using Specific and Random Primers

Fragaria vesca cDNAs obtained were analyzed by PCR applying 37 random primers (10 mer) and degenerate primers related to biochemical pathways. To determine the structure of degenerate oligonucleotides, different nucleic and protein sequences were aligned using Clustal V software. PCR reactions were carried out in 50 µl final volumes using 0.5 units of Taq polymerase, 10 mM Tris-HCl pH 8.1, 50 mM KCl, 250 mM dNTPs, 3 mM $MgCl_2$ and 2 mM primers, 0.5 ng of cDNA for a total of 45 cycles applying the following thermal profile: 94°C for 1.10 min, 44°C for 1.40 min, and 72°C for 2.0 min., the final extension step was done at 72°C for 5 min. Amplified fragments were separated through 1.6% agarose gel stained with ethidium bromide. All PCR reactions were repeated three times to confirm their repeatability.

Elution, Cloning, Sequencing and Hybridization of cDNA Fragments

Differentially expressed bands obtained were excised from the gel and eluted using "Quantum Prep Freeze'N Squeeze DNA Gel Extraction Spin Column" (Biorad). Recovered fragments were cloned using pGEM-T Easy Vector Systems (Promega). Cloned cDNAs were then sequenced using a Sequenase kit applying Sp6 primer.

Sequences were analyzed applying FASTA, BLASTP, SIGSCAN, PROSCAN programs and compared with the EMBL Nucleotide Sequence Database.

GENESCAN software was also used to locate CDS (Coding Sequence) region in the sequences isolated. The sequences isolated were hybridized with *Fragaria vesca* and *Rhizoctonia* DNA previously extract as described by Martelli et al. (2002), to confirm the origin of the expressed sequence.

RESULTS AND DISCUSSION

The procedure used to obtain infected plants was extremely efficient, in fact the 100% of treated plants were positive in the test performed.

The procedure described for RNA extraction and cDNA synthesis proved to be very useful to isolate good quality RNA and to generate cDNA from the tissue analyzed.

Only 7 of the 37 primers tested were able to detect differentially expressed sequences. 84 differentially expressed fragments were collected. All fragments were cloned to produce a specific cDNA library. From the 84 fragments isolated, 18 fragments were selected for the sequence analysis. The selection was carried out taking into account the following parameters: differentially expressed fragments between inoculated plant and tester plants; differential fragments present at all sampling times. To confirm the origin of the selected fragments all of them were hybridized with the *Rhizoctonia* DNA. Results obtained showed that only 10 fragments could be definitely classified as *Fragaria vesca* fragments.

In Table 1 we report the homology of the fragments selected. Seven of them showed similarity with ESTs previously isolated from several plant species. The others showed high homology with sequences already isolated and having direct or indirect action against fungus. In particular the sequences labelled as 6A and 7AP showed homology with glucanase and chitinase respectively, enzymes with a direct action against fungus. Results showed the complete homology of the 7 AL isolated fragment with a beta-galactosidase. The amplicon was isolated from an inoculated resistant plant. The action of this protein could be classified as having indirect activity against fungus. In particular the gene produces a protein that causes the necrosis of the cell around the infection point. The necrotic areas create a physical barrier to the fungus development.

CONCLUSIONS

The experimental methods applied proved suitable to study host pathogen interaction in strawberry and the experimental design had an important role in producing the results described. By applying a step by step strategy of analysis, it was possible to isolate expressed sequences involved in the *Fragaria vesca – Rhizoctonia fragariae* interaction and several differentially expressed fragments were isolated. A specific cDNA library was created that will be useful to understand more about the expression of the genes and their control. The results obtained improve knowledge about the indirect beta-galactosidase action against fungus. In particular, future research activity will be directed to assess the beta-galactosidase action against other fungi.

Literature Cited

Bachem, C.W., Van der Hoeven, R.S., de Bruijn, S.M., Vreugdenhil, D., Zabeau, M. and Visser, R.G. 1996. Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development: Plant J. 9:745-753.

Hancock, J.F. 1999. Strawberries. CAB International, Wallingford. UK.

Liang, P. and Pardee, A.B. 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257:967-971.

Martelli, G., Sabina, M.R., Sunseri, F. and Greco, I. 2002. Phylogenetic studies on *Fragaria* spp. by using Random Amplified Hybridized Fragment Polymorphism (RAHFPs). Proc. 4th International Strawberry Symposium 9-14 July 2000 Tampere (Finland). Acta Hort. 567:65-69.

Park, J.M., Park, C.J., Lee, S.B., Ham, B.K., Shin, R. and Paek, K.H. 2001. Over expression of the tobacco *Tsi1* gene encoding an REBP/AP2-type transcription factor

enhances resistance against pathogen attack and osmotic stress in tobacco. Plant Cell 13:1035-1046.
Yi, S.Y. and Hwang, B.K. 1998. Molecular cloning and characterization of a new basic peroxidase cDNA from soybean hypocotyls infected with *Phytophthora sojae* f. sp. glycines. Mol. Cells 8:556-564.
Yoshida, S., Ito, M., Nishida, I. and Watanabe, A. 2001. Isolation and RNA gel blot analysis of genes that could serve as potential molecular markers for leaf senescence in *Arabidopsis thaliana*. Plant Cell Physiol. 42:170-178.

Tables

Table 1. Sequence homology.

Code	Protein	Homology %	Accession
6A	Putative glucanase protein	42	P52407
	Putative chitinase protein	53	S65019
7AP	Transport membrane protein	90	Q98ER5
	Putative glucanase protein	67	P52407
7AL	Beta-galactosidase	100	Q57170
	Lac z pectin	95	Q47336
1B	Ipotetical protein	96	Q85F46
4B	*Oryza sativa* genomic DNA	85	OSJN00256
	Ipotetical protein	78	Q8L358
5C	Ipotetical protein	89	Q08791
6CP	Ipotetical protein	75	Q85F16
	Oryza sativa cromosomic	80	AP003280
6CL	Ipotetical protein	98	Q8 36P5
3D	Ipotetical protein	81	TU5834
	Permease protein	63	RT6952
8D	Putative chitinase protein	48	ML58L3
	Ipotetical protein	79	QOL250