Identification of differentially expressed root genes upon rhizomania disease

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SUMMARY

Rhizomania is one of the most devastating sugar beet diseases. It is caused by *Beet necrotic yellow vein virus* (BNYVV), which induces abnormal rootlet proliferation. To understand better the physiological and molecular basis of the disorder, transcriptome analysis was performed by restriction fragment differential display polymerase chain reaction (RFDD-PCR), which provided differential gene expression profiles between non-infected and infected sugar beet roots. Two distinct viral isolates were used to detect specific or general virus-induced genes. Differentially expressed genes were selected and identified by sequence analysis, followed by reverse Northern and reverse transcriptase PCR experiments. These latter analyses of different plants (*Beta vulgaris* and *Beta macrocarpa*) infected under distinct standardized conditions revealed specific and variable expressions. Candidate genes were linked to cell development, metabolism, defence signalling and oxidative stress. In addition, the expression of already characterized genes linked to defence response (pathogenesis-related protein genes), auxin signalling and cell elongation was also studied to further examine some aspects of the disease. Differential expression was retrieved in both *B. vulgaris* and *B. macrocarpa*. However, some candidate genes were found to be deregulated in only one plant species, suggesting differential response to BNYVV or specific responses to the BNYVV vector.

INTRODUCTION

Comparative transcript profiling provides essential information that can be used to explain a large range of biological differences between organisms, tissues, developmental processes (Whitelaw *et al.*, 2000), cell fate (Whitelaw *et al.*, 2002) and pathological

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situations (Liang and Pardee, 2003). A large range of methods can be used for transcript comparisons, from subtractive hybridization (Diatchenko *et al.*, 1996) to produce expression libraries, to DNA microarrays to profile global changes (Duggan *et al.*, 1999). The latter approach is applied in particular to completely sequenced organisms but is quite restrictive when applied to partially characterized genomes. Tools have been developed to assess comparative analysis of as yet uncharacterized genomes. Within such methods, restriction fragment differential display polymerase chain reaction (RFDD-PCR) is of particular interest because this method permits the comparison of more than two samples and allows the identification of up- and down-regulated genes (Gravesen *et al.*, 2000). It is derived from the RNA differential display method (Liang and Pardee, 1992; Liang *et al.*, 1992) but is more informative as it mainly provides nucleotide sequences of mRNA coding regions rather than just 3′ untranslated regions.

Rhizomania is a devastating viral disease of sugar beet (*Beta vulgaris*) and is present almost worldwide. The disease is induced by the phytovirus *Beet necrotic yellow vein virus* (BNYVV), which is transmitted by the soil-borne vector *Polymyxa betae* in a persistent manner because of resting spores retaining the virus. Rhizomania studies have been extensively focused on the characterization of the function of virally encoded genes (Chiba *et al.*, 2008; Klein *et al.*, 2007; Link *et al.*, 2005; Richards and Tamada, 1992; Tamada, 1999; Vetter *et al.*, 2004) but little is known about the cellular activities that are modulated during the disease and how root proliferation is initiated. Histological characterization (Pollini and Giunchedi, 1989) and hormonal changes (Pollini *et al.*, 1990) have been reported without information about the intimate molecular mechanisms present at the basis of such physiological alterations. Analyses of sugar beet infected by fungi or viruses have permitted the characterization of the pathogenesis-related protein 2 gene (*PR-2*) as well as the *PR-3* gene, which are induced during sugar beet infections or stresses (Burketova *et al.*, 2003; Coutts *et al.*, 1994; Fleming *et al.*, 1991; Nielsen *et al.*, 1993, 1994, 1996). As sugar beet is far

from being a model plant, complete characterization of its genome will not be available for many years. Therefore, we used a differential display strategy to compare non-infected and BNYVV-infected root systems from susceptible *B. vulgaris* plants and characterize some differentially expressed genes. We also used *Beta macrocarpa* in our studies. Unlike *B. vulgaris*, *B. macrocarpa* leaves can be rubinoculated with BNYVV, thereby avoiding variation associated with the presence of the vector *P. betae*. B-type and P-type isolates that respectively contain four and five RNA species were used to infect plants. Subsequent gene expression profiling of selected genes was performed using reverse Northern and reverse transcriptase PCR (RT-PCR) on distinct plant materials. These analyses were also performed to visualize the expression of other well-characterized genes linked to defence response (e.g. PR-protein genes) or related to sugar beet hormonal changes (e.g. auxin signalling) and root elongation (e.g. extensin gene).

RESULTS

Identification of differentially expressed genes by RFDD-PCR

In order to study *B. vulgaris* root transcript profiles in response to typically BNYVV natural infections mediated by the *P. betae* vector, healthy plants (H) cultivated in soil mixtures containing aviruliferous *P. betae* were compared with infected plants grown in soil with viruliferous *P. betae* strains harbouring B-type (A2) or P-type (A4) BNYVV isolates. Two distinct viral isolates were used in this study in order to discriminate between genes generally induced in response to all types of BNYVV and genes specifically modulated by one viral isolate. Characteristic symptoms of BNYVV infection were detected on leaves (chlorosis and dwarfism) and on roots (root system disorders and rootlet proliferation) after 8 weeks. Total RNAs from roots of infected and healthy plants were extracted and first analysed by Northern blotting for the presence of viral RNA 1, 2, 3 and 5 (Fig. 1). Total RNAs from *Chenopodium quinoa* leaves mechanically inoculated with a BNYVV P-type isolate was used as a viral control (C, Fig. 1). RNA 4 detection was omitted to better distinguish RNA 5, as RNA 4 length (1467 nt) is very close to RNA 5 length (1350 nt). RNA 4 detection was without interest here as this RNA species is present in all naturally infected plant because it is involved in virus transmission and pathogenesis (Rahim *et al.*, 2007; Tamada and Abe, 1989). Our results confirmed that A2 and A4 sets of roots were respectively infected by B-type and P-type BNYVV isolates (A2, A4, Fig. 1). No BNYVV infection was detected on plants grown in aviruliferous soil (H, Fig. 1). Identical amounts of total RNAs from healthy (H) and infected (A2 and A4) root samples were analysed by RFDD-PCR in duplicates with nine tested primer sets (0-extension primers and displayPROBE primers). Although
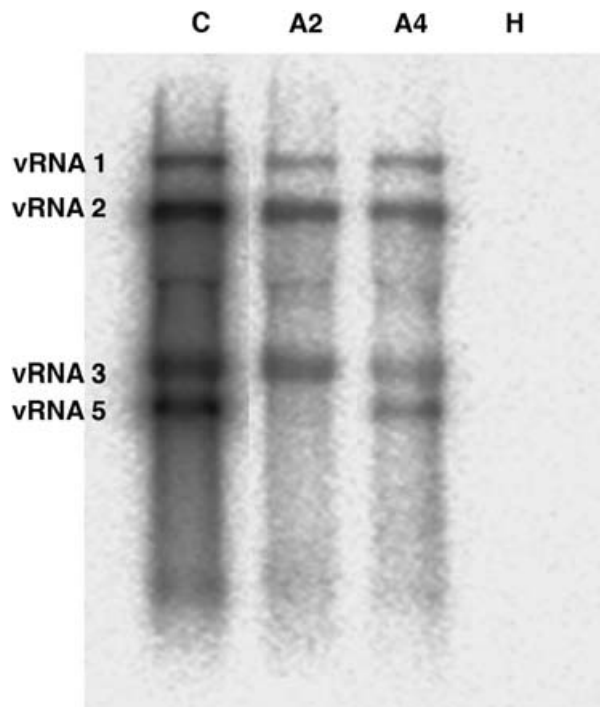

Fig. 1 BNYVV RNA detection within total RNAs from *Beta vulgaris* roots infected by B-type (A2) or P-type (A4) BNYVV viral isolates. Total RNAs from non-infected roots (H) were also tested. Viral RNA 1, 2, 3 and 5 were detected by Northern blotting with specific riboprobes. RNA 4 probe was omitted to allow the visualization of the RNA 5 species. Positions of viral RNAs were determined with the detection of viral RNAs within total RNAs from *Chenopodium quinoa* leaf lesions induced by a P-type BNYVV isolate (C). Positions of viral species are indicated on the left.

the majority of bands revealed no differential expression profiles between non-infected and BNYVV-infected plants, discriminative bands were also found and selected (Fig. 2). Reproducibility was estimated by comparison of the duplicated experiments. In total, 53 differentially expressed bands were originally isolated from RFDD-PCR gels, and 42 bands were successfully re-amplified and yielded colonies after transformation. The 42 selected bands were sequenced, 14 of which consisted of BNYVV viral RNA sequence (e.g. *2b*, *4b*, *6b*, *10b*, *14b*, Fig. 2), thus leaving 28 bands of interest providing 50 sequences valid for further analysis. The 50 sequences were compared against non-redundant databases (NCBI) using tBlastx and against the *Arabidopsis thaliana* database (TIGR) using WU-Blast 2.0. On the basis of sequence identity, putative function and GO annotations were assigned to each sequenced cDNA. The results are summarized in Table 1.

B. vulgaris candidate gene expression patterns

Differential display assays are known to produce bands consisting of more than one sequence, thus providing false-positive
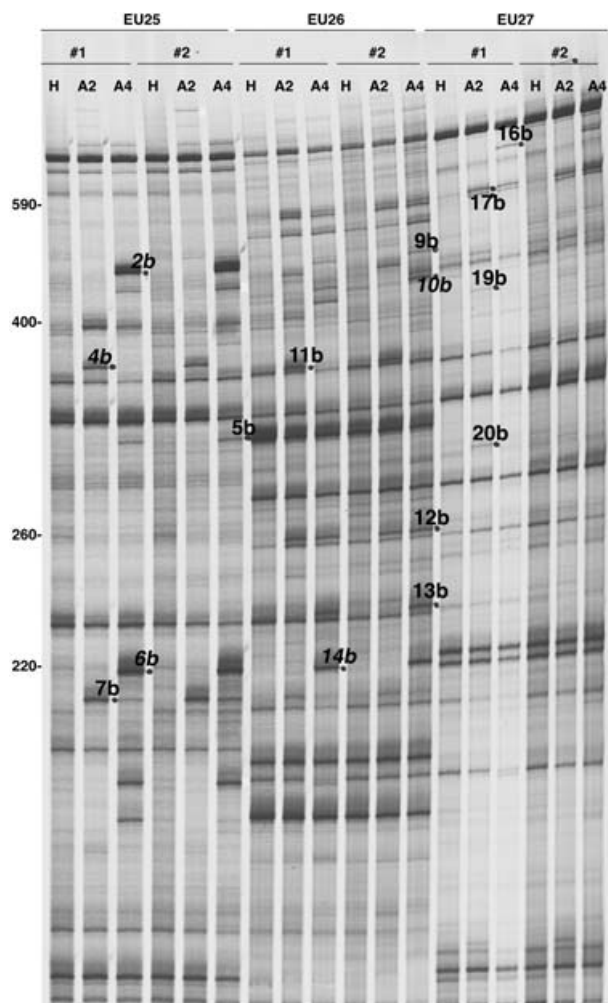
Fig. 2 Autoradiogram obtained from RFDD-PCR analysis showing the amplification products of RNAs from *Beta vulgaris* healthy roots (H) used as reference, and from roots infected by a B-type BNYVV isolate (A2) or a P-type BNYVV isolate (A4). Amplification reactions were made in duplicates (nos. 1 and 2) using the labelled 0-extension primer in combination with one of the three displayPROBE primers (EU25, EU26 and EU27) tested in this example. Differentially expressed bands (detected here in infected samples) are indicated by dots and FDD name reported in Table 1. Names in italic correspond to BNYVV amplified sequences. Numbers on the left refer to the size of PCR fragments (base pairs).

Fig. 3 Expression patterns of genes selected after the initial characterization. Reverse Northern analysis consisted of replicate membranes harbouring PCR products of cDNA clones hybridized with ^{32}P-labelled cDNA probes derived from *Beta vulgaris* healthy (H panels) or infected (A4 panels) root mRNAs. The identifier of each cDNA indicated on the top is also reported in Table 1. A viral sequence (V) was used as a positive control for hybridizations.

clones among true differentially expressed candidate genes. The 50 cDNAs isolated previously were analysed by reverse Northern procedures both to validate the result of the RFDD-PCR experiment and to distinguish between differentially expressed sequences and contaminating sequences (Fig. 3). This method permitted the multiple screening of candidates, which requires small amounts of RNA to produce only two labelled cDNA probes. PCR products were transferred in duplicate onto nylon membranes, one of which was hybridized with cDNA probes obtained
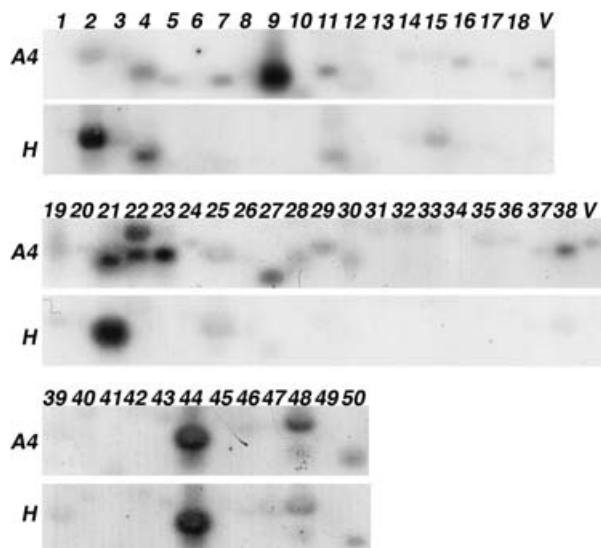
from healthy roots (H panels, Fig. 3), and the other with cDNA probes obtained from P-type BNYVV-infected roots (A4 panels, Fig. 3). The detection of the viral control only with A4 cDNA probes validated the experiment (V, Fig. 3). Signals were integrated and compared between healthy and infected tissues. Using such a method for the confirmation of transcript profiling, one needs to consider the possible absence of detection due to a low abundance of some mRNAs and/or the low representation of full-length radiolabelled cDNA that may not hybridize on the sequence amplified by RFDD-PCR. Twenty clones were not or were only poorly detected (nos. 1, 6, 8, 12, 13, 14, 20, 26, 31–34, 36, 40–42, 45–47, 49; Fig. 3, see also Table 1 for details) whereas five clones presented no differential transcript accumulation (nos. 3, 25, 43, 44, 50). For most of the remaining clones (18/25) the nature of the variation (increase, decrease) was identical between cDNA-AFLP and reverse Northern blotting. Transcript profiling was confirmed for cDNAs encoding a ubiquitin (no. 11), a plasma membrane H$^+$-ATPase (no. 17), a germin-like (*GLP*, no. 22), a peroxidase (no. 29) and metabolism genes (nos. 19, 38), as well as sequences with unknown function (nos. 10, 16, 18), many ribosomal RNAs (nos. 5, 7, 24, 28, 35, 37) and most intriguingly virus, phage or protozoan sequences (nos. 23, 27, 30). For the last clones (7/25), the nature of the variation differed between the two procedures. Differences between RFDD-PCR results and reverse Northern analysis were detected for genes encoding a heat shock 70 protein (*HSC70*, no. 2), a glutathione S

Table 1 Candidate transcripts detected by RFDD-PCR analyses and differentially expressed between healthy (H) and BNYVV-infected (A2 or A4) sugar beet roots

No.*	FDD†	H‡	A2	A4	Name§	Size (bp)¶	Predicted function (Organism)**	Accession no.††	AT locus‡‡	GO terms§§
1	1a		x	x	18V15	584	No significant similarity	—		
2	2a			x	18V21	464	Cytosolic heat shock 70 protein (*Spinacia oleracea*)	AF034618	At3g12580	Pro, RS, Rox
3					18V25	455	Phospholipase D alpha 1 (*Arabidopsis thaliana*)	NM_112443	At3g15730	Met, ST
4	5a		x	x	18V56	262	Glutathione S transferase (*Arabidopsis thaliana*)	AF349527	At4g02520	RS, Rox
5	6a			x	18V61	185	26S rRNA (*Chenopodium rubrum*)	AM263434		
6					18V62	181	Zinc-containing alcohol dehydrogenase (*Ralstonia eutropha*)	YP_297879		
7					18V63	181	23S rRNA (*Pseudomonas stutzeri*)	X87289		
8	7a	x			19S12	233	Large-subunit rRNA (*Thaumatomonas* sp. *TMT002*)	DQ980477		
9					19S13	874	Large-subunit rRNA (*Thaumatomonas* sp. TMT002)	DQ980477		
10	8a		x	x	19V12	61	No significant similarity	—		
11	13a		x	x	19V65	266	Ubiquitin UBQ10 (*Arabidopsis thaliana*)	DQ793132	At4g05320	Pro, RS
12	14a			x	19V72	196	No significant similarity	—		
13	17a		x	x	20V16	576	23S rRNA gene (*Rhizobium leguminosarum*)	CP000133		
14	18a			x	20V21	441	Amino acid transporter AAP4 (*Arabidopsis thaliana*)	NM_125780	At5g63850	Trans, RS
15					20V26	443	Genomic DNA, Os12g0190400 gene (*Oryza sativa*)	AP008218		
16	19a	x			20V34	369	Unknown protein (*Arabidopsis thaliana*)	NM_105087	At1g64140	Un
17					20V36	369	Plasma membrane H$^+$-ATPase (*Triticum aestivum*)	AY829002	At2g18960	Trans, RS, ST
18	20a		x	x	20V46	260	No significant similarity	—		
19	5b			x	25V51	313	Carbamoyl-phosphate synthase (*Arabidopsis thaliana*)	NM_102730	At1g29900	Met
20					25V55	313	FLS2 Leu-rich repeat protein kinase (*Arabidopsis thaliana*)	NM_124003	At5g46330	ST, RS
21	7b		x	x	25V75	213	2-Dehydro-3-deoxyphosphoheptonate aldolase (*Arabidopsis thaliana*)	NM_120162	At4g39980	Met, RS
22	9b			x	26V12	469	Germin-like protein (*Mesembryanthemum crystallinum*)	M93041	At1g09560	RS
23					26V13	483	LBVV-pol gene for L protein (*Lettuce big-vein virus*)	AB075039		
24	11b	x			26V31	365	Large-subunit rRNA (*Thaumatomonas* sp. TMT002)	DQ980477		
25	12b		x	x	26V46	255	Glucose-6-phosphate 1-dehydrogenase (*Arabidopsis thaliana*)	AK227918	At5g40760	Met
26	13b			x	26V52	235	26S ribosomal RNA (*Maytenus fournieri*)	AF222393		
27	16b			x	27V21	89	Genomic DNA (*Staphylococcus aureus* phage phi13)	AF424783		
28					27V24	700	23S rRNA (*Acidovorax avenae* subsp. *citrulli*)	CP000512		
29					27V26	700	Peroxidase, per2 gene (*Beta vulgaris*)	AJ276131	At5g19890	RS, Rox
30	17b		x	x	27V31	585	Microtubule-associated protein (*Trypanosoma cruzi*)	AF158722		
31					27V33	585	16S rRNA (*Streptomyces glomeroaurantiacus*)	AB249983		
32					27V36	585	16S rRNA (*Streptomyces* sp. 4_C7_41)	EF540496		
33	18b		x	x	27V42	575	16S rRNA (*Streptomyces* sp. 4_C7_41)	EF540496		
34					27V44	575	16S rRNA (*Streptomyces* sp. 4_C7_41)	EF540496		
35	19b	x			27V51	414	26S rRNA (*Afrostyrax* sp. Cheek 5007)	AF479115		
36					27V52	414	Chloroplast DNA (*Cyanidioschyzon merolae* strain DBV201)	AY286123		
37	20b	x			27V61	302	26S rRNA (*Medusagyne oppositifolia*)	AF479120		
38					27V62	302	ʟ-allo-threonine aldolase (*Malus x domestica*)	AY742299	At1g08630	Met
39	1c	x			22V12	368	18S ribosomal RNA (*Allantion* sp. ATCC 50734)	AF411265		
40	3c	x			23V11	594	TIP1 Palmitoyltransferase (*Arabidopsis thaliana*)	NM_122042	At5g20350	Cell, RS
41					23V13	52	No significant similarity	—		
42					23V16	584	16S rRNA (*Streptomyces* sp. 4_C7_41)	EF540496		
43	4c		x	x	23V21	489	No significant similarity	—		
44					23V22	494	Phenylalanine ammonia lyase (*Beta vulgaris*)	AJ810175	At2g37040	Met, RS, Rox
45					23V24	492	U2 snRNP auxiliary factor large subunit (*Arabidopsis thaliana*)	NM_104771	At1g60900	Rna
46	12c	x			24S21	406	26S rRNA (*Portulaca grandiflora*)	AF479093		
47					24S22	406	BvMS1 Methionine synthase (*Beta vulgaris*)	AB221011	At5g17920	Met
48					24S23	406	No significant similarity	—		
49					24S24	338	26S rRNA (*Nicotiana tabacum*)	AF479172		
50	10c			x	24V31	134	Mitochondrial genomic DNA (*Beta vulgaris*)	BA000024		

*Identifier of each cDNA.

†Number of the band detected on denaturing gels.

‡Expression profile: x in the H column refers to a repressed gene during the infection, and 'x' in the A2 and/or A4 columns refers to up-regulated genes.

§Name of the pGEM-T cloned sequence.

¶Size of the cloned cDNA.

**The best Tblastx result with E value below 1 e^{-3}.

††Accession number of the Tblastx homologue.

‡‡The best-matched *Arabidopsis thaliana* (AT) orthologue.

§§If the AT orthologue is identified, global gene function as described by the Gene Ontology Consortium. GO terms: response to abiotic or biotic stimulus (RS), response to oxidative stress (Rox), RNA binding and processing (Rna), protein binding, folding and modification (Pro), metabolism (Met), cell organization and biogenesis (Cell), signal transduction (ST), transport (Trans), unknown process (Un).

transferase (*GST*, no. 4), a 2-dehydro-3-deoxyphosphoheptonate aldolase (no. 21), unknown function sequences (nos. 15, 48) and ribosomal RNAs (nos. 9, 39). RNAs from prokaryotes or unrelated species that may differ within the soils were cloned as well. A *Varicosavirus* rhabdoviral-related sequence (no. 23) might be explained by the presence of *Olpidium brassicae* in the soil, but no infection was detected by Northern blots (data not shown). Such sequences with no plant sequence identities (*A. thaliana* Locus, Table 1) reflected plant RNA contaminations with soil microorganism RNAs.

Expression patterns of candidate genes in response to early BNYVV infection

To analyse the early expression of candidate cDNAs in response to premature BNYVV root infection, new complementary approaches were performed on the cultivated sugar beets (*B. vulgaris* ssp. *vulgaris*) and on the related wild species (*Beta macrocarpa*). In the first experiment, *B. vulgaris* healthy plantlets (H) cultivated in sand mixed with aviruliferous *P. betae* soil were compared with infected plantlets (B) grown in sand mixed with viruliferous *P. betae* soil harbouring B-type BNYVV isolates. This bioassay, developed in the SESVanderHave laboratory, was the method of choice to provide standardized and rapid infections of young root systems. TAS-ELISA assays were performed to quantify BNYVV multiplication in roots 7, 14, 21 and 28 days after infection (Fig. 4a). This analysis allowed us to detect an initial infection as soon as 7 days post-infection (dpi), and an optimal infection at 14 dpi (B, Fig. 4a). These two time points were selected for further transcript analyses. In the second experiment, *B. macrocarpa* control plantlets (H) were compared with systemic infected plantlets (I) obtained after leaf infection by a B-type BNYVV inoculum. Indeed, *B. macrocarpa* was chosen as it can be systemically infected after leaf rub inoculation without the need of the vector *P. betae*. Hence, the *B. macrocarpa*/BNYVV system allows for verification of our findings and for the selection of genes that are specifically associated with BNYVV infection from those that may be associated with the presence of *P. betae* or other microbes that were present in BNYVV/*P. betae*-exposed versus non-exposed *B. vulgaris*. BNYVV leaf rub inoculation of *B. macrocarpa* led to successful infection of the roots (Fig. 5a). These infected roots were harvested as soon as visible systemic symptoms (leaf blisters and chlorosis) appeared onto non-inoculated leaves (approximately 13 dpi). Entire root systems from infected and non-infected plants were submitted to immunoprinting to detect and precisely localize BNYVV infection within roots (Fig. 5a). This method distinguished between infected tissues (i) and non-infected tissues (h) on the unique early-infected root system (I, Fig. 5a). The control plant served only to determine the threshold chemiluminescence signal level (H, Fig. 5a). These specific tissues were extracted from the
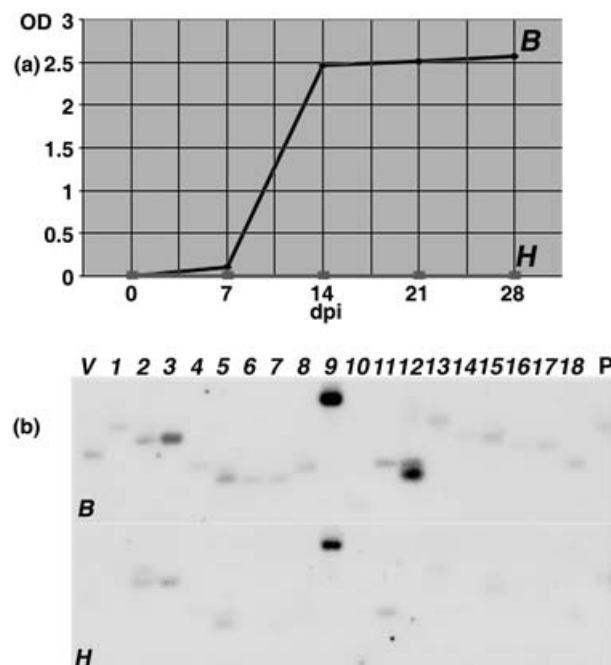

Fig. 4 (a) BNYVV detection in *Beta vulgaris* roots infected by a B-type BNYVV isolate (B) using standardized conditions (bioassay, SESVanderHave laboratory) or in control roots (H). Viral contents were monitored by ELISA analysis for BNYVV CP protein detection. Results are expressed as an optical density value (OD). (b) Differential expression of RFDD-PCR candidate genes after *B. vulgaris* standardized infections. Reverse Northern hybridizations were performed with ^{32}P-labelled cDNA probes derived from *B. vulgaris* healthy (H) or infected (B) root RNAs extracted 14 days after treatment. The identifier of each cDNA indicated on the top is also reported in Table 1. A viral sequence (V) and a *B. vulgaris* beta-1,3-glucanase (accession no. BQ591809) *PR-2* sequence (P) were used as controls.

corresponding frozen roots and total RNAs were prepared for subsequent analyses. This method allowed us to study gene expression by comparison of infected and non-infected samples from the same individual.

Expression profiles performed by reverse Northern blots were restricted to the set of 18 cDNA candidates (nos. 1–18, Table 1), due to the low amounts of total RNAs. Reverse Northern hybridizations consisted of comparisons between non-infected and infected roots of *B. vulgaris* (Fig. 4b) and *B. macrocarpa* (Fig. 5b) RNA extracts harvested respectively at 14 and 13 dpi. The detection of the viral control only with infected cDNA probes validated the experiment (V, Figs 4b and 5b). In addition, a *B. vulgaris* beta-1,3-glucanase sequence (*PR-2*, accession no. BQ591809), known to be induced by plant stress and infection, was added (P, Figs 4b and 5b), and also the *B. vulgaris* housekeeping ubiquitin sequence (accession no. BQ583989) to confirm ubiquitin gene activation (no. 11, Table 1) in *B. macrocarpa* (U, Fig. 5B). Signals were integrated and compared between healthy
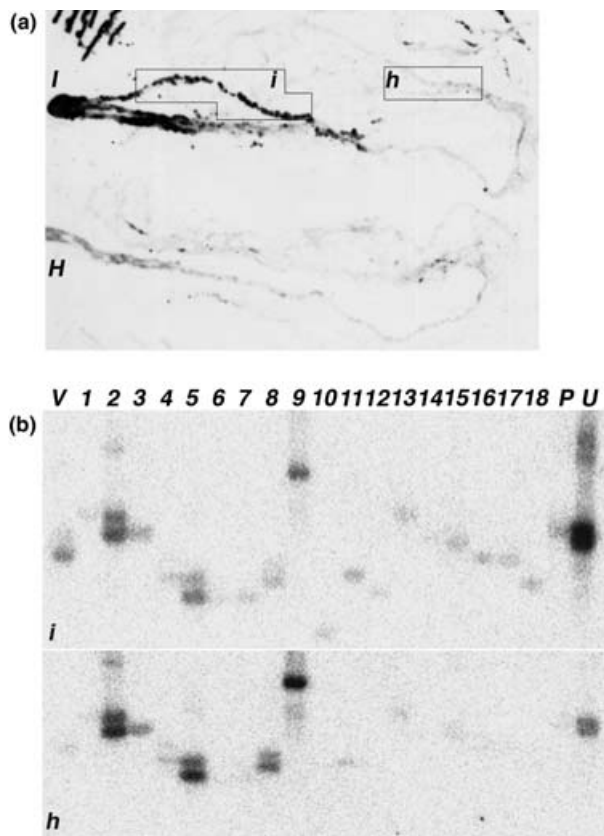
Fig. 5 (a) BNYVV localization in *Beta macrocarpa* roots after plant systemic infection. Viral contents of *B. macrocarpa* roots harvested 13 days after infection (I) or control roots (H) were monitored by immunoprinting and permitted the localization of infected tissues (i) and non-infected tissues (h) selected for subsequent total RNA extraction. Background on control roots (H) corresponds to the processing of chemiluminescent reagent by cellular peroxidases. (b) Differential expression of RFDD-PCR genes after *B. macrocarpa* systemic infection. Reverse Northern hybridizations were performed with cDNA probes derived from *B. macrocarpa* healthy (h) or infected (i) root RNAs. Identifiers of cDNA clones are indicated on the top. A viral sequence (V) was used as a positive control, as well as a *B. vulgaris* beta-1,3-glucanase *PR-2* (P) sequence (accession no. BQ591809) and a ubiquitin (U) sequence (accession no. BQ583989).

and infected tissues, and between new infection procedures and previous *B. vulgaris* root infections (Figs 3, 4b and 5b). Reverse Northern experiments confirmed RFDD-PCR analysis based on the induction in both infected species of 23S rRNAs (nos. 7 and 13), unknown protein-encoding genes (nos. 10, 12, 15, 16 and 18), and genes encoding a ubiquitin (no. 11), an amino acid permease (*AAP*, no. 14) and an H⁺-ATPase (no. 17). The *GST* (no. 4) and unknown plant function sequences (nos. 1 and 6) were found to be induced only in the *B. vulgaris* bioassay experiment. For the remaining clones, the nature of the variation differed between the two species, showing up-regulation in *B. vulgaris* (Fig. 4b)

but repression in *B. macrocarpa* (Fig. 5b). This expression pattern was observed for *HSC70* (no. 2), the phospholipase D gene (*PLD*, no. 3) and ribosomal RNAs (nos. 5, 8 and 9).

We extended some of our analyses only for cDNAs with putative function of interest. Semi-quantitative RT-PCR was performed on total RNAs from infected (B) and control (H) *B. vulgaris* rootlets collected at 7 dpi and from infected (i) and healthy (h) tissues extracted on the same *B. macrocarpa* plant at 13 dpi (Fig. 6). Signals were quantified and expression ratio calculated between infected and non-infected values (ratio B/H and ratio i/h, Fig. 6). We analysed the early expression of the well-studied *B. vulgaris* beta-1,3-glucanase gene (*PR-2*, accession no. BQ591809) and the basic class IV chitinase gene (*PR-3*, accession no. CF542771) previously detected in rhizomania-infected sugar beet roots (Burketova *et al.*, 2003). These two genes were found to be activated in both infected species (2.5- and 3.5-fold increase for *PR-2*; 2.7- and 4.0-fold increase for *PR-3*, Fig. 6). Among the previously identified cDNAs, some of them were found to be activated in both infected species. Up-regulation was confirmed for genes encoding a ubiquitin (no. 11), an H⁺-ATPase (no. 17) and a glucose-6-phosphate 1-dehydrogenase (*G6PDH*, no. 25). However, *GLP* (no. 22) was found activated in *B. vulgaris* but repressed in *B. macrocarpa*. Finally, two genes, *HSC70* (no. 2) and *AAP* (no. 14), did not reveal a significant difference.

These RT-PCR experiments also provided the opportunity to analyse other genes of interest. The auxin binding protein 1 gene (*ABP1*, accession no. BQ 584486) was analysed as auxin hormonal changes were previously reported in rhizomania syndrome (Pollini *et al.*, 1990). Moreover, the extensin gene (accession no. BQ583860) was also studied. This gene belongs to the hydroxy-proline-rich glycoprotein (HRGP) family, key regulatory elements of cell-wall development (Cassab, 1998) that can be induced by auxin, or biotic or abiotic stresses (Merkouropoulos and Shirsat, 2003). *ABP1* and the extensin gene were activated in both infected species (2.5-fold increase in *B. vulgaris* and 2.0-fold increase in *B. macrocarpa*, Fig. 6). Finally, leaf blisters observed on systemically infected *B. macrocarpa* leaves were similar to the phenotypes observed on tomato leaves infected by *Potato spindle tuber viroid* (Qi and Ding, 2003). This led us to analyse expression variations of an expansin gene (accession no. BQ586143), a member of a gene family involved in root initiation and elongation and modulated by hormonal changes (Cho and Cosgrove, 2002). This expansin gene was found to be up-regulated in infected *B. vulgaris* roots (5.0-fold increase, Fig. 6) but it was not detected in *B. macrocarpa* root tissues.

DISCUSSION

To study the molecular mechanisms responsible for rhizomania syndrome of sugar beet, which results in root proliferation, we initiated RFDD-PCR analyses to compare the root RNA contents of infected plants and healthy plants. We have reproduced the natural infection process, growing the plants in soil containing the aviruliferous and viruliferous *P. betae* vector to obtain healthy and infected plants, respectively. Viral isolates containing four and five RNA components were used to obtain B-type (A2) and P-type (A4) BNYVV-infected plants, respectively (Fig. 1). Differential display patterns were then produced from infected and healthy plants (e.g. Fig. 2) and 53 bands were selected. Among the 42 bands successfully cloned and sequenced, 14 were assigned to BNYVV viral sequences (names in italic, Fig. 2), thus leaving 28 bands of interest (FDD names, Table 1) providing 50 sequences that were further characterized (Table 1). Many sequences corresponded to rRNA or DNA sequences either from unicellular eukaryotes (nos. 8, 9, 24, 30, 36, 39, Table 1) or prokaryotes (nos. 6, 7, 13, 27, 28, 31–34, 42) or unrelated virus (no. 23). The presence of soil-dependent differential profiles was confirmed by the detection of prokaryotic RNA species with no plant sequence identities (*A. thaliana* Locus, Table 1). Therefore, to avoid such uncontrolled variations among total RNA samples, we performed similar reverse Northern analyses or semi-quantitative RT-PCR amplifications with plant rootlets naturally infected under standardized conditions (Fig. 4) or root portions selected on the same systemically infected plant (Fig. 5), which allowed us to focus only on genes mainly deregulated by the BNYVV infection and not by the vector or soil microorganisms.

Plant rRNAs were also identified (nos. 5, 26, 35, 37, 46, 49, Table 1). Within such rRNA sequences, differential expression of only three sequences (nos. 5, 35, and 37) was slightly visualized (Fig. 3) and was therefore not considered as significant. No signal or differential expression was found for unknown candidate nos. 41, 43, 48 and 50 as well as for sequences with plant putative function, i.e. nos. 20, 36, 40, 44, 45 and 47 (Figs 3 and 4b), which may be considered either as contaminating fragments during gel recovery or as false positive amplicons generated by RFDD-PCR. Differential expression of unknown function sequences nos. 10, 12, 15, 16 and 18 as well as genes encoding for ubiquitin (no. 11, Figs 3, 4b, 5b and 6), H^+-ATPase (no. 17, Figs 3, 4b, 5b and 6), *GLP* (no. 22, Fig. 3; *B. vulgaris*, Fig. 6), *AAP* (no. 14, Figs 4b and 5b) was detected by at least two approaches (reverse Northern and/or semi-quantitative RT-PCR) and corresponded to gene activation in response to BNYVV infection. Genes corresponding to *HSC70* (no. 2, Fig. 4B), *PLD* (no. 3, Fig. 4B), *GST* (no. 4, Fig. 4B), *G6PDH* (no. 25, Fig. 6) and genes encoding a carbamoyl-phosphate synthase (no. 19, Fig. 3), a peroxidase (no. 29, Fig. 3), and the L-allo-threonine aldolase (no. 38, Fig. 3) were confirmed by only a single experiment (reverse Northern or semi-quantitative RT-PCR). To explain such contradictions between experiments, it should be recalled that large gene families exist in plant genomes, and we could not exclude the possibility that different members of the same gene family with distinct expression profiles were cross-hybridized by reverse Northern and alternatively amplified by RFDD-PCR or RT-PCR (e.g. *HSC70*). Therefore, the reverse Northern results may also reflect the differential regulation of members of a gene family rather than one specific gene. Further Southern blot and Northern blot experiments will be performed to identify the differentially regulated gene(s).

Comparison of *B. vulgaris* and *B. macrocarpa* differential expression patterns permitted the identification of genes that were differentially expressed because of the presence of BNYVV versus those genes that were differentially expressed possibly because of the presence of the vector *P. betae*. For example, one could argue that the *GLP* gene is induced by *P. betae*, as this gene was confirmed to be up-regulated in the *B. vulgaris* experimental system (no. 22, Figs 3 and 6) but not in that of *B. macrocarpa* (no. 22, Fig. 6). An alternative explanation is that *B. vulgaris* and *B. macrocarpa* differ in their responses to BNYVV.

Differential expression of the ubiquitin gene was found to be induced in the three different reverse Northern analyses and was also confirmed by RT-PCR. In maize kernels grown *in vitro*, the Ubi-1 promoter is stimulated by the plant hormone indole-acetic acid but not by pathogen infection (Muhitch and Shatters, 1998). Ubiquitination plays major roles in the regulation of plant defence and plant signalling pathways (Devoto *et al.*, 2003; Dreher and

Callis, 2007). Until now, it has not been known whether virus-mediated over-expression of ubiquitin mRNA favours viral infection or plant defence, as both scenarios are possible (Angot *et al.*, 2007). Transcriptome analyses of lesions induced after pathogen inoculation of wild-type and 'lesion mimics' *spl11* mutant revealed the up-regulation of several genes in which *GST* and *GLP*, as well as *ATPase*, expansin, extensin, phenylalanine ammonia-lyase (*PAL*) and PR-protein encoding genes are retrieved (Zeng *et al.*, 2006). In our RFDD-PCR approach, many stress-response genes were found to be activated as well (the *PLD*, *GST*, *PAL* and *peroxidase* genes).

ABP1 was activated (Fig. 6), reinforcing the role of auxin hormonal signalling during *B. vulgaris* root infections. The ABP1 protein mediates cell expansion (Timpte, 2001). Auxin is also known to induce the expression of expansins that act together with H$^+$-ATPases to extend the plant cell wall but also to participate in cellular processes involved in growth and development (Choi *et al.*, 2006). In the present study, a member of the H$^+$-ATPases gene was of particular interest: it was found to be induced by RFDD-PCR and its differential expression was confirmed by the three reverse Northern analyses and by RT-PCR. Acidification is required for cellular expansion of cell walls by expansin proteins. Interestingly, one expansin gene tested here was also found to be induced in *B. vulgaris*-infected roots (Fig. 6). Recently, auxin has been shown to promote disease susceptibility (Navarro *et al.*, 2006) and many pathogens use alternative strategies to take advantages of this property, from tobacco mosaic virus (Padmanabhan *et al.*, 2005, 2008) to *Pseudomonas syringae* (Chen *et al.*, 2007). Such deregulation can, however, be counterbalanced as it has been recently demonstrated that IAA-amino synthetase GH3-8 allows plants to inhibit expansin expression and promotes basal immunity to reduce pathogen invasion (Ding *et al.*, 2008).

The extensin gene was shown to be induced during root initiation (Keller and Lamb, 1989) as well as by biotic and abiotic stresses (Merkouropoulos and Shirsat, 2003; Merkouropoulos *et al.*, 1999). The hormones auxin, salicylic acid and methyl jasmonate activate the extensin gene (Merkouropoulos and Shirsat, 2003), expression of which has also been reported in programmed cell death (Asai *et al.*, 2000). In our experiment, the extensin gene was found to be activated in roots of both species tested (Fig. 6).

Other putative up-regulated cDNA candidates were also linked to auxin signalling as well as to cellular differentiation, responses to oxidative stress or defence signalling as revealed by Gene Ontology Annotations (Table 1). Among them, *G6PDH* and the carbamoyl-phosphate synthase encoding gene are of particular interest. Indeed, during the infection process, *G6PDH* gene induction could benefit the virus as the enzyme could participate in the reduction of redox-stress-induced apoptosis (Fico *et al.*, 2004). Moreover, carbamoyl-phosphate synthase participates

within arginine and pyrimidine biosynthesis (Kolloffel and Verkerk, 1982; O'Neal and Naylor, 1976; Slocum, 2005). Such compounds can lead to the production of nitric oxide and cGMP second messenger (Delledonne, 2005). Both molecules are involved in plant development and defence response and act together with auxin for root morphogenesis (Jacobi *et al.*, 2006; Pagnussat *et al.*, 2003, 2004). Further experiments will be required to confirm the differential expression of these two genes upon BNYVV root infection.

It is clear that our transcriptome analyses of *Beta* species did not provide consistent results. For comparisons performed by the use of natural infection methods, soils were different and brought significant variability. Using infected and non-infected tissues from the same plant (i.e. *B. macrocarpa*), soil variability was resolved but amounts of RNA were not sufficient to conduct complete analyses. However, some candidate genes whose differential expression was well and truly confirmed highlighted the predominant role of auxin in root disease development. New approaches using transgenic plants expressing inducible BNYVV proteins will permit more efficient studies of initial and late events occurring in rhizomania syndrome.

EXPERIMENTAL PROCEDURES

Plant growth and infections

For *B. vulgaris* ssp. *vulgaris* root infections, susceptible plants were cultivated in soil mixtures containing viruliferous *P. betae* harbouring a B-type BNYVV isolate (A2) or P-type BNYVV isolate (A4) originating from soils collected from fields at Amifontaine (France) or Yèvre-la-Ville (France), respectively. Control plants were grown in virus-free *P. betae* soil (H) coming from Rothamsted (UK). *P. betae*-containing soils were mixed with sterilized compost (1:10) and ten sugar beet plants were grown in each compartment to allow the multiplication of *P. betae*. After this amplification step, plants were removed, 20 new seeds were sown and only ten plantlets of similar size were conserved after germination and allowed to grow for 8 weeks (six- to eight-leaf stage) until visible symptoms appeared on infected plants. Plants were harvested, roots were carefully washed and pooled prior to being weighed and subsequently ground in liquid nitrogen and stored at −80 °C.

For standardized *B. vulgaris* infections (bioassay developed in SESVanderHave laboratory, Tienen, Belgium), five sets of 35 sugar beet seedlings were grown in individual tubes containing coarse sand mixed with 10% of viruliferous *P. betae*-containing soil from Germany with a B-type BNYVV isolate (B). Five other groups of 35 control seedlings were grown in sand mixed with aviruliferous *P. betae*-containing soil (H). Roots of 35 plantlets were harvested at 0, 7, 14, 21 and 28 days after treatment, immediately frozen in liquid nitrogen and conserved at −80 °C.

For each time point, 50 mg of fresh root tissue was reserved for virus quantification by a TAS-ELISA at 405 nm using primary (H3) and monoclonal (MAFF 9) antibodies (Neogeneurope) directed against BNYVV coat protein (CP).

For *B. macrocarpa* systemic infections, one mature leaf of a 3-week-old seedling was rub inoculated with Stras1234, a B-type BNYVV inoculum (B), as described previously (Lauber *et al.*, 1998). Plants were harvested and roots collected as soon as visible systemic symptoms appeared on leaves (approximately 13 days after inoculation). Control plantlets were mock inoculated with sterile water (H). Entire root systems from infected and non-infected plants were put between a set of nitrocellulose filter and a plastic sheet and then submitted to a pressure tissue print. Roots were then frozen and stored at −80 °C on the plastic sheet until RNA extraction; meanwhile, the nitrocellulose membrane was submitted to an immunodetection procedure using specific BNYVV CP antisera.

Total RNA isolation, mRNA purification and Northern blot hybridization

RNAs were extracted from 100 mg of frozen material using an RNeasy® Plant Mini Kit (Qiagen), according to the manufacturer's recommendations (including DNase treatment). Plant mRNAs were purified with the NucleoTrap® mRNA Mini Kit (Macherey-Nagel) as described by the supplier's protocol. Northern blot analyses were performed using 7 μg of total RNAs from *B. vulgaris* non-infected or infected roots or 2 μg of total RNAs from *Chenopodium quinoa* leaf lesions induced by a P-type BNYVV isolate. Viral RNAs were detected with specific riboprobes complementary to viral RNA 1, 2, 3 and 5 as described previously (Lauber *et al.*, 1998; Link *et al.*, 2005).

RFDD-PCR

The analysis was carried out as described in detail in the protocol from the displayPROFILE™ kit (Qbiogene). Double-stranded cDNAs were produced in duplicate using 1 μg of total RNAs and $T_{25}V$ ($T_{25}A$, G or C) primer. Purified DNA was digested with *Taq*I endonuclease for 2 h at 65 °C and completed by the ligation of adaptor mixture containing an extension protection group for 3 h at 37 °C using T4 DNA ligase. The template was PCR amplified using a 0-extension primer ^{33}P-labelled complementary to the EP adaptor in combination with a 3-extension displayPROBE primer recognizing the standard adaptor and the three nucleotides adjacent to the *Taq*I site. For each template, PCR reactions using the labelled 0-extension primer in combination with one of the nine displayPROBE primers tested (EU18, EU19, EU20, EU22, EU23, EU24, EU25, EU26 and EU27) were performed as follows: ten cycles of 94 °C for 30 s, 60 °C (−0.5 °C per cycle) for 30 s, and 72 °C for 1 min, followed by 15 cycles of 94 °C for 30 s, 55 °C for

30 s and 72 °C for 1 min. PCR products were denatured and size fractionated in a 6% polyacrylamide gel (acryl/bis: 19:1) containing 8 M urea run for 3 h at 60 W. RFDD-PCR gels were vacuum blotted and dried on Whatman 3MM paper for 1 h and subsequently exposed to X-ray films. Bands of interest were selected and cut from the blotted gel on paper. The small paper cuttings were heated in distilled water for 15 min at 95 °C to elute the DNA from the paper. Samples were then amplified again by PCR and purified. After phosphorylation of the DNA 5′ ends, amplicons were cloned into pGEM®-T vector (Promega) and six positive clones were sequenced for each pGEM®-T construction. Sequences were compared against all sequences, using the tBlastx program with the NCBI database (http://www.ncbi.nlm.nih.gov/blast/) and the WU-Blast 2.0 program with the TIGR *A. thaliana* database (http://tigrblast.tigr.org/er-blast/). Candidates with appropriate *A. thaliana* orthologues were kept for further analyses. A biological function was then assigned, according to the Gene Ontology Annotations (GO terms) provided by the TAIR Search tools (http://www.arabidopsis.org/tools/bulk/go/) for each AT Locus identifier.

Reverse Northern hybridizations

Inserts of sequenced cDNA clones were amplified by PCR with SP6 and T7 primers that were complementary to pGEM®-T vector sequences flanking both sides of cDNA inserts. One hundred nanograms of purified PCR products was analysed in duplicate by electrophoresis on a 2% agarose gel and transferred onto a nylon membrane. Five micrograms of total RNAs or purified mRNA from infected or non-infected roots was used to prepare complex probes by reverse transcription in the presence of [α-^{32}P] dCTP. Half of the membrane was hybridized with control probes, the other half with probes corresponding to infected roots. Signals were quantified using a Fuji MAS1000 BioAnalyser and MacBAS software.

Semi-quantitative RT-PCR

cDNA were synthesized using 1 μg of total RNAs and oligo(dT)$_{25}$ primer with Superscript™ II reverse transcriptase (Invitrogen) according to the manufacturer's recommendations. PCR amplifications were performed on 1–9 μL of the 10× diluted cDNA solution using *Taq* DNA Polymerase (Invitrogen) and consisted of 28 cycles of 94 °C for 15 s, 50 °C for 30 s and 72 °C for 1 min. Control reactions to normalize RT-PCR amplification were run with primers derived from the L11 ribosomal protein gene (accession no. BE590348, G. Weyens, personal communication). PCR products were separated onto a 1.5% agarose gel stained with EtBr and image processing was carried out with a Bio-Rad GelDoc apparatus. Signals were analysed with Quantity One software (Bio-Rad) and quantified with ImageJ 1.38w software (http://rsb.info.nih.gov/ij/).

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