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Infection with *Rhizoctonia solani* Induces Defense Genes and Systemic Resistance in Potato Sprouts Grown Without Light

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ABSTRACT

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Rhizoctonia solani is an important soilborne and seedborne fungal pathogen of potato (*Solanum tuberosum*). The initial infection of sprouts prior to emergence causes lesions and may be lethal to the sprout or sprout tip, which results in initiation and compensatory growth of new sprouts. They emerge successfully and do not suffer significant damage. The mechanism behind this recovery phenomenon is not known. It was hypothesized that infection may induce pathogen defense in sprouts, which was investigated in the present study. Tubers were sprouted in cool and moist conditions in darkness to mimic conditions beneath soil. The basal portion of the sprout was isolated from the apical portion with a soft plastic collar and inoculated with highly virulent *R. solani*. Induction of defense-related responses was monitored in the apical portion using microarray and quantitative polymerase chain reaction techniques at 48 and 120 h postinoculation (hpi) and by challenge-inoculation with *R. solani* in two experiments. Differential expression of 122 and 779 genes, including many well-characterized defense-related genes, was detected at 48 and 120 hpi, respectively. The apical portion of the sprout also expressed resistance which inhibited secondary infection of the sprouts. The observed systemic induction of resistance in sprouts upon infection with virulent *R. solani* provides novel information about pathogen defense in potato before the plant emerges and becomes photosynthetically active. These results advance our understanding of the little studied subject of pathogen defense in subterranean parts of plants.

Additional keywords: disease resistance, ecology, genomics, infection cycle, resource allocation, stem canker, transcriptome.

Rhizoctonia solani Kühn (teleomorph *Thanatephorus cucumeris* [Frank] Donk) infects at least 200 plant species and is one of the most common soilborne pathogens in crop plants (29,58,59). Strains of *R. solani* belong to at least 14 different, genetically defined populations of anastomosis groups (AG) determined by anastomosis between hyphae of strains belonging to the same AG (16,58). Potato (*Solanum tuberosum* L.) is mostly infected with isolates of AG-3 (2,5,17), especially in the potato production areas under cool climates such as northern Europe (38). The various types of damage to the subterranean parts in infected plants eventually materialize as malformed tubers, increased proportion of small and overlarge tubers (5,48, 54,55,66), and considerable reduction of the marketable yield (5,18).

Asexual, vegetatively growing, multinucleate hyphae of *R. solani* grow on the plant surface, attach within 12 h, and form T-shaped branches in flattened hyphae. Penetration into the epidermal tissues and cortex takes place with thin and densely located infection pegs that form beneath clumps of hyphae (35). Further growth occurs inter- or intracellularly, and is associated with secretion of extracellular enzymes (29). Consequently, the infected tissues collapse and form brown lesions known as stem canker (5,18). This disease develops mainly prior to emergence (32,56,63). The pathogen continues to grow on roots and stolons and forms sclerotia on them, which is stimulated by senescence of the plant towards the end of the growing season. Sclerotia remaining in soil at harvest provide inoculum for infection of new plants in the next growing season (54). Sclerotia are also formed on tubers and known as black scurf. These sclerotia provide means for *R. solani* to infect new crops when black scurf-infested tubers are used as seed (18,55,66).

There have been few studies reporting on the interaction of *R. solani* with potato sprouts prior to emergence. The seminal studies of Richards (49) and Sanford (53) showed that the hyphae colonize densely a region just below the apex, which often kills the tip of the sprout (49). The plant responds with initiation of new sprouts from the base of the damaged one. In these new sprouts severe symptoms are usually not observed and they emerge successfully (53), which is unexpected considering that the pathogen has already infected plant tissues and presumably gained more energy and resources for further infection. It has previously been reported in bean (*Phaseolus vulgaris* L.) (28,60,65,67) and rice plants (*Oryza sativa* L.) (22,37) that infection with virulent isolates of *R. solani* activates many defense-related genes and results in production of pathogenesis-related proteins. Similar studies have not been reported on potato. Furthermore, in bean plants defense-related genes are induced systemically outside the infection site upon infection with *R. solani* (28). However, it is less known whether the observed induction of defense-related genes and proteins actually results in elevated levels of resistance to infection.

The aim of this study was to test whether infection with *R. solani* can activate defense-related genes and induce resistance systemically in potato sprouts. The sprouts were grown without light and in cool temperature to mimic the conditions in soil. Systemically induced changes in gene expression following primary infection were tested and challenge-inoculation carried out to verify whether the possibly observed differential expression of genes was associated with resistance to secondary infection with *R. solani*.

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*The *e*-Xtra logo stands for "electronic extra" and indicates that the online version contains a table showing a list of differentially expressed potato genes.

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MATERIALS AND METHODS

Inoculation. Strain RS11 of *R. solani* was recently isolated from potato in Finland and found to be one of the most virulent isolates among the 98 tested (38). Strain RS11 was grown on potato dextrose agar (PDA; Biokar, France) at room temperature (18 to 20°C) in the dark for 5 days. *R. solani* grows vegetatively and does not form spores under the in vitro and in vivo growth conditions used in this study.

Certified healthy seed potatoes of cv. Saturna were obtained from The Finnish Seed Potato Center Ltd. (Tyrnävä, Finland). Dormant tubers were surface-sterilized with 1% NaOCl for 5 min and rinsed four times (5 min each) with autoclaved tap water. Subsequently, they were placed in a dark room at 20°C for presprouting and protection from light. When sprout growth had started, tubers were moved to propagators and placed as a single layer on the bottom. The incubators were kept in a controlled growth cabinet (Sanyo Scientific, Bensenville, IL) at constant 18°C temperature without light. Growth of sprouts was periodically checked under photosynthetically inactive black light (365 nm). When sprout length reached approximately 5.0 cm, a collar made of 0.15-mm thick low density polyethylene (LDPE) plastic (Pentti Laiho Ky, Nastola, Finland) was positioned to the middle of the sprout to isolate basal and apical portions (Fig. 1A). The basal portion of the sprout (1 sprout per tuber) was inoculated with RS11 by placing a plug (5 mm diameter) of PDA with the fungus on the tuber next to the sprout (Fig. 1A and B). Challenge inoculation of the apical portion was done 120 h after inoculation of the basal portion of the sprout. At this time, a PDA plug with the fungus was placed inside the plastic collar so that it was in contact with the sprout. Control sprouts had a plastic collar but were "mock-inoculated" with plugs of fungus-free PDA. Propagators were sprayed gently with tap water when needed to maintain humidity. All handling and observations (see later) were done under photosynthetically inactive light in a dark room where the growth cabinet was located (room temperature 18°C).

Visualization of fungal growth. Fungal growth at the inoculated base of the sprout was monitored daily. Following chal-

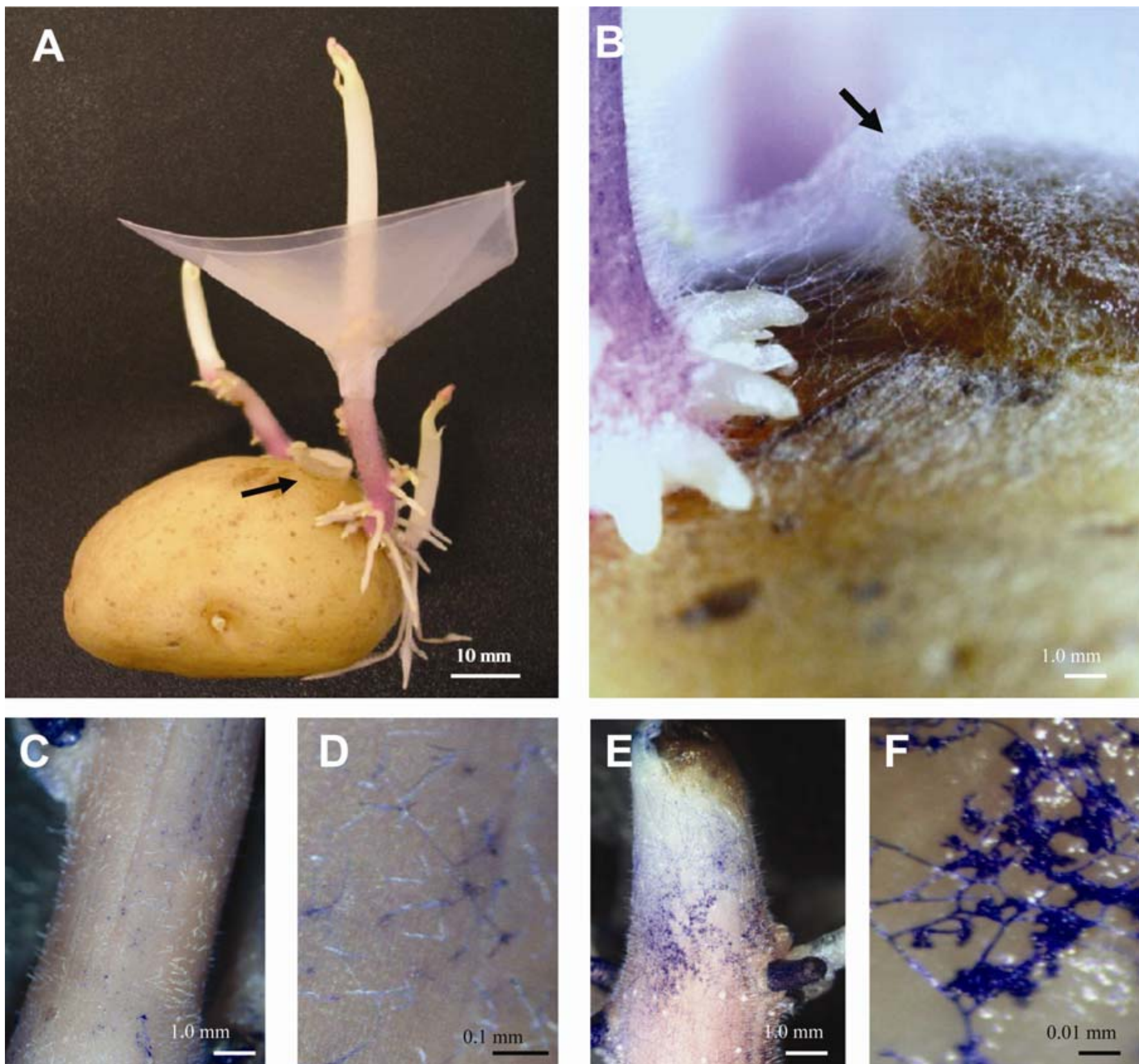

Fig. 1. Experimental set-up for inoculation and study of induction of systemic resistance in potato sprouts inoculated with *Rhizoctonia solani*. **A,** The apical portion of the sprout was isolated from the basal portion with a plastic collar to prevent growth of hyphae from the basal portion that was inoculated using an agar plug containing *R. solani* (indicated by an arrowhead). The apical portion was sampled for gene expression analysis. **B,** The hyphae of *R. solani* grew from the agar plug (arrowhead) to the base of the sprout and colonized it within 48 h. **C and D,** Induction of systemic resistance was tested in the apical portion of the sprout by challenge-inoculation with *R. solani* 120 h after inoculation of the basal portion. Scarce growth of hyphae and no infection structures were observed up to 21 days following challenge-inoculation. **E and F,** In the apical portion of the mock-inoculated control sprouts (i.e., not inoculated with *R. solani*), an abundance of fungal hyphae and infection structures were observed 21 days postinoculation. **F,** T-shaped branches and clumps of hyphae are observed on the flattened hyphae indicating formation of infection structures (35). **D** and **F** are close-ups of **C** and **E**, respectively. Fungal hyphae were stained with lactophenol cotton blue.

lenge-inoculation of the sprout top, the apical portion of the sprout was monitored at 3-day intervals, and hyphal growth and symptom development were observed for 21 days.

To observe the growth of hyphae in more detail, the sprout was stained in lactophenol cotton blue followed by rinsing with tap water. Stained sprouts were studied under Leica MZFLIII stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany) and photographed with digital camera using ViewFinder 1.0.135 and StudioLite 1.0.136 software (Pixera Corporation, Los Gatos, CA).

Extraction of RNA. From each treatment and time point (Fig. 2), the apical portions were sampled from five sprouts, pooled, snap-frozen in liquid nitrogen, and stored at –80°C until RNA was isolated. The frozen material was crushed and ground under liquid nitrogen. Total RNA was extracted from 0.5 g of the homogenate using the Trizol reagent (14) and purified with Qiagen RNeasy Kit (Qiagen, Hilden, Germany) or by lithium chloride precipitation (52). Quality of RNA was verified spectrophotometrically.

Microarray analysis of gene expression. RNA samples were analyzed on microarray slides containing 11,412 validated potato cDNA clones according to the scheme presented in Figure 2. Samples i48hpi and i120hpi collected from the inoculated sprouts 48 h postinoculation (hpi) and 120 hpi, respectively, were analyzed against their mock inoculated counterparts, m48hpi and m120hpi, respectively. Additionally, sample m48hpi was analyzed against sample 0 which consisted of five sprouts sampled just before inoculation of other sprouts. To detect any changes in gene expression associated with growth and development of the sprouts during the experiment, samples 0, m48h, and m120h were analyzed against each other. The entire experiment was carried out twice as explained previously, starting from surface sterilization and sprouting of tubers. In both experiments, hybridization of RNA samples was repeated using dye-swapping.

Extracted total RNA was amplified, repurified, and labeled with N-hydroxysuccinimide (NHS) ester dyes (Cy3 and Cy5) using Amino Allyl MessageAmp II aRNA Amplification Kit according the manufacturer's instructions (Ambion, TX). The method amplifies the mRNA and includes DNase treatment, which eliminates the influence of any possible trace amounts of DNA in the RNA samples extracted from plants. The labeled RNA (4 µg) was hybridized on TIGR Potato cDNA Array (10K version 4) obtained from the National Scientific Foundation's (NSF) Potato Functional Genomics Project (TIGR Solanaceae Genomics Resource, Rockville, MD). The hybridization scheme (pairs of samples compared on the same array) is presented in Figure 2. Hybridization and stringency washes were done according to the method developed by TIGR (30) with minor modifications. Microarray slides were incubated in prehybridization buffer (5× sodium chloride–sodium citrate buffer (SSC), 0.1% sodium dodecyl sulfate (SDS), and 1% bovine serum albumin [BSA]) prewarmed to 42°C for 1 h, rinsed five times in fresh deionized water, and dried in a slide centrifuge. The samples labeled with Cy3 and Cy5 were combined and spun for 5 min at 13,000 rpm in a table centrifuge. Supernatants were transferred to new Eppendorf tubes, 20 µl of poly(dA) (1 µg/µl) and 1 µl of herring sperm DNA were added, and samples were dried using vacuum centrifugation at 60°C. Subsequently, 60 µl of prewarmed (42°C) hybridization buffer (50% formamide, 5× SSC, and 1% SDS) was added. Probes were denatured for 3 min at 95°C and placed on ice. Arrays were hybridized in a humid hybridization chamber at 42°C for 18 h. Coverslips were removed on the following day by merging the slide into washing buffer I (1× SSC and 0.2% SDS). Stringency washing was done at room temperature with washing buffer I for 4 min, washing buffer II (0.1× SSC and 0.2% SDS) for 10 min, and twice with 0.1× SSC for 10 min. Finally, slides were washed twice with deionized water for 10 min, dried in a slide centrifuge, and analyzed. Technical replications of hybridizations were done as described previously using dye-swapping.

Microarrays were scanned with GenePix 4200 AL (MDS Analytical Technologies, Toronto, Canada) using pixel resolution of 10 µm. Image analysis was carried out with GenePix Pro 6.0 software (Axon Instruments, Foster City, CA). Spot intensities were characterized by the mean of the foreground and median of the local background pixel values. Scanned and segmented images were visually checked and spots which showed anomalies in the hybridization were excluded from the analysis. Data normalization and statistical tests were computed using R software (47) with limma package (57). Spots whose foreground area was less than 30 pixels were not included in the analysis. Background-subtracted foreground signals were used for computing the ratios between Cy5 and Cy3 signals. Ratios were transformed into logarithmic domain and lowess normalization was applied for each slide. Normalized log-ratio values from duplicated spots were averaged on each array.

A linear model was fitted to the normalized log-ratio values. The model was parameterized so that there was a separate parameter for mock (m) and induced (i) sample at two time points (48 and 120 h); these conditions are denoted below as m48h, m120h, i48h, and i120h. Time point 0 was considered as a reference. Two arrays were used for measuring the difference between time point 0 and m48h. Four parameter values were estimated for each gene, one for each condition of interest. Differences between the conditions were investigated using four contrasts (i) m48h versus m120h, (ii) m48h versus i48h, (iii) m120h versus i120h, and (iv) i48h versus i120h (Fig. 2). Differentially expressed genes were detected by testing the contrasts for each gene and applying an empirical Bayes variance shrinkage method. False Discovery Rate (FDR) method was used for correcting the *P* values due to multiple tests and the genes with FDR < 0.05 were selected as differentially expressed. The microarray analyses complied with MIAME recommendations.

Genes with statistically significant changes in their expression level were grouped according to their known or predicted biological functions using the gene ontology listing (GO files) of TIGR and relevant research reports (3,41,62). Overrepresented classes among differentially expressed genes were found by making the Fisher exact test for each class.

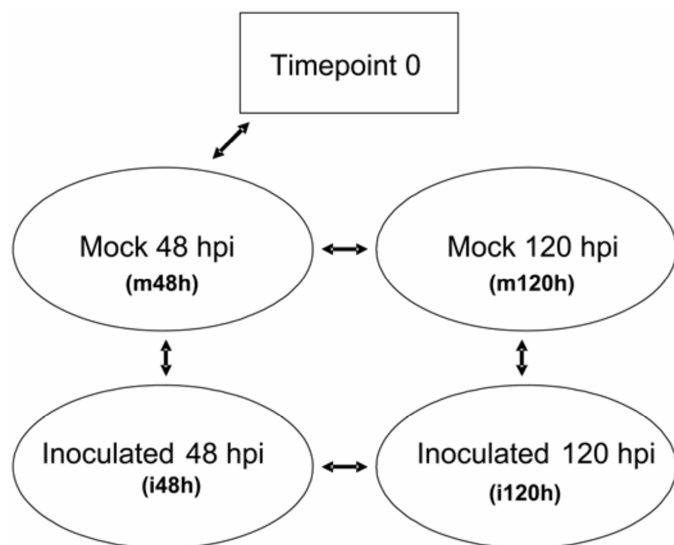

Fig. 2. The microarray hybridization scheme used for comparison of gene expression in the apical portion of potato sprouts. The basal portion of sprouts was inoculated with *Rhizoctonia solani* (i48h and i120h) or mock-inoculated (m48h and m120h) and samples collected 48 or 120 hours postinoculation (hpi), respectively. Each sample contained a pool of RNA from five sprouts. Samples at time point 0 were taken from five sprouts just before inoculation and mock-inoculation of other sprouts. Two independent biological experiments were carried out.

Quantitative real-time polymerase chain reaction (qRT-PCR). Gene expression was also tested by qRT-PCR. Primers for the genes to be tested were designed based on the sequences of the cDNAs spotted on the array (provided by TIGR) using Primer Express v2.0 software (Applied Biosystems, Foster City, CA). The potato actin gene was used as a reference. Specificity of primers was verified by alignment with the original Gene Bank sequence using standard Blast (National Center for Biotechnology Information [NCBI]). Expression levels of the potato actin gene (X55746.1) were tested in all samples to be used as a reference for data normalization. One microgram of total RNA was treated with DNaseI (Promega Corporation, Madison, WI) and reverse transcribed using *Maloney murine leukemia virus* (M-MLV) reverse transcriptase according the manufacturer's instructions (Promega). The cDNA was diluted five-fold for analysis. The reaction mixture was prepared and two-step qRT-PCR carried out with LightCycler 480 following instructions of the LightCycler 480 SYBR Green I Master Kit (Roche Diagnostics GmbH, Mannheim, Germany). The PCR program included 5 min pre-incubation at 95°C, followed by 45 cycles of 10 s at 95°C, 5 s at 60°C, and 5 s at 72°C. A melting curve analysis was made at the end of the program by elevating the temperature of the denatured (5 min at 95°C) product from 65 to 97°C.

Expression fold changes of the analyzed genes were calculated with E-method (Roche Applied Sciences) according to the efficiencies of clone-specific primers that were designed based on the potato cDNA sequence data available from TIGR. The qRT-PCR analysis of each sample included three technical replicates for each gene. The fold change for each gene was computed as an average of three qRT-PCR runs (technical replicates).

Results from the two types of gene expression analysis were compared by plotting the fold change values of genes obtained by qRT-PCR as a function of fold change values of probe intensities obtained by the microarray analysis.

RESULTS

Differential expression of genes. Changes in gene expression were assessed in the apical portions of potato sprouts following previous inoculation of the basal portion with the highly virulent isolate of *R. solani* (RS11, AG-3; 38). The basal and apical portions of the sprout were isolated with a soft but tight plastic collar (Fig. 1A) to prevent growth of mycelia from the basal portion to the apical portion that was analyzed. The method of isolation worked well and no hyphae were detected in the apical portion of the sprouts at the time of sampling, as observed under microscope following staining with lactophenol cotton blue (data not shown).

The first sampling of the apical portion of five sprouts was made at 48 hpi when *R. solani* was colonizing the surface of the basal portion of the sprout but had not yet formed detectable infection structures (Fig. 1B). A total of 77 genes appeared to be upregulated and 45 genes showed reduced expression when the pooled RNA of inoculated sprouts (i48h) was compared with mock-inoculated sprouts (m48h) on microarray slides containing 11,412 validated potato cDNA clones (Table 1). The second sampling of the apical portions of additional five sprouts was made 120 hpi when T-shaped branches were observed on the flattened hyphae, and clumps of hyphae were formed on the basal portion of the sprout, which indicated formation of infection structures (35). At this time, expression of 453 and 326 genes was found to be upregulated and down-regulated, respectively, in the apical portion of inoculated sprouts (Table 1). Samples taken 48 and 120 hpi were directly compared on the array to determine how expression differed in inoculated sprouts between these time points (Fig. 2). Of the 77 genes that were upregulated at 48 hpi, 76 genes were still upregulated at 120 hpi. Phenylalanine ammonialyase (PAL) gene was the only one upregulated at 48 hpi that no longer showed significantly higher expression at 120 hpi compared with the controls. These results were nearly identical for two independent experiments, consistent with the carefully standardized growth conditions, treatments, and other procedures.

To verify that changes in gene expression patterns were not influenced by physiological age of the plant during the experiment, samples from mock-inoculated sprouts were compared (0 versus m48h, and m48h versus m120h; Fig. 2). Just a few genes exhibited a slightly altered expression level over the 120 h, but none of these differences were statistically significant (data not shown). Therefore, it was concluded that physiological changes associated with growth and development of the sprout per se did not influence gene expression patterns within the time needed for the experiment.

Genes with statistically significant changes in expression levels (FDR < 0.05) were grouped according to their known or predicted biological function (gene ontology [GO]). Overrepresented classes among differentially expressed genes were detected using the Fisher exact test where a small *P* value of a gene class indicates a potentially meaningful biological role for that GO class. The test indicated nine gene ontology classes with $P < 0.05$ (GO:0009536, GO:0009579, GO:0009607, GO:0015979, GO:0030154, GO:0030246, GO:0016049, GO:0016740, and GO:0019825; codes according to the TIGR database). Additional sources (3,41,62) were used to make the final classification of the genes (Table 1). A total of 27 and 79 genes showing upregulated expression in the inoculated sprouts at 48 and 120 hpi, respectively, were classified to the category of disease defense and

TABLE 1. The number and gene ontology-based categorization of differentially expressed genes in the uninoculated apical portion of potato sprouts following inoculation of the basal portion

	Sampling time[a]			
	48 hpi		120 hpi	
Category	Induced	Suppressed	Induced	Suppressed
Primary and secondary metabolism	19	12	103	94
Energy	1	3	20	11
Disease defense and cell rescue	26	6	79	24
Replication and transcription	7	2	43	35
Signaling	4	2	29	29
Cellular transport and organization	0	0	23	17
Development, growth and cell structure	3	10	17	36
Protein synthesis, modification and destination	1	0	20	11
Classification not clear/function unknown	15	10	119	69
Total no.	76	45	453	326

[a] The apical parts of sprouts were sampled 48 h postinoculation (hpi) and 120 hpi of the basal portion with *Rhizoctonia solani* and gene expression compared with mock-inoculated sprouts at the same time points using microarray analysis. Only genes whose differential expression was consistently observed in two independent experiments are included (false discovery rate <0.05).

cell response (Table 1). With the exception of PAL (mentioned previously), the 27 genes falling into this category and detected at 48 hpi remained significantly upregulated at 120 hpi, along with 53 additional defense-related genes. The detected defense-related genes encoded chitin-hydrolyzing enzymes such as acidic chitinases of classes II, III, and IV, members of the pathogenesis-related (PR) protein groups 1, 2, 3, 4, 8, 9, 10, and 11 (including 1,3-β-glucanase and lignin-catalyzing peroxidases), osmotin-like proteins, defense-associated signaling kinases, host protein protecting substances, and enzymes leading to phytoalexin accumulation (Table 1).

A total of 24 genes whose expression was systemically upregulated in the inoculated sprouts and whose involvement in pathogen defense has been implicated in previous studies were chosen for analysis with qRT-PCR. The samples from both time points of the two experiments were analyzed (Table 2). Data were normalized using the expression levels of the actin gene, which remained constant between the samples, time points, and experiments (threshold 23.24 ± 0.44 cycles; n = 24).

Data from qRT-PCR and microarray analyses were compared by plotting the fold change values obtained in qRT-PCR as a function of the fold change values of signal intensities obtained in the microarray analysis (Fig. 3). The results from these analyses were consistent for all but two tested genes. Differential expression of one gene was detected only by microarray analysis and it was omitted. On the other hand, the expression fold change of a putative class IV chitinase gene (clone STMFB59; Table 2) was considerably higher in the microarray analysis than qRT-PCR. Therefore, the two values for this gene were not considered while fitting a regression line to the data (Fig. 3). The slope of the line was 1.5 indicating that qRT-PCR was more sensitive than microarray analysis in detecting differences of gene expression. The 24 genes for which upregulation was detected with both methods are listed in Table 2.

Systemic induction of resistance. Since sampling for gene expression required removal of the apical portion of the sprout, two experiments, additional to those for microarray and qRT-PCR analyses, were carried out under identical growing conditions to monitor possible induction of systemic resistance to *R. solani* up to 21 days post-challenge-inoculation (dpi). The aim of these additional experiments was to test whether the observed changes in the transcriptome in the apical portion of sprouts were associated with detectable levels of resistance to *R. solani*. The upper part of the sprout above the plastic collar was challenge-inocu-

TABLE 2. Quantitative real-time reverse-transcription polymerase chain reaction analysis of systemic induction of 24 defense-related genes in the uninoculated apical portion of potato sprouts following inoculation of the basal portion in two experiments (I and II)

Clone[a]	GenBank accession number	Target gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	48 hpi I fold change	48 hpi II fold change	120 hpi I fold change	120 hpi II fold change
STMEB78	BQ118564	Glucan endo-1,3-beta-glucosidase	CACATTGCTTCTGGGATGGA	TTTAACATCAGGCCAGAAATCTTTAA	2.67	4.47	5.33	3.81
STMJE14	BQ517508	Acidic endochitinase precursor (EC 3.2.1.14)	TGGCCTATTCGATTACGTTTGG	CCGCGCTACCACCAGAGTAT	3.02	3.22	2.61	2.50
STMJD93	BQ517484	Class II chitinase (EC 3.2.1.14)	GCAGCTAACTCGTTTCCAGCTT	AAAGGCAGCCATTTCCTTCTT	1.11	1.56	1.65	1.13
STMFB59	BQ121945	Class IV chitinase	TCGCTCATGTTACACATGAAACTG	GGGTACTCTGTGTTGGTCTCATCA	1.19	1.36	1.45	1.36
STMFB72	BQ121967	Peroxidase precursor (EC 1.11.1.7)	TGCCCCTGACCCTTCAATAG	CATCCCCGTTTTGTGGACAT	2.57	1.71	6.26	4.38
STMGQ39	BQ507437	Phenylalanine ammonia-lyase (fragment)	GAGTCGTGGACAGGGAATACG	AGCTTCTGCATCAAAGGGTAGGT	2.20	3.16	1.69	1.36
STMEY55	BQ121547	TSI-1 protein (PR-10)	TTGTACCTAAATTGTTGTCACATGATG	GATGCTTCCAGCACCACCAT	2.10	2.20	4.49	1.66
STMEV57	BQ121120	Wound-induced protein 1	CCAGGCGTGAACCCAAGTAA	CGTATTCGGTTCCGTTGTTCTC	2.42	2.96	2.92	1.85
STMFB93	BQ121995	Basic PR-1 protein precursor PR-1	AACCTAGCTGCCGCTTTCC	TCTCATCGACCCACATCTTCAC	3.86	2.93	5.77	3.70
STMFB44	BQ121920	Prb-1b PR-1	AACCTAGCTGCCGCTTTCC	TCTCATCCACCCACATCTTCAC	3.17	3.59	6.96	6.42
STMJF08	BQ517643	Putative xyloglucanase inhibitor	ACCATTGCCAATGAAGAAGTAAATC	GGGTCACCAGTTGGAGGATAATC	2.80	2.27	3.39	2.30
STMIS95	BQ515591	Putative glutathione S-transferase T1	TTGAATTGCACCAAACCAGAGA	TTTGGAGGAGACAACCTTGGA	0.96	1.30	1.19	1.09
STMIM43	BQ514525	Heat shock protein (*Lycopersicum esculentum*)	GCGGCCGCATCACAAG	GTTTGATTCCATCACATTGCTTCT	1.30	2.86	3.10	2.18
STMHR02	BQ511358	Putative WRKY-type DNA binding protein	GGTAACGTATTGAATACGCCATCTAC	CCATCTGCATCTCCATGTCCTT	2.25	2.29	3.15	2.51
STMHE53	BQ509684	MAP kinase phosphatase	GCTTTGCCTTCATCACCTTCA	ACTGCTGGTTGCATAAGAAGAGAA	2.00	3.34	3.57	2.72
STMIV34	BQ516009	Receptor-like protein kinase	AGGTCCAGTACCTGTCATGAGTCA	GGGAACGGCATTGTTTCG	1.98	2.78	2.52	2.49
STMHZ47	BQ512612	STH-2 (*Lycopersicon esculentum*) PR-10	GCTTTGGTTGTTGATGCTGACA	CATCTCCCTCAGTCTCAACATTCTT	3.14	3.20	7.54	5.03
STMIT70	BQ515720	Osmotin-like protein	TTGCCAGACCGGTGATTGT	GCTAGGGTGTTTGGCGATTTAC	2.89	2.81	5.75	3.64
STMCF50	BQ112158	Lipase-like protein	CAAAGGAATGGTTCAGCAAGAAG	AAACCTCAGCCACAACTATGTCATAC	8.81	1.96	4.53	5.12
STMHT35	BQ511717	Proteinase inhibitor II	GTGATGAGCCCAAGGCAAAT	GCCAATCCAGAAGATGGACAA	2.03	1.40	3.90	4.43
STMJB23	BQ517030	Aminotransferase 2	TCCTGCCCTCTTCCTTGTTG	CATTCGTCCATACGGAAATCAA	6.41	5.05	9.52	2.04
STMIM75	BQ514583	Beta-fructofuranosidase (EC 3.2.1.26 EC 3.2.1.26)	CAAGACACAAGTCCAAAATTATGCA	TTGTCGGGCTTGATCCACTTA	2.19	5.62	9.62	3.17
STMHZ50	BQ512617	Caffeoyl-CoA O-methyltransferase	ACAGGAAGCTGGCAAATTTCA	GGATTTCGTATTGGAACTCAACAA	1.88	3.63	2.73	2.77
STMEJ12	BQ119353	asparagine synthetase (*Triphysaria versicolor*)	GTTGCTTCTGTCACTGCTCGAT	AAGTTGTGCTCCCCATTGCT	3.26	3.76	6.87	4.36
	X55746.1	*S. tuberosum* PoAc100 gene for actin	GTACGTCGCTATTCAGGCAGTCTT	CAGAATCCAGCACAATACCTGTTG				

[a] Clone names are according to the TIGR-SGR database. Fold changes of gene expression are means of three technical replicates and presented relative to mock-inoculated sprouts. The potato actin gene was used as an endogenous control. At 48 and 120 h postinoculation (hpi), the apical portion of sprouts sampled at 48 and 120 hpi of the basal part, respectively. I and II, two independent biological experiments.

lated with *R. solani* 120 h after inoculation of the lower part (Fig. 1A and B). The results of both experiments consistently revealed high levels of systemically induced resistance to secondary infection with *R. solani*. It was exhibited as a strong inhibition of hyphal growth and negligible formation of infection structures on 14 of the 16 sprouts tested (87.5%), as shown in Figure 1 (C and D) following staining of the hyphae with lactophenol cotton blue. However, on two sprouts hyphal growth was more abundant. In contrast, when the lower part was not previously inoculated, the upper part of the sprout remained susceptible to infection. In these controls, the upper part of 13 of the 16 sprouts (81.3%) was heavily colonized by the mycelium of *R. solani* and an abundance of hyphal clumps indicated efficient formation of infection structures (Fig. 1E and F). Fungal growth was particularly strong in a zone 3 to 7 mm below the sprout tip and on the emerging laterally growing stolons initiated from this zone (Fig. 1E), which was consistent with the pioneering studies and photographic documentation by Richards (49). He found that *R. solani* initially attacked "the sinus or re-entrant angle of the hook-shaped bud, at which point considerable quantities of mycelium was collected."

DISCUSSION

The results of this study show that potato sprouts developing without access to photosynthetically active light can respond to *R. solani* infection by induction of a systemic defense response. This induction correlates with significant reduction of subsequent infection by the same pathogen. Systemic induction of resistance was associated at the molecular level with induction of a large number of defense-related genes as soon as 48 and 120 hpi. At 48 hpi, the base of the sprout was colonized by *R. solani* but no apparent infection structures were detected. However, systemic induction of defense-related genes at this early stage of the infection process was observed, which showed that potato sprouts are capable of recognizing the invading hyphae of *R. solani* quite sensitively and quickly. To our knowledge, induction of efficient pathogen defense has not been characterized previously in the subterranean parts of the potato plant, including sprouts prior to emergence. This study also provides first indications of the potato genes that respond to infection with virulent strains of *R. solani*, an important pathogen damaging potato crops in all production areas (38,68, and references therein).

The results of this study might help to explain a long-known phenomenon characteristic of the first phase of disease caused by *R. solani* on potato. After initial infection and damage of the first sprouts, new sprouts suffer much less from infection of *R. solani* (49). As noted by Sanford (53), "these secondary sprouts appear to possess a remarkable degree of resistance, notwithstanding, the primary sprouts were very susceptible and severely attacked under apparently identical conditions." Many authors have noted the phenomenon but the mechanism behind it has received less attention (4,31,48,54,70). In the present study, we did not test induced resistance and defense gene expression in the secondary sprouts, and our experimental layout does not therefore fully correspond to the normal infection process on developing potato plants. However, the experiments mimicked seedborne infection and the data showed that induction of defense begins early, prior to development of visible infection structures and damage on sprouts. It may be hypothesized that under normal growth conditions the speed of growth of *R. solani* towards the sprout tip may exceed the speed at which sufficiently strong defense is induced in the sprouts that get infected first, which allows *R. solani* to cause heavy infection and death of the tip in the initially infected sprouts. In the experiments of the present study, we could observe that the initially infected sprout responds with systemic induction of defense because the growth of *R. solani* to the apical portion of the sprout was physically prevented. The possibility therefore remains that the level of defense is elevated in the secondary sprouts that emerge from the basal portion of the initially infected sprout. The secondary sprouts may be primed for defense and/or express higher levels of resistance than the primary sprout from the beginning of their development and hence escape the disease caused by *R. solani* for this reason. This hypothesis needs to be tested in future studies.

It is remarkable that significant resistance to *R. solani* developed in potato sprouts that were not exposed to photosynthetically active light. The different hypotheses explaining how light affects pathogen or herbivore resistance make a common presumption that allocation to defense versus growth and storage is the function of competition between these three end-points for the limited resources (50). Activation of defense involving a large number of genes, as observed in this study, should therefore be constrained for resources and rendered less efficient in lack of light (44). Prior to emergence, the seed tuber is the only source of energy for sprouts, whereas they can obtain additional energy via photosynthesis after emergence. This idea is supported by the fact that development of stem canker ceases at stem bases beneath the soil following plant emergence (32,56,63,70). However, the results of this study indicate that potato sprouts are able to defend against *R. solani* also without light. The sprouts can quickly sense invasion by *R. solani*, mobilize resources from the seed tuber, and hence, activate an efficient defense response before they obtain additional energy via photosynthesis. It seems that potato is well-equipped and adapted to combat hostile invaders in the underground parts of the plant even in absence of light and active photosynthesis.

A total of 779 genes in potato sprouts showed statistically significant, altered expression in the apical, noninfected portion 5 days (120 hpi) after inoculation of the basal portion. These data indicate efficient systemic signaling in the sprouts. The number of differentially expressed genes might have been much larger than detected in this study because the TIGR 10K potato microarray

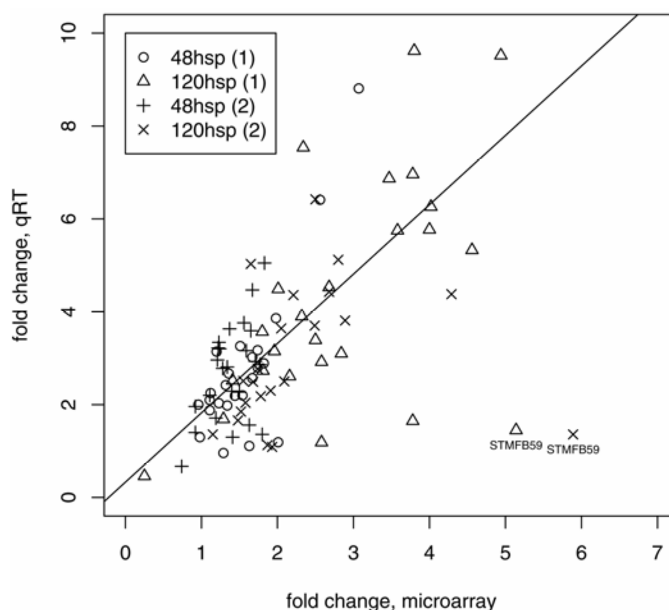

Fig. 3. Comparison of the results of microarray analysis and quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) analysis on systemic induction of 24 defense-related genes in potato sprouts. The fold change values obtained in qRT-PCR were plotted as a function of the fold change values of signal intensities obtained in the microarray analysis for 24 systemically induced defense-related genes analyzed at two time points (48 and 120 h postinoculation) in the two independent biological experiments 1 and 2. Results from the two analyses were consistent except for a class IV chitinase gene (STMFB59) that showed a much higher fold change in microarray analysis than qRT-PCR. It was excluded prior to fitting the illustrated regression line to the data.

contains probes for approximately one-third of the total estimated number of potato genes. The upregulated transcripts mainly belong to the diverse groups of genes that are also activated by other pathogens in potato and pathogen infection in other plant species. Examples include potato infected with *Phytophthora infestans*, *Pectobacter carotovorum* (*Erwinia carotovora*), or viruses (23,42,45,51,62), other species of Solanaceae infected with a wide range of pathogens (24), rice challenged with *R. solani* (68), and canola (74), and wheat (26) infected with many economical important fungal pathogens. Products of these induced genes prevent pathogen invasion and damage of plant tissues by strengthening cell walls, generating reactive oxygen species and other molecules for signaling and with direct antimicrobial effects, and producing phytoalexins and lytic enzymes that can destroy the intruder (24,43).

A large number of the activated defense-related genes observed in this study encode chitin-hydrolyzing enzymes belonging to the PR protein groups 3, 4, 8, and 11 (64) that have no endogenous substrates in higher plants (1). According to the microarray data, six chitinase genes were activated 48 hpi and all continued to show high levels of expression until 120 hpi, which suggested an important role in the response to *R. solani*. Several chitinase isoforms have been identified in plants (20). They are induced during invasion of many fungi and presumed to play an active role in defense against necrotrophic fungal pathogens (64). Chitinases can break down fungal cell walls by catalyzing hydrolysis of chitin, but the specific hydrolytic activity on colloidal chitin may vary within a range of two orders of magnitude depending on the form of chitinase and substrate (12). The ability of purified chitinase to digest cell walls of *R. solani* has been demonstrated in vitro and in vivo (6,7).

Systemic activation of the 1,3-β-glucanase (PR-2) gene was observed in potato sprouts concomitant with induction of the acidic chitinases belonging to classes 2, 3 (acidic endochitinase precursor), and 4 defined by van Loon and van Strien (64). Previously, pronounced elevation of the enzymatic activity levels of chitinase and 1,3-β-glucanase has been detected in potato leaves infected with *P. infestans* (36). Many studies indicate that protection against *R. solani* is enhanced by co-expression of chitinases and the 1,3-β-glucanases providing another important fungal cell wall hydrolyzing enzyme activity (46). Concomitant expression of osmotin-like proteins with chitinases was also observed in potato sprouts in this study. In a previous study on potato suspension cultures, co-expression of these proteins was hypothesized to enhance host defense (61) because overexpression of osmotins may delay symptom development following infection of potato plants with *P. infestans* (39). Co-activation and synergistic action of the different enzymes and defense-related proteins may be needed to speed up destruction of the hyphae of *R. solani*. The newly synthesized chitin in the cell walls of young hyphae is more sensitive to enzymatic degradation (40) and defense is hence best achieved by early actions against the young hyphae. This hypothesis is consistent with the observation that little fungal growth was observed in the apical portion of potato sprout expressing systemically induced resistance.

On the other hand, *R. solani* produces polygalacturonase and various pectin methylesterases to degrade plant cell walls (8,33). The resultant degradation products can act as elicitors and induce expression and systemic accumulation of defense-associated molecules, such as the salicylic acid (SA) dependent basic PR-1 protein, defense associated signaling kinases, lignin catalyzing peroxidases (PR-9), host protein protecting substances, and enzymes for phytoalexin accumulation including PAL. Induction of the respective genes was detected in potato sprouts infected with *R. solani* in this study. Accumulation of transcripts for PAL was high at 48 hpi but no longer significantly different from controls at 120 hpi, which indicates that induction of the phenylpropanoid pathway took place quickly after infection. In bean plants, infection with pathogenic *R. solani* causes strong systemic induction of PAL and another defense-related enzyme, 1,3-β-D-glucanase (67). The glucan endo-1,3-β-D-glucosidase activity that was detected at the mRNA transcript level also in this study catalyses the hydrolysis of 1,3-β-D-glucosidic linkages in 1,3-β-D-glucans of the fungal cell walls. The released α-1,3-glucans induce host defense, including elevated protection against *R. solani* (71,72). Since induction of these PR-proteins is SA-dependent, and SA can be used to induce resistance to *R. solani* in cowpea (19), SA probably plays an important role also in the systemic activation of defense in the potato sprouts infected with *R. solani*. Activation of SA-dependent PR proteins may also occur due to abiotic stress (9), but this could be excluded in the case of this study because no significant activation was observed in the control plants.

Many differentially expressed defense-related genes detected in potato sprouts in this study have been shown by previous authors to provide protection against *R. solani* when over-expressed in transgenic plants. Constitutive expression of chitinases, 1,3-β-glucanases, and ribosome inactivating proteins from the respective transgenes, alone or in different combinations, has improved resistance to *R. solani* in potato and other important crop plants, including rice, canola, tobacco, and cucumber (11,21,40,46,69). The protective effects have been observed as reduced incidence of infection, lower seedling mortality, and alleviated severity of symptoms.

Besides their intrinsic scientific value, the data of this study encourage development of new means for the control of *R. solani* in potato. At present, the control relies on the use of healthy seed potatoes, dressing of black scurf-infested seed lots with chemical fungicides, and practicing crop rotation to avoid build-up of inoculum in soil during continuous potato cultivation in the same field (59). As it is now known that potato sprouts can be induced for resistance to *R. solani* before emergence, activation of resistance might be further exploited using biocontrol agents (10 and references therein). The observed induction of defense-related genes can be achieved following inoculation of plants with nonpathogenic binucleate *Rhizoctonia* or hypovirulent isolates of *R. solani*. The "priming" of defense by the nonpathogenic isolates is one key for their potential in biological control of pathogens (34,73). Soilborne nonpathogenic binucleate *Rhizoctonia* isolates colonize potato plants in the field (38) and can be used for biocontrol purposes in potato (71,72). In tomato, hypovirulent *R. solani* can induce systemic defense and alleviate the symptoms caused by virulent *R. solani* (15).

Responses of plants are initially rather similar in the compatible and incompatible interactions with microbes (24). Defense responses are activated also in roots invaded by arbuscular mycorrhizal (AM) fungi, although the opposite may be experienced in some cases (27). In bean and alfalfa, the responses induced by AM fungi have not been sufficient to protect the roots against infection with *R. solani* (27,28), whereas in potato AM fungi can protect the plants against *R. solani* and increase yields under experimental conditions (75). Whether the mechanism is associated with resistance induction, competition, or other mechanisms has not been reported. In low input production systems AM fungi are pivotal for supplying potato plants with phosphorous (25), which in turn is of high demand for ensuring good processing quality of tubers and high starch yields (13). Therefore, future studies should develop applicable means for the use of living biocontrol agents or other resistance-inducing elicitors for early priming of defense in potato sprouts and protection of them against the first phase of disease caused by *R. solani*.

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