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Analysis of differentially expressed genes in a susceptible and moderately resistant potato cultivar upon *Phytophthora infestans* infection

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SUMMARY

To gain deeper understanding of the host–pathogen interaction in the system potato–*Phytophthora infestans*, subtractive hybridization in combination with cDNA array hybridization was used. Leaflets of a moderately resistant and a susceptible potato cultivar were inoculated with *P. infestans*. The infection of the potato leaves was quantified by real-time quantitative PCR. Using infected and control tissue, two cDNA libraries highly enriched for *P. infestans*-induced genes were prepared. Within 531 clones randomly picked and sequenced from the libraries, 285 unigenes were found, from which 182 clones were selected for further analysis by cDNA array hybridization. Sixteen hours post inoculation genes were not induced significantly, whereas 72 h post inoculation induction of gene expression was clearly detectable. In both cultivars, 143 genes were induced moderately (≥ two-fold), and 35 of the selected genes appeared to be strongly induced (≥ seven-fold). Among these clones were mainly genes associated with stress and/or defence mechanisms. The strongest gene induction was found in 4-week-old susceptible plants. In the moderately resistant cultivar, transcripts of a number of genes accumulate with plant age; as a result, induction of gene expression upon infection was less pronounced. Down-regulation of three genes was observed in both cultivars, upon infection. Transcript levels of these three genes increased in uninfected plants within 4 weeks of growth. Other differences in defence responses of the two cultivars could be determined and their effects are discussed.

INTRODUCTION

Two different types of late blight resistance are known: the qualitative and quantitative resistance (reviewed in Wastie, 1991). Qualitative resistance is mediated by R-genes, which leads to a race-specific hypersensitive response. Because the pathogen is very efficient in building new virulences, the R-gene approach leads no longer to comprehensive resistance (Fry and Goodwin, 1997)—a situation that might change with the new insights gained by cloning the *R1* gene (Ballvora *et al.*, 2002) and *RB*, an R-gene from *Solanum bulbocastanum* that has not yet been overcome by any of the *P. infestans* pathotypes (Song *et al.*, 2003). By contrast, quantitative resistance is controlled by many interacting genes that do not prevent infection, but slow down the development of the pathogen at individual infection sites on the plant. This type of resistance lasts longer because it is race-nonspecific and polygenic (Colon *et al.*, 1995; Turkensteen, 1993).

The recognition and defence of *P. infestans* by its host, and the ability of the pathogen to avoid or overcome the host's defences, implies a complex, dynamic communication network between the interacting organisms. The induction of biochemical response pathways requires the up- or down-regulation of genes. Several studies have demonstrated that infection with *P. infestans* leads to transcriptional activation of various genes in potato (Avrova *et al.*, 1999; Beyer *et al.*, 2001; Birch *et al.*, 1999; Choi *et al.*, 1992; Fritzemeier *et al.*, 1987; Schröder *et al.*, 1992; Taylor *et al.*, 1990; Zhu *et al.*, 1995).

Comprehensive studies of the differential gene expression may lead to a better understanding of the plant–pathogen interactions in quantitative resistance mechanisms of the plant. Therefore, we compared the differences in induction of gene expression between the susceptible potato cultivar Indira and the moderately resistant potato cultivar Bettina, which exhibits quantitative resistance against *P. infestans* (Dr V. Zinkernagel, TUM-Weihenstephan, Germany, personal communication). We constructed two cDNA libraries highly enriched for *P. infestans*-induced genes derived either from the susceptible or from the moderately resistant cultivar. The differential expression of 182 genes derived from both cDNA libraries was studied using cDNA arrays. The influence of plant age on the level of resistance was analysed using 2- and 4-week-old potato plants with respect to early, 16 h post inoculation (hpi), and late, 72 hpi, defence response. Differences in defence responses of the two cultivars could be clearly determined.

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RESULTS

P. infestans infection

In order to follow the infection process in detail, the amount of *P. infestans* mRNA within the samples was estimated using quantitative RT-PCR (qRT-PCR). The *P. infestans* quantification was performed with the same mRNA used in subsequent hybridization experiments. Primers for the qRT-PCR were designed to I-G10, a *P. infestans*-derived gene exhibiting 84% amino acid identity to an elongation factor 1-beta gene from *Candida albicans* (X96517) (see sequence analyses).

The average Ct-values of the infected leaflets are shown in Table 1. Sixteen hours post inoculation only small amounts of the pathogen were observed in both cultivars. In the susceptible cultivar an infection with *P. infestans* was clearly detectable at 72 hpi, whereas owing to the incompatible reaction very low amounts of the pathogen were detected at 72 hpi in Bettina. qRT-PCR quantification data correspond very well with the number of visible lesions on infected leaves (data not shown).

Between the 2- and 4-week-old variants within the same cultivar no major difference in the amount of *P. infestans* mRNA could be determined (Table 1).

Construction of cDNA libraries

In order to obtain a comprehensive set of genes for expression analyses, two cDNA libraries were generated. The cDNA libraries were constructed by vertis Biotechnologie AG, Freising, Germany, using subtractive hybridization. For the first cDNA library (Bettina-lib), 2-week-old potato cultivar Bettina was used to cover genes in a very young plant. The tester was prepared from a mixture of RNA from leaflets inoculated with *P. infestans* for 16 h and for 72 h. The driver was prepared from a mixture of RNA from control leaflets and of RNA from *P. infestans* to eliminate pathogen-derived genes. For the second cDNA library (Indira-lib), only 4-week-old potato cultivar Indira was used. In preliminary experiments a stronger gene induction was observed in 4-week-old compared with 2-week-old Indira plants. The tester consisted of RNA from leaflets that were incubated with *P. infestans* for 72 h. The driver was again prepared from a mixture of RNA from control leaflets and from *P. infestans*.

Table 1 Average Ct-values of the potato leaflets inoculated with *P. infestans* and the non-template control (NTC).

Cultivar	Age	Hours post inoculation	Average Ct-value	Amount of pathogen cDNA [ng]/250 ng total RNA from potato leaflets
NTC	—	—	29.8	0.000 ± 0.000
Bettina	2 weeks	16	26.2	0.012 ± 0.001
	2 weeks	72	25.6	0.019 ± 0.010
	4 weeks	16	26.1	0.013 ± 0.005
	4 weeks	72	25.5	0.020 ± 0.009
Indira	2 weeks	16	26.3	0.011 ± 0.007
	2 weeks	72	19.8	1.868 ± 0.438
	4 weeks	16	25.2	0.025 ± 0.010
	4 weeks	72	20.2	1.327 ± 0.052

Sequence analyses

To gain insight into the contents of the cDNA libraries, 138 clones from the cDNA library Indira-lib and 393 clones from the cDNA library Bettina-lib were randomly picked and partially sequenced. BLASTn and BLASTx searches were performed for each clone (Altschul *et al.*, 1990). E-values greater than e-10 were considered not statistically significant (no similarities found). In the cDNA library Indira-lib, 92 of the 138 sequenced clones encoded individual genes, whereas 201 unigenes could be derived from 393 clones of the Bettina-lib. In order to decrease redundancy in the Bettina-lib, identified redundant genes were removed from this cDNA library by subtractive hybridization. The 92 unigenes picked from Indira-lib consisted of 31 clones with homologies to stress- and/or defence-related genes. Thirty unigenes appeared to be involved in general metabolism and 31 genes code for unclassified proteins or represent clones for which no similarities could be found to any database sequence. From the 201 unigenes picked from Bettina-lib, 120 consisted of unclassified proteins and clones with no statistical similarity. Only 36 matched stress- and/or defence-related genes and 45 resembled general metabolic genes (Table 2).

Only five genes were found in both cDNA libraries that exhibit 100% sequence identity: four different PR-proteins and one phenylalanine–ammonia–lyase (PAL) isoform. Two pairs of the selected cDNAs encoded the same genes (B-H6 and B-C2: unknown protein, I-B4 and I-D11: thaumatin-like protein) but matched different regions of the sequences.

For further analyses, the 92 unigenes from Indira-lib and 90 unigenes from Bettina-lib were selected according to their putative biological function and spotted on to nylon filters. The 182 cDNAs were classified into nine functional categories according to partial sequence identities to known enzymes and proteins (Table 3).

Table 2 Details of the contents of the cDNA libraries Indira-lib and Bettina-lib.

Indira-lib	138 clones	92 unigenes: 31 unknown function
		31 defence- and/or stress-related
		30 general metabolism
Bettina-lib	393 clones	201 unigenes: 120 unknown function
		36 defence- and/or stress-related
		45 general metabolism

Table 3 Functional classifications of the chosen genes from the cDNA libraries Indira-lib and Bettina-lib.

	Indira-lib number	Bettina-lib number
Photosynthesis/chloroplast	0	5
Cellular metabolism	6	6
Signalling	5	4
Transporters	1	1
Transcription	4	13
Growth/development	6	2
Hormone-related	3	2
Stress/defence	33	17
No similarities found	17	16
Unknown protein	17	24
Total	92	90

By adding RNA from *P. infestans* to the driver, pathogen-derived sequences should have been eliminated from the cDNA libraries. However, two of the 92 selected clones from Indira-lib appeared to be derived from the pathogen; I-G10 matches an elongation factor1-beta gene from the fungus *C. albicans* and I-H4 matches a glucose-related protein GRP 78/BIP from the oomycete *Phytophthora cinnamomi*. In order to identify further pathogen-derived genes present in the 182 selected cDNAs, the printed cDNA arrays were hybridized with ^{33}P-labelled cDNA prepared from total RNA from *P. infestans*. Only three clones hybridized with the pathogen: I-G2 (protein with unknown function from *Arabidopsis thaliana*), I-G10 and I-H4.

Expression analyses

To determine what proportion of the 182 unigenes represented transcripts that accumulate during infection, the expression for each gene was studied with cDNA array hybridization.

The printed cDNA arrays were hybridized with ^{33}P-labelled probes prepared from total RNA. The RNAs had been isolated from leaves of 2- and 4-week-old potato plants at 16 and 72 hpi with *P. infestans* or from respective water controls (Fig. 1). Because no significant changes were observed at 16 hpi, we concentrated on the 72-h variants.

Scatter plots of normalized signal intensities of *P. infestans* infected plants vs. control plants gave an overview of changes in the expression of the selected genes in 2- (Fig. 2A,C) and 4-week-old plants (Fig. 2B,D) at 72 hpi.

In total, only four of the 182 clones were not induced significantly (induction ≤ two-fold). According to their expression patterns, the induced genes can be grouped into three different classes. Class I: the majority of the clones (143) exhibited a weak to moderate up-regulation (between two- and seven-fold). Class II: 35 clones revealed a medium (≥ seven-fold) to strong induction

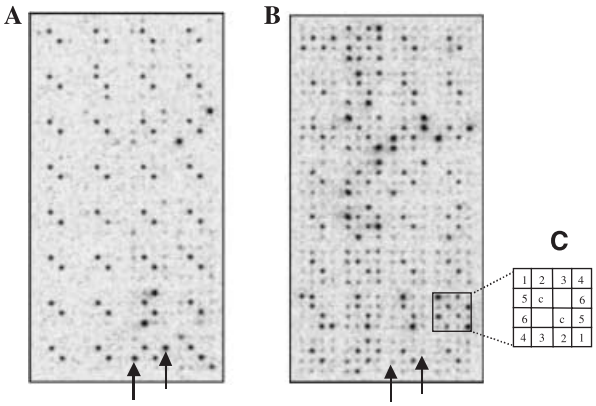

Fig. 1 Representative hybridization profiles corresponding to control (A) and infected plants (B). Filters containing duplicates of 182 cDNAs and control clones were hybridized with ^{33}P-labelled cDNA prepared with total RNA from 4-week-old potato cultivar Indira treated with water (A) or *P. infestans* (B) for 72 h. Increased abundance upon *P. infestans* attack is clearly observable for many clones. The arrow points to a cDNA for which the expression is reduced after the infection. Duplicate spots are indicated by arrows within the same cDNA array. The diagram (C) indicates the configuration of the subarrays; c refers to the control DNA fragments (see Experimental procedures). A list of the cDNA spotted on the filter set can be obtained upon request.

(≥ 15-fold) in at least one experimental condition. Class III: three genes are down-regulated (Table 4).

The highest proportion of the class II cDNAs came from the cDNA library Indira-lib and clones encoded mainly stress- and/or defence-related genes. Among these are many PR-proteins (Selitrennikoff, 2001, and references therein). For example, mRNA levels of clone I-B4 encoding a thaumatin-like protein and I-E7 matching a β-1,3-glucanase were at least seven-fold higher in all four experimental conditions compared with the control plants. Other PR-protein homologues (I-F7, I-F10, I-G7, I-G12, I-H2, and I-H7) were seven- to 48-fold more abundant in two to three experimental conditions. The medium to strong gene induction was observed most often in the 4-week-old Indira plants. Four-week-old Bettina plants showed a weaker gene induction compared with the 2-week-old variant. This could be explained by the observation that transcripts of a number of the analysed genes accumulated to higher levels in the noninfected 4-week-old compared with the noninfected 2-week-old variant (mostly stress- and/or defence-related; see encircled genes in Figs 2D and 3A). The up-regulation of these genes after infection was thereby compensated by high expression levels present in the noninfected plants and as a result a weaker gene induction was observed compared with the 2-week-old Bettina plants (Table 4).

In the scatter plots of the 4-week-old potato plants three genes are found that accumulate significantly in the control plants and are down-regulated upon *P. infestans* infection (class III; Figs 2B,D and 3B). In 2-week-old plants the genes appear to be weakly induced. Affected were only genes derived from the cDNA

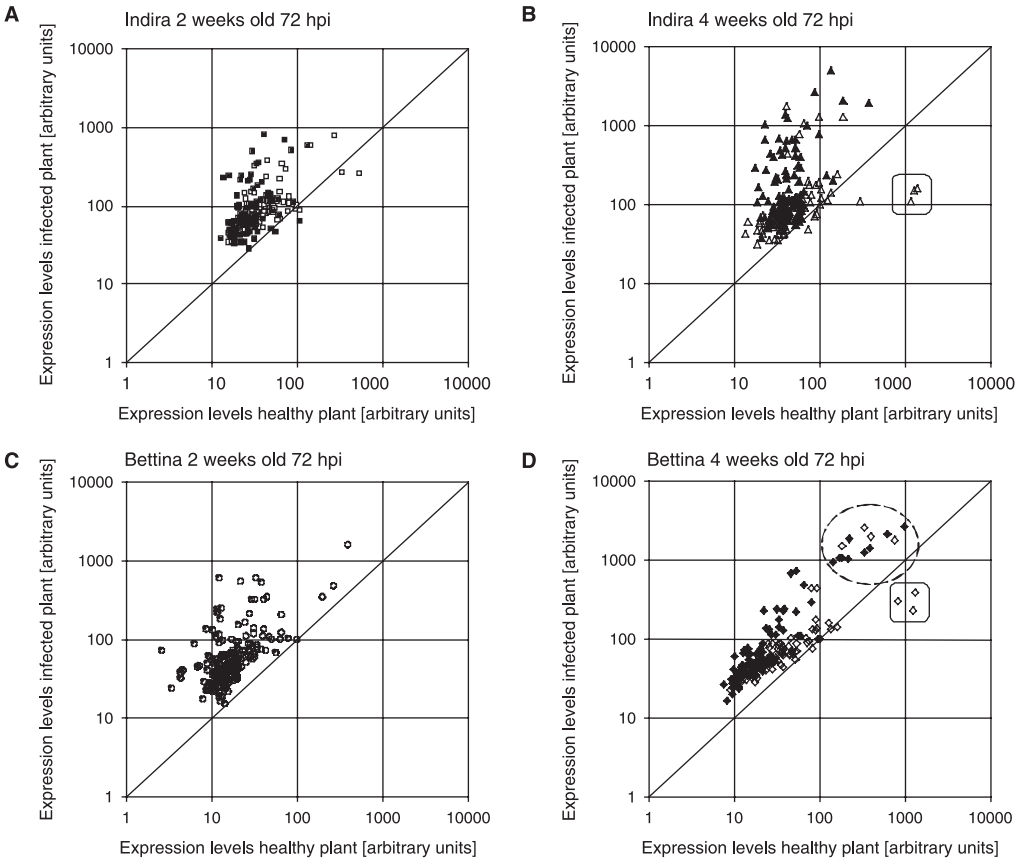

Fig. 2 Scatter plot of changes in expression levels of 2- and 4-week-old potato cultivars Bettina and Indira 72 h after inoculation with *P. infestans*. Marks above the diagonal represent induced genes, marks below the diagonal represent repressed genes. White symbols mark genes from the cDNA library Bettina-lib; black symbols represent genes from the cDNA library Indira-lib. The square highlights three genes that are down-regulated in both cultivars (Fig. 3B,D). The circle surrounds a group of genes that have high expression levels in the control as well as in the infected Bettina plants (Table 4).

library Bettina-lib. The down-regulated clones were, in detail: clone B-A2 for which no significant similarities to any database sequence were found was down-regulated eight-fold in the 4-week-old Indira and three-fold in the 4-week-old Bettina. B-B5 encoding a proteinase inhibitor 1 (Z12611) was repressed nine-fold in the susceptible and three-fold in the moderately resistant cultivar. The mRNA of B-F7 with highest homologies to a leucine aminopeptidase (X77015) was down-regulated 11-fold in Indira plants and five-fold in Bettina.

In 2-week-old potato plants, 60% of the analysed genes appear to be more strongly induced in the resistant cultivar than in the susceptible cultivar. For some of the genes the highest induction was observed in the 2-week-old Bettina plants at 72 hpi (Tables 4 and 5). Among these are proteins involved in signalling (B-C7 encoding a 14-3-3 protein tft 18 gene), translative modification (I-A10 matching a CAAX prenyl protease and I-A9 matching a GlcNAC transferase), proteolysis (B-F10 with highest homology to an aspartic protease 2), energy metabolism (I-A11 encoding a hexose transporter and I-A8 matching a citrate binding protein), stress and defence mechanisms (B-H1 encoding a salicylic acid carboxyl methyltransferase, B-H7 with highest homology to a heat shock protein, and I-H7 encoding the PR-protein pr1b1), as well as four clones with unknown function or no similarities to any database sequence (B-D5, I-A12, I-E12, and I-H6).

Among the cDNAs found in the cDNA library Bettina-lib were three resistance-mediating genes: *R1*, which is associated with late blight in potato and which has recently been cloned by combining positional cloning with a candidate gene approach (Ballvora *et al.*, 2002); a homologue to the resistance gene cluster, which was described with potato virus x and potato cyst nematode (van der Vossen *et al.*, 2000); and *Cf-9* resistance gene cluster, which confers resistance to leaf mould in tomato (Jones *et al.*, 1994). Upon infection, weak induction of expression of the three resistance genes could be observed in both cultivars (two- to four-fold, Table 6). The clone B-E7 encoding a *Cf-9* resistance gene cluster revealed the highest induction compared with the other identified resistance-mediating genes. The presence of R-genes in Indira is contrary to the statement that this cultivar does

Table 4 Genes with medium (≥ seven-fold) to strong (≥ 15-fold) induction and down-regulated genes caused by *P. infestans* in at least one experimental condition.

				Indira		Bettina	
Clone*	GenBank Accession no.	Homology†	E-value	2 weeks 72 hpi	4 weeks 72 hpi	2 weeks 72 hpi	4 weeks 72 hpi
Genes with minor induction in 4-week-old Bettina due to high expression levels in the control plants				Fold induction§			
B-B6	CK640702	Lipid transfer protein precursor, *C. annuum*, AF118131	4e-98	5.0	13.2	7.6	5.0
B-B8	CK640704	No similarities found	—	5.0	16.9	10.1	8.3
B-B10	CK640706	Unknown protein, *A. thaliana*, NP_194876‡	2e-39	9.7	41.8	24.8	7.6
B-F1	CK640742	Chitinase, *S. tuberosum*, U02606	0.0	3.8	6.9	7.8	2.4
I-B6	CK640792	Unknown protein, *A. thaliana*, NP_194878‡	6e-23	5.0	29.0	10.8	3.7
I-B12	CK640798	Lipid desaturase, *L. esculentum*, X94944	e-134	15.0	29.5	18.4	5.0
I-D9	CK640818	Class II chitinase, *S. tuberosum*, U44969	0.0	18.7	10.9	13.9	6.3
I-F10	CK640840	*pr-p2, L. esculentum*, X58548	0.0	4.3	35.8	19.2	8.0
I-G12	CK640849	Osmotin-like protein, *S. commersonii*, X72928	0.0	9.3	5.1	13.6	2.7
I-H2	CK640851	*pr 1a, L. esculentum*, AJ011520	6e-58	5.3	44.7	20.5	3.6
I-H7	CK640854	*pr1b1, S. phureja*, AJ493450	0.0	15.9	34.5	47.8	3.4
I-H8	CK640855	Root-specific metal transporter, *L. esculentum*, AY196091	0.0	16.2	20.1	18.1	5.8
I-H11	CK640858	Copper amine oxidase, *A. thaliana*, AAB87690‡	e-21	7.4	15.2	12.3	6.5
Genes induced mainly in Indira				Fold induction§			
I-B10	CK640796	Vetispiradiene synthase, *S. tuberosum*, AF043300	0.0	2.6	8.7	2.0	3.7
I-C6	CK640804	Sesquiterpene synthase, *L. hirsutum*, AF279456	2e-91	2.1	11.7	1.7	6.1
I-C9	CK640807	Peroxidase, *L. esculentum*, X94943	0.0	1.2	17.1	2.8	5.6
I-C10	CK640808	*LeMir, L. esculentum,* U70076	0.0	3.0	12.0	2.9	5.2
I-C11	CK640809	Catechol oxidase precursor, *V. faba*, S24758	3e-15	3.1	13.9	3.6	5.2
I-E8	CK640826	β-1, 3-glucanase precursor, *S. tuberosum*, AY170826,	0.0	10.7	13.1	4.1	6.2
I-F2	CK640832	Biotic cell death-ass. protein, *N. glutinosa*, AY170826	4e-83	4.5	16.7	2.7	5.3
I-F7	CK640837	NtPRp27, *S. tuberosum*, AY185207	0.0	6.6	14.0	7.0	4.0
Genes induced mainly in Bettina				Fold induction§			
I-A8	CK640782	Citrate binding protein, *S. tuberosum*, AB061262	0.0	3.2	5.6	14.0	6.1
I-A9	CK640783	GlcNAc transferase, *N. tabacum*, AJ295005	e-38	0.8	2.6	7.5	3.2
I-A10	CK640784	CAAX prenyl protease, *A. thaliana*, AAL07084‡	5e-30	1.6	1.4	8.7	1.8
I-A11	CK640785	Hexose transporter, *S. tuberosum*, AAF74567‡	3e-20	8.2	10.1	27.8	7.6
Genes with medium to strong induction in both cultivars				Fold induction§			
B-B11	CK640707	Auxin-induced mRNA, *N. tabacum*, X56265	e-114	4.8	10.5	6.4	5.5
B-E1	CK640731	Glutathione S-transferase, *S. tuberosum*, J03679	0.0	3.3	13.6	4.7	4.8
I-A7	CK640781	Unknown protein, *A. thaliana*, NP_172940‡	6e-28	0.6	7.2	8.5	3.7
I-B4	CK640790	Thaumatin-like protein, *N. tabacum*, X15224	4e-44	7.2	14.0	9.2	14.4
I-D8	CK640817	Endochitinase, *L. esculentum*, Z15138	0.0	2.8	7.9	4.5	5.2
I-D11	CK640820	Thaumatin-like protein, *N. tabacum*, X15224	4e-80	10.0	14.4	7.3	2.1
I-E7	CK640825	β-1, 3-glucanase, *L. esculentum*, X74906	0.0	9.8	30.5	15.1	13.2
I-F6	CK640836	No similarities found	—	9.5	5.4	7.2	3.6
I-G7	CK640863	PR-protein, *O. sativa*, AAD38293‡	2e-54	2.0	12.5	6.2	10.5
I-H10	CK640857	PEP Carboxylase kinase, *S. tuberosum*, AF453448	0.0	5.9	7.9	9.1	7.7
Down-regulated genes				Fold repression¶			
B-A2	CK640686	No similarities found	—	1.3	8.5	0.6	2.7
B-B5	CK640701	Proteinase inhibitor 1, *S. tuberosum*, Z12611	e-116	2.2	8.7	0.6	3.4
B-F7	CK640747	Leucine aminopeptidase, *S. tuberosum*, X77015	0.0	0.4	10.7	0.3	5.4

*cDNA clones derived from the cultivar Bettina are abbreviated B-, clones derived from Indira are abbreviated I-, respectively.

†Accession numbers from GenBank referring to the DNA sequence are given, where available.

‡Accession number refers to protein sequence.

§Fold induction calculated by dividing the expression level in the infected plant by the expression level in the control plant.

¶Fold repression calculated by dividing the expression level in the control plant by the expression level in the infected plant.

Table 5 Genes with highest induction levels in the 2-week-old potato cultivar Bettina at 72 hpi.

Clone*	GenBank Accession no.	Homology†	E-value	Indira 2 weeks 72 hpi	Indira 4 weeks 72 hpi	Bettina 2 weeks 72 hpi	Bettina 4 weeks 72 hpi
				Fold induction§			
B-A7	CK640691	Ripening regulated protein, *L. esculentum*, AF204786	e-114	2.9	1.2	4.6	1.9
B-C7	CK640715	14-3-3 protein tft18 gene, *L. esculentum*, X98864	3e-75	0.8	1.7	3.6	2.1
B-C10	CK640718	DNA J-like protein, *A. thaliana*, AAF24498	e-38	1.6	1.5	4.4	0.9
B-D5	CK640725	No similarities found	—	1.8	1.2	4.4	1.8
B-F10	CK640749	Aspartic proteinase 2, *G. max*, BAB64296‡	8e-59	1.9	1.4	5.6	1.7
B-H1	CK640763	SA carboxyl methyltransferase, *A. belladonna*, AB049752	3e-31	1.0	1.4	4.4	2.9
B-H7	CK640769	Heat shock protein, *L. esculentum*, X54029	0.0	1.1	0.8	2.6	1.4
I-A12	CK640786	No similarities found	—	1.3	3.1	6.9	2.6
I-E12	CK640830	No similarities found	—	2.5	2.1	6.6	3.0
I-H6	CK640853	Unknown protein, *A. thaliana*, B84905	9e-19	1.9	3.4	6.5	2.4

*cDNA clones derived from the cultivar Bettina are abbreviated B-, clones derived from Indira are abbreviated I-, respectively.
†Accession numbers from GenBank referring to the DNA sequence are given, where available.
‡Accession number refers to protein sequence.
§Fold induction calculated by dividing the expression level in the infected plant by the expression level in the control.

Table 6 Gene induction of three resistance-mediating genes in a susceptible and moderately resistant potato cultivar 72 h after inoculation with *P. infestans*.

Clone*	GenBank Accession no.	Annotation†	E-value	Indira 2 weeks 72 hpi	Indira 4 weeks 72 hpi	Bettina 2 weeks 72 hpi	Bettina 4 weeks 72 hpi
				Fold induction‡			
B-E7	CK640737	*Cf-9* resistance gene cluster, *L. pimpinellifolium*, AJ002236	2e-82	2.9	4.2	2.6	2.4
B-E8	CK640738	*R1* gene, *S. tuberosum*, AF447489	e-129	2.0	1.8	1.9	2.6
B-G3	CK640754	Resistance gene cluster, *S. tuberosum*, AF265664	0.0	3.7	3.1	3.1	2.6

*cDNA clones derived from the cultivar Bettina are abbreviated B-.
†Accession numbers from GenBank referring to the DNA sequence are given, where available.
‡Fold induction calculated by dividing the expression level in the infected plant by the expression level in the control.

not carry any of the *Solanum demissum* R genes *R1–R11* (http://www.europotato.org).

DISCUSSION

The two subtractive cDNA libraries utilized proved to be highly enriched for *P. infestans*-induced genes. Only 3% of 182 analysed cDNAs did not exhibit differential expression patterns relative to controls. This allowed us to access a vast source of *P. infestans*-induced genes. By using arrays with partially sequenced genes, a direct correlation between expression patterns and potential functions could be made and assigned to most of the clones. Some of the cDNAs having homology to unclassified *Arabidopsis* proteins, e.g. I-A7 and B-B10, and others for which no similarities could be found with any database sequence, e.g. I-A12 and B-B8, were induced after an infection with *P. infestans*. These findings may help to elucidate the function of these proteins.

Five genes encoding different PR-proteins and one PAL isoform were found in both cDNA libraries exhibiting 100% sequence identity. This small overlap between the two cDNA libraries may reflect the fact that only a subset of genes was compared that derive from distinct traits for which the two genotypes differ. Furthermore, differences in plant age or the limited sample size could have an impact on the number of genes shared in both libraries.

In this study, for the analysis of differential gene expression, 72 hpi was a better point of time than 16 hpi because no significant changes in gene induction were found in both cultivars at this early stage of the infection process. In 2-week-old plants, the susceptible cultivar revealed a weaker gene induction upon *P. infestans* attack compared with the moderately resistant cultivar; at the same time Indira was affected by the pathogen more severely than Bettina. For some of the genes the highest induction was observed in the 2-week-old Bettina plants at 72 hpi (Tables 4 and 5). Because Bettina-lib was generated from 2-week-old

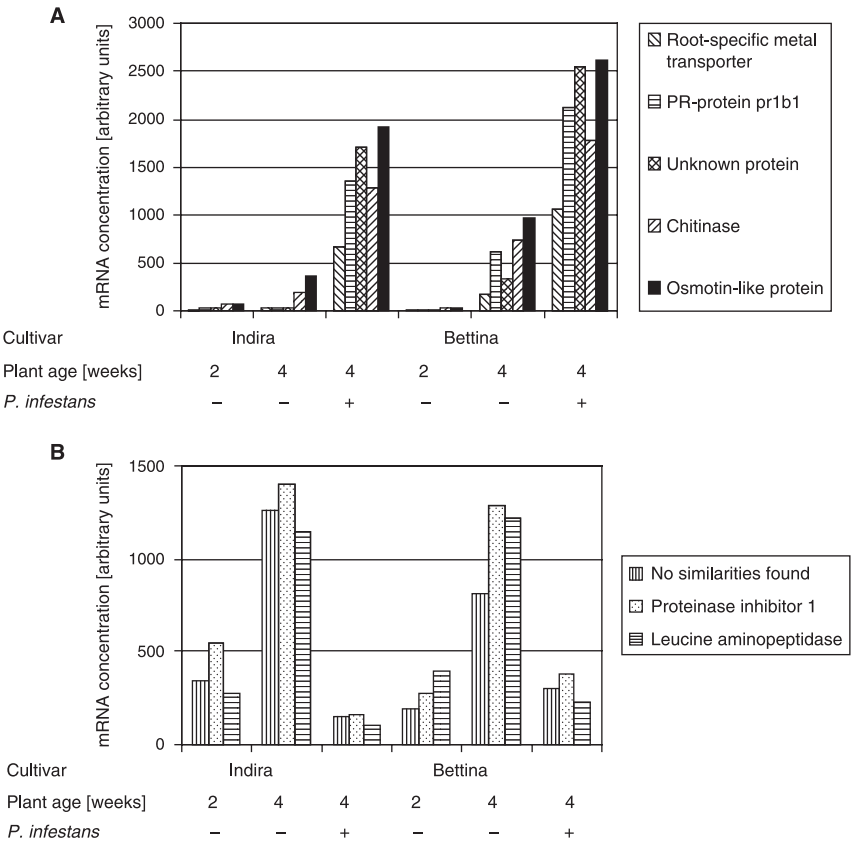

Fig. 3 Comparison of different expression patterns of selected genes. Plants were 2 or 4 weeks old. Detached leaflets were either dipped in water (control, –) or *P. infestans* sporangium suspension (infected, +). Leaflets were harvested 72 h after the treatment. Expression levels were deduced from cDNA array hybridization experiments. (A) Induction of gene expression in response to *P. infestans* and accumulation of transcripts in control plants. (B) Repression of gene expression.

plants, an inadvertent selection for clones most strongly expressed in 2-week-old plants at 72 hpi is possible. However, three of ten genes with highest induction levels in the 2-week-old Bettina were derived from Indira-lib.

The transcripts of some genes, e.g. encoding PR-1, PR-2, PR-3 and PR-5 proteins, accumulated substantially in noninfected 4-week-old Bettina (Fig. 3A) and as a result a weak gene induction was observed. Owing to lower transcript levels in noninfected 4-week-old Indira plants these genes are induced more strongly upon *P. infestans* infection (Table 4). Vleeshouwers *et al.* (2000) reported a correlation between high levels of PR-1, PR-2 and PR-5 mRNA and the level of resistance against *P. infestans* of different potato cultivars, an observation that is consistent with our findings.

Most of the moderately up-regulated cDNAs came from the cDNA library Indira-lib. From this study, it is not obvious whether gene induction was caused as a direct response to the pathogen or as an indirect response to wounding caused by the growth of *P. infestans* within the leaf.

Some of the genes showed medium to strong induction in only one certain cultivar or at onetime point (Tables 4 and 5), whereas others were induced in both cultivars without regarding to the level of resistance. For example, I-C9 exhibits up-regulation only in 4-week-old Indira at 72 hpi and clone I-B12 was strongly induced in both 2-week-old cultivars and 4-week-old Indira at 72 hpi, whereas only moderate induction was observed in 4-week-old Bettina, which in this case was due to high expression levels of the gene already in noninfected 4-week-old Bettina (Table 4). I-B12 exhibits 87% nucleotide identity to a lipid desaturase-like protein from *Lycopersicon esculentum* and the highest identity score for I-C9 (90%) was obtained when compared with the peroxidase from *L. esculentum*. Gadea *et al.* (1996) described both genes to be induced upon virus infection and ethylene treatment and concluded that the desaturase-like gene and the peroxidase could be involved in the solidification of plant cells.

I-H8 was one of the clones with high expression levels already in the noninfected 4-week-old Bettina; in the other three experimental conditions the clone was strongly induced. I-H8 encodes a protein with 97% nucleotide identity to a root-specific metal transporter from *L. esculentum* belonging to the Natural Resistance-Associated Macrophage Protein (NRAMP) family, which functions as a divalent-metal efflux pump in the membrane (Bereczky *et al.*, 2003). Depletion of divalent metals in the phagosomal lumen improved resistance against intracellular bacteria in mice (Forbes and Gros, 2001). Bereczky *et al.* (2003) reported a novel function for NRAMP1 in providing iron in vascular parenchyma in iron-deficient tomato plants. To our knowledge, no member of the NRAMP family has ever been described in potato in the context of *P. infestans*. Another clone with high expression levels in the

noninfected 4-week-old Bettina is I-G12, which shows highest homology (97% nucleic acid level) to an osmotin-like protein from *Solanum commersonii.* Woloshuk *et al.* (1991) found an inhibition of *P. infestans* caused by an osmotin-like protein by lysis of the sporangia and inhibition of hyphal growth. In several studies with transgenic potato plants expressing genes for an osmotin-like protein, a delayed development of disease symptoms after an inoculation with *P. infestans* could be observed (Li *et al.*, 1999; Liu *et al.*, 1994; Zhu *et al.*, 1995).

In the 4-week-old Indira at 72 hpi the clone I-C10 with 94% nucleic acid homology to the *LeMir* gene from *L. esculentum* is induced moderately. In tomato, the gene is induced early after infection of tomato with root-knot nematodes (*Meloidogyne javanica*) (Brenner *et al.*, 1998). Because of the up-regulation caused by different pathogens this gene might be a general defence gene.

The clone I-F2 shows highest homology (85% nucleic acid level) to a biotic cell death-associated protein from *N. glutinosa* with unknown function and 43% amino acid homology to a miraculin homologue from *Youngia japonica*. Miraculin exhibits homology to *LeMir* (54% amino acid identity); both proteins belong to the soy bean trypsin inhibitor family but may have a different function because of different structures (Brenner *et al.*, 1998). Like I-C10, I-F2 was found to be moderately induced only in the 4-week-old susceptible cultivar Indira, suggesting a possible correlation of susceptibility and gene induction.

In 4-week-old potato plants of both cultivars three genes were found that accumulate significantly in the noninfected plants and exhibite a down-regulation upon *P. infestans* infection (class III; Figs 2B,D and 3B). These clones appear to be weakly induced in 2-week-old plants, which explains why they are components of Bettina-lib. The down-regulation of these clones was not revealed until gene expression in 2- and 4-week-old plants was compared.

The genes that have highest induction levels in the 2-week-old Bettina are mainly involved in energy metabolism, post-translational modifications or do not yet have a distinct function. In particular, the strong induction of I-A11 encoding a hexose transporter responsible for the amylolytic breakdown of starch (Weber *et al.*, 2000) and I-A8 encoding a citrate binding protein indicate that in the moderately resistant cultivar the energy-providing pathways are more active than in the susceptible cultivar. Probably, this is necessary to provide the plant with enough resources for building up defence factors already in the noninfected plant.

Clone B-C7, which is induced only in the moderately resistant cultivar, has 89% nucleotide identity to a 14-3-3 protein from *L. esculentum*. 14-3-3 proteins are phosphoserine-binding proteins that bind a variety of transcription factors and other signalling proteins, and play important roles in the regulation of plant development and stress responses (Roberts, 2003). 14-3-3 proteins are part of the defence reaction by regulating the proton pump (H^+-ATPase) to initiate the hypersensitive response (Roberts *et al.*, 2002).

In conclusion, numerous differences between the gene induction in the susceptible and moderately resistant potato cultivars could be found. Many genes have been observed to be up- or down-regulated upon *P. infestans* infection. An examination of a wider range of potato genotypes with similar levels of resistance will be required to answer the question of whether our findings represent a general feature of the potato–*P. infestans* interaction or if they apply only to the cultivars Bettina and Indira. In addition, a comparison of gene induction caused by *P. infestans* and different biotic or abiotic factors will be useful to differentiate between general defence mechanisms and specific defence mechanisms against *P. infestans*, which might also lead to helpful markers for potato breeders. In order to complete the information on the function and nature of the genes that respond to *P. infestans* infection, studies on the protein level as well silencing and over-expression of candidate genes will be helpful.

EXPERIMENTAL PROCEDURES

Plant material and treatment

To compare gene induction after *P. infestans* infection the following potato cultivars were chosen: Indira, which has a field resistance of 3 according to the international 1–9 scale and does not carry any of the *Solanum demissum* R-genes *R1–R11*; and Bettina, which has a field resistance of 5 and contains an uncharacterized R-gene (http://www.europotato.org, Anon., 2003). The potato cultivars were grown from tubers in a greenhouse at 18 ± 4 °C under a 16-h day length. Following the conditions for first infections in the field, the third and fourth leaves of 2- and 4-week-old plants were used for experiments. Detached leaflets were inoculated using a mixture of the *P. infestans* strains 31 (virulence genes 1, 3, 4, 5, 7, 8, 10, 11, mating type A1), 38 (virulence genes 1, 3, 4, 7, 8, 10, 11, mating type A1) and 57 (virulence genes 1, 3, 4, 7, 8, 10, 11, mating type A1) obtained from the Bayerische Landesanstalt für Landwirtschaft, Freising, Germany. Sporangia suspensions were diluted to 10^4 sporangia/mL, whereas each isolate contributed equally to the mixture. Control leaflets were treated with water. The plant material was incubated on Petri dishes containing water agar in a growth chamber at 16 °C under a 16-h light period. Inoculation with the mixture of the three *P. infestans* strains caused no qualitative resistance reaction in both potato cultivars. Samples were taken 16 and 72 h after the inoculation, frozen in liquid nitrogen and stored at −80 °C.

Total RNA isolation, purification and *in vitro* RNA synthesis

Total RNA from the leaves and the *P. infestans* strains grown on V8 medium was isolated by using TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. After adding 1/3

volume of 8 M LiCl the RNA was precipitated overnight at 4 °C and harvested by centrifugation at 12 000 ***g*** for 20 min.

Plasmid pMM14 was used to synthesize the RNA used as a spiking control for the array hybridization experiments. The plasmid is composed of the 3′ untranslated region of the luciferin-binding protein from the dinoflagellate *Gonyaulax polyedra* (Mittag *et al.*, 1994), cloned in pTZ18R (Pharmacia) and was kindly provided by Dr M. Mittag (University Jena, Germany). RNA was *in vitro* synthesized from the T7-promotor according to the instructions of the supplier of the T7-polymerase (MBI Fermentas).

Quality of all RNAs was verified by gel electrophoresis and quantity was determined spectrophotometrically.

Quantitative real-time one-step RT-PCR

The following primers were designed to the clone I-G10 having homologies with the *C. albicans* elongation factor 1-beta gene (accession no. X96517) and were synthesized commercially (Thermo Hybaid). The sequence of the 5′ primer is CGACGATGAC-CTCTTCGGTGATGA, and the sequence of the 3′ primer AGTC-CTCGAAACCCTCAATGGCGT.

Gene-specific RT-PCR amplification was carried out with the one-tube QuantiTect SYBR® Green RT-PCR Kit (Qiagen) using 250 ng purified total RNA from the potato cultivars. Twenty-five-microlitre reactions were pipetted in each well of 96-well optical reaction plates closed with optical caps (Applied Biosystems). Each sample was run in triplicate in addition to nontemplate controls containing water instead of RNA. The RT-PCR were performed in an automated ABI Prism 7700 sequence detector (PE Biosystems) following an initial incubation of 50 °C for 30 min, 95 °C for 15 min and then 35 cycles of 94 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s during which the fluorescence data were collected. In order to calculate the input amount of target mRNA from *P. infestans* in potato leaflets, a standard curve was created using mRNA from the pathogen. For each sample, a specific Ct-value is created, which is defined as that cycle number at which a statistically significant increase in the reporter fluorescence can first be detected. The Ct-values were plotted vs. the log of the initial amount of mRNA. For all calculations the average value was used. No signal above the level of the nontemplate control was observed. PCR products were verified by gel electrophoresis, to confirm that the signal was the result of product amplification and not due to primer dimers.

cDNA library construction

Two cDNA libraries highly enriched for *P. infestans*-induced genes were prepared, one from the moderately resistant potato cultivar Bettina, the other from the susceptible cultivar Indira. The libraries were constructed by vertis Biotechnologie AG, Freising, Germany, using an innovative subtractive hybridization technique. In short, with RNA derived either from the infected plants (tester) or from the control plant (driver), first-strand cDNA synthesis was performed using M-MLV-RNase H^- reverse transcriptase.

In the case of the Bettina library (Bettina-lib), 2-week-old plants were used. The tester cDNA was prepared from a mixture of RNA (1 μg each) from leaflets that were incubated with *P. infestans* for 16 h and for 72 h. The driver was prepared from a mixture of RNA (1 μg each) from control leaflets treated with water for 16 h and 72 h and of RNA from a mixture of all three *P. infestans* strains (30 ng). For the Indira library (Indira-lib), 4-week-old potato cultivar Indira was used. The tester consisted of RNA from leaflets that were incubated with *P. infestans* for 72 h (2 μg). The driver cDNA was prepared from a mixture of RNA from control leaflets treated with water for 72 h (2 μg) and from *P. infestans* (30 ng). For cDNA synthesis, different oligo(dT)-linker primers were used for the preparation of the tester and the driver cDNA. The linker primer used for synthesis of the tester cDNA contained a *Not*I recognition site.

Second strand synthesis was performed with Klenow DNA-polymerase using different random linker primer for the preparation of the tester and driver cDNA. The resulting S0 cDNAs were then amplified with long and accurate PCR (LA-PCR) according to Barnes (1994). For subtraction, single stranded (ss) cDNA was prepared from the tester (sense strand) and driver (anti-sense strand) S0 cDNAs. A ten-fold excess of anti-sense ss-driver was then hybridized with the sense ss-tester. Reassociated tester/driver ds-cDNA was separated from the remaining ss-tester cDNA (subtracted cDNA) by passing the mixture over a hydroxylapatite column. After hydroxylapatite chromatography, the ss-tester cDNA was amplified with the tester-specific primers using LA-PCR. After a second round of subtractive hybridization using S1 sense cDNA as tester, the S2-cDNA was obtained.

In the case of the Indira library, the S2 cDNA was cloned into plasmid pCR®2.1 using the TA Cloning® Kit Vector (Invitrogen) and transformed into One Shot® TOP10F′ Chemically Competent *Escherichia coli* cells (Invitrogen). In the case of the Bettina library, the S2 cDNA was directionally cloned in plasmid pBluescript II SK (–) (Stratagene). For directional cloning, the S2 cDNA was first subjected to a limited exonuclease treatment to generate *Eco*RI overhangs at the 5′-ends, was then digested with *Not*I and ligated into *Eco*RI and *Not*I digested pBS II sk+ vector. Ligations were electroporated into T1 Phage resistant DH10B™ (Invitrogen) electro-competent cells.

PCR amplification of cDNA inserts

Colonies were randomly picked from the plated subtractive cDNA libraries and cultured in 1 mL of LB-Amp medium in 96-well plates at 37 °C. The cDNA inserts in the clones were amplified by PCR in a Perkin-Elmer GeneAMP PCR System 9600 thermocycler using primers GACTGGAAAGCGGGCAGTGAG (forward) and

TGCTGCAAGGCGATTAAGTTG (reverse) flanking the cloning site of the pCR®2.1 Vector (Invitrogen) and pBluescript II SK (–) (Stratagene). PCR reactions (30 μL) contained 27 μL distilled water, 0.05 μL each of the primers (100 μM each), 3 μL 10× Advantage Buffer (Amersham Biosciences), 0.125 U home-made *Taq* (Pluthero, 1993) and 1 μL bacterial culture. Cycling conditions were 2 min at 95 °C, 35× (15 s at 95 °C, 30 s at 55 °C, 3 min at 72 °C). Quality of all PCR products was verified by gel electrophoresis.

Sequence analysis

M13 rev, M13(–20) fwd and T7 primer were used to generate partial 5′ sequence information of the cDNA clones with the ABI PRISM® BIG DYE Terminator Cycle Sequencing Ready Reaction Kit version 1.0 (Applied Biosystems). PCR reactions were carried out in a Perkin-Elmer GeneAMP PCR System 9600 thermocycler and analysed using an ABI PRISM® 377 DNA sequencer (Perkin-Elmer). Sequence data were analysed using the Sequencing Analysis Software version 3.2 ABI PRISM® (Perkin-Elmer). Runs with low-quality peak shape were discarded. Similarity searches were made with BLAST programs at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/).

Preparation, hybridization and washing of cDNA macroarrays

Before spotting, all amplified cDNA fragments were concentrated through MultiScreen®-PCR Plates (Milipore) to approximately 0.4 μg/μL. cDNA arrays were performed by the transfer of 30 ng of PCR products in duplicates to positively charged nylon membranes (Pall Biodyne B, DuPont) using the nano plotter NP1c (GeSiM). Plasmid pMM14 (Mittag *et al.*, 1994) was printed on each membrane as spiking control. The membranes were washed in 0.2 M NaOH, 0.2 M SDS at 65 °C for 15 min and in 30 mM Tris pH 7.5 at room temperature for 15 min.

First-strand cDNA synthesis and concurrent labelling with ^{33}P-modified nucleotides were performed on total RNA by oligo-dT priming. Ten micrograms of total RNA was mixed with 40 pg of spiking RNA prepared from pMM14 plasmid and 2 μg of $(dT)_{12–18}$ (Roche) in 8 μL of DEPC treated water. The sample was denatured at 72 °C for 2 min and subsequently cooled to 42 °C. Reverse transcription was performed in a total volume of 22.5 μL using REVERTAID M-MuLV reverse transcriptase (Fermentas). Incubation was for 1 h at 42 °C in the presence of 0.33 mM each of dATP, dGTP and dTTP, 3.3 μM dCTP, and 10 μCi of [^{33}P]dCTP (3000 Ci/mmol, ICN Biomedicals). The RNA was hydrolysed for 30 min at 65 °C after the addition of 150 mM NaOH and 10 mM EDTA. The unincorporated nucleotides were removed using SEPHADEX G-50 (Amersham Biosciences). The incorporation of label was checked by scintillation counting.

The sample was denatured at 95 °C for 5 min. Arrays were prehybridized for 2 h in Church buffer (Church and Gilbert, 1984) containing 0.25 M $NaPO_4$ pH 7.2, 7% SDS, 1 mM EDTA, 250 μg tRNA (Roche), 2.5 μg oligo $d(A)_{40–60}$ (Amersham Biosciences). Hybridization was in the same buffer containing 40 μg tRNA (Roche) and 0.4 μg oligo $d(A)_{40–60}$ (Amersham Biosciences) at 65 °C overnight. Membranes were washed twice in 2× SSC, 0.1% SDS and twice in 40 mM $NaPO_4$ pH 7.2, 0.1% SDS at 65 °C for 20 min. The filters were exposed to imaging plates (Kodak) for at least 24 h.

To analyse the data statistically, hybridization was conducted twice to obtain more than three data points for each clone.

Data acquisition and analyses

Hybridization signals on the imaging plates were detected using STORM 860 (Molecular Dynamics) with a resolution of 50 μm. The image data obtained were imported into the program package Array Vision (Imaging Research, Canada) for spot detection and quantification of hybridization signals. The signals derived from hybridization of the cDNA transcribed from the spiking RNA to pMM14 plasmid were defined for reference determination. For normalization, the individual spot was divided by the reference and by the internal control (clone B-G5, encoding a DNA J-like protein from *S. tuberosum*, X94301), which exhibited equal gene expression levels in all experiments.

Database accession numbers

The new cDNA sequences reported here have been deposited in the GenBank with the accession numbers CK640685-CK640865 and CK656422.

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