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ORIGINAL PAPER

Rapid transcriptional response of apple to fire blight disease revealed by cDNA suppression subtractive hybridization analysis

John L. Norelli · Robert E. Farrell Jr. · Carole L. Bassett · Angela M. Baldo · Donna A. Lalli · Herb S. Aldwinckle · Michael E. Wisniewski

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Abstract Fire blight, caused by the bacterium *Erwinia amylovora*, is a destructive disease of many tree and shrub species of the Rosaceae. Suppression subtractive cDNA hybridization (SSH) was used to identify genes that are differentially up- and down-regulated in apple (*Malus* x *domestica*) in response to challenge with *E. amylovora*. cDNA libraries were constructed from *E. amylovora*- and mock-challenged apple leaf tissue at various time intervals after challenge treatment, ranging from 0.25 to 72 h post-inoculation (hpi), and utilized in SSH. Gel electrophoresis of PCR-amplified SSH cDNAs indicated a greater quantity and size diversity in the down-regulated EST population at early times after challenge (1 and 2 hpi) compared to early up-regulated sequences and to sequences down-regulated at later (24 and 48 hpi) times after challenge. A total of 468 non-redundant *Malus* ESTs isolated by SSH in response to *E. amylovora* challenge were characterized by bioinformatic analysis. Many of ESTs identified following *E. amylovora* challenge of apple were similar to genes previously reported to respond to bacterial challenge in *Arabidopsis thaliana*. The results indicate that there was a substantial early (1 and 2 hpi) transcriptional response in apple to fire blight disease involving both the down- and up-regulation of host genes. Additionally, genes identified responding to fire blight challenge early (1 and 2 hpi) differed from those identified later (25, 48, and 72 hpi) in the infection process.

Keywords Rosaceae · Plant pathogenic bacteria · Tree Fruit diseases · Gala · Expressed sequence tag · Gene regulation

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J. L. Norelli and R. E. Farrell, Jr. contributed equally to the design and execution of the experiments in this paper.

J. L. Norelli (✉)
Appalachian Fruit Research Station, USDA-ARS,
2217 Wiltshire Rd.,
Kearneysville, WV 25430, USA
e-mail: jay.norelli@ars.usda.gov

R. E. Farrell Jr.
Biology Department, Pennsylvania State University,
York, PA, USA

C. L. Bassett · D. A. Lalli · M. E. Wisniewski
Appalachian Fruit Research Station, USDA-ARS,
Kearneysville, WV, USA

A. M. Baldo
Plant Genetic Resources Unit, USDA-ARS,
Geneva, NY, USA

H. S. Aldwinckle
Department of Plant Pathology, NYSAES, Cornell University,
Geneva, NY, USA

Introduction

Plants respond to infectious pathogens, abiotic stresses, and injury in a genetically programmed manner that involves crosstalk between stress signaling pathways (Fujita et al. 2006; Noselli and Perrimon 2000). Pathogen-derived disease is an abnormal condition resulting from a continuous interaction between host and microorganism. This interaction is critical in determining successful infection by the pathogen or in leading to the ability of the host to resist

infection. Understanding the steps that define the host–pathogen interaction will be an important factor contributing to the development of new strategies to increase disease resistance in economically important crops.

Fire blight, caused by the bacterium, *Erwinia amylovora*, is a destructive disease of many Rosaceae tree and shrub species, including *Malus* x *domestica* (apple), *Pyrus communis* (pear), and *Cydonia oblonga* (quince). The pathogen can infect blossoms, leaves, stems, immature fruits, woody branches, and root crowns (van der Zwet and Beer 1999). It enters the host through natural openings, such as blossom nectaries or wounds; multiplies in intercellular spaces; invades cortical parenchyma; rapidly spreads through the plant via vascular tissues; and can either cause cell death that results in the necrosis of woody tissues or can reside in symptomless tissue (Vanneste and Eden-Green 2000). Fire blight disease was first reported in 1780 on pomaceous fruit trees in New York and has spread throughout North America, through most of Europe, to the Middle East, and to New Zealand (Baker 1971; Bonn and van der Zwet 2000; Griffith et al. 2003). Annual losses to fire blight and costs of control in the USA are estimated at over $100 million.

Pathogenesis by *E. amylovora* is dependent on (1) genes encoding a type III secretion system required for both hypersensitive resistance in non-host plants and pathogenicity (Hrp-TTSS), (2) genes encoding enzymes involved in the synthesis of extracellular polysaccharides, and (3) genes facilitating the growth of *E. amylovora* in its host (Oh and Beer 2005). The Hrp-TTSS of *E. amylovora* plays a key role in translocating virulence effector proteins into plant host cells (Barny 1995; He et al. 2004; Oh and Beer 2005; Wei et al. 1992). In other bacterial plant pathogens, Hrp-TTSS effector proteins are involved in suppressing basal resistance responses against microorganisms, in redirecting normal host metabolism to facilitate pathogen multiplication, in causing cell necrosis and in triggering host resistance when recognized by host disease resistance (R) proteins (Abramovitch et al. 2006; DebRoy et al. 2004; de Torres et al. 2006; Martin et al. 2003; Nomura et al. 2006; Thilmony et al. 2006; Truman et al. 2006). The *E. amylovora* disease-specific protein (Dsp) A/E is an effector required for pathogenicity, being involved in both the suppression of basal resistance and the induction of cell necrosis (Bogdanove et al. 1998; Boureau et al. 2006; DebRoy et al. 2004; Gaudriault et al. 1997). Although several other effector proteins have been identified in *E. amylovora*, i.e., EopB1, AvrRpt2$_{EA}$, and HopC1$_{EA}$, their precise role in pathogenesis has not yet been determined (Nissinen et al. 2007; Oh and Beer 2005; Zhao et al. 2005, 2006).

The interaction of *Pseudomonas syringae* pv. *tomato* DC3000 with *Arabidopsis thaliana* is the model system for studying bacterial plant diseases mediated by a Hrp-TTSS (Collmer et al. 2002). High-density oligonucleotide microarrays have been used to characterize the *A. thaliana* transcriptome following *P. syringae* pv. *tomato* DC3000 challenge (Thilmony et al. 2006; Truman et al. 2006). Transcripts associated with several metabolic pathways, including plastid-based primary carbon metabolism, pigment biosynthesis, aromatic amino acid metabolism, phenylpropanoid biosynthesis, ABA and abiotic stress responses, and salicylic acid and defense responses were modulated in *A. thaliana* within 10–12 h post-*P. syringae* pv. *tomato* DC3000 challenge (Thilmony et al. 2006; Truman et al. 2006). Approximately 2,800 genes are differentially regulated in response to bacterial pathogen inoculation in *A. thaliana* (Thilmony et al. 2006), and the expression of approximately 900 genes is modulated by Hrp-TTSS effector proteins within 12 h post-pathogen challenge (Truman et al. 2006).

Less is known about host responses to infection by *E. amylovora* and the biological mechanisms of resistance and susceptibility. Atypical of other Hrp-TTSS-mediated plant pathogenesis, *E. amylovora* induces lipid peroxidation, electrolyte leakage, and modulation in antioxidant status in its susceptible hosts, an effect that is more characteristic of the oxidative burst generally associated with hypersensitive resistance in non-hosts than susceptibility in a host (Venisse et al. 2001). Three pathogenesis-related protein (PR) genes of apple, *PR-2*, *PR-5*, and *PR-8*, are known to be induced in response to inoculation with *E. amylovora* (Bonasera et al. 2006b). Additionally, infection of apple by *E. amylovora* results in decreased photosynthetic efficiency prior to cell necrosis (Bonasera et al. 2006a; Heyens and Valcke 2006).

A key objective for better understanding fire blight disease is characterizing apple's transcriptional response to infection by *E. amylovora*. Among the various methods available, we chose to use suppression subtractive hybridization (SSH) to characterize the apple transcriptome during *E. amylovora* infection. This technology has been successfully applied to the analysis of several tree species transcriptomes during development and in response to stress (Bassett et al. 2006; Degenhardt et al. 2005; Ranjan et al. 2004; Yakovlev et al. 2006). To gain insight into the temporal dynamics of pathogenesis, RNAs isolated from *E. amylovora*- and mock-challenged leaf tissue were collected at several time points ranging from 0.25 to 72 h post-challenge, converted to cDNAs, and subjected to forward and reverse SSH. We report the identification of 468 apple genes that are up- or down-regulated during fire blight challenge.

Materials and methods

Plant material

One-year-old apple trees of 'Gale Gala' grafted onto 'EMLA 26' rootstock (Adam's County Nursery, Aspers,

PA, USA) were root-pruned to fit into a 15-cm, 2.5-L pot, potted as per Bassett et al. (2006), cut back to six to ten dormant scion buds (ca. 15 cm from graft union), and trained to a single shoot. One group of 60 trees was grown together in a single plant growth chamber at 24±2°C, 16 h day length, ambient RH (ca. 75±15%) and photosynthetic photon flux density of ca. 400 μmol m^{-2} s^{-1}. Immediately prior to treatment, trees were visually evaluated for growth vigor and divided into 18 equal vigor blocks of three replicate trees for each challenge treatment sample time.

Challenge treatments and sampling

Challenge treatments consisted of transversally bisecting the first ten leaves from the shoot apex with scissors dipped in (1) a suspension of 1×10^9 colony forming units of *E. amylovora* strain Ea273 ml^{-1} 50 mM phosphate buffer pH 6.5 (*E. amylovora*-inoculated), prepared as per Norelli et al. (2003), and (2) 50 mM phosphate buffer pH 6.5 (mock-inoculated). Leaf tissue samples were collected at 0.25, 1, 2, 6, 12, 24, 48, 72, and 96 h post-inoculation (hpi) from the first five inoculated leaves from the shoot apex on three replicate trees of both challenge treatments. A 4–6-mm wide strip of tissue was cut parallel to the original inoculation cut, frozen immediately in liquid nitrogen, and stored at −80°C.

RNA isolation

Prior to RNA isolation, samples were pooled from the replicate trees. RNA was isolated from 0.1–0.15 g leaf tissue using the Concert Plant RNA Reagent (Invitrogen #451002), according to the manufacturer's protocols, evaluated for quality by electrophoresis, treated with RNAse-free DNAse, and stored at −80°C. To confirm samples were DNA-free after DNAse digestion, RNA samples were PCR-amplified with DNA primers (Table 1S) designed to amplify an intron with flanking exon sequence of the *Malus* sorbitol-6-phosphate dehydrogenase gene (Genbank: D11080).

cDNA suppression subtractive hybridization library construction and sequencing

SSH libraries were constructed using SuperSMART cDNA Synthesis Kit (BD Bioscience Clontech #K1054-1) and Clontech PCR-Select cDNA Subtraction Kit (BD Bioscience Clontech #K1804-1) as per Bassett et al. (2006). The tester and driver treatments used in both forward and reverse SSH are summarized in Table 1. After two cycles of PCR amplification, SSH cDNAs were non-directionally cloned using a PCR 2.1-TOPO TA Cloning Kit (Invitrogen #K4500), following the manufacturer's protocol. White colonies were isolated from selective media and transferred to 96-well plates. Clones were sequenced by the Nucleic Acid Facility at the US Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center (Wyndmoor, PA, USA) using an M13F primer.

EST sequence editing and analysis

DNA sequence traces were base-called using the PHRED algorithm (CodonCode Aligner v.1.5.2), and sequence with a quality value <200 was deleted (Ewing et al. 1998). The proprietary sequences for the cloning vector and adaptors used in the SSH procedure were added to a downloaded copy of the National Center of Biotechnology Information (NCBI) UniVec database (NCBI VecScreen, The UniVec Database 2007). The resulting dataset was then provided to crossmatch for vector screening (Phred, Phrap, Consed 2007). Match parameters were adjusted until adaptor was successfully identified without blocking non-adaptor sequences (−minmatch 18 −minscore 12). The remaining unmasked sequences were then compared with sequences in the Genbank database using BLAST algorithms by: (1) blastx comparison with the NCBI non-redundant database (NCBI BLAST 2006); (2) blastn comparison with the NCBI EST database; (3) blastn comparison with the mitochondrion genomes of *A. thaliana* (Genbank: NC_001284), *Brassica napus* (NC_008285), *Oryza sativa* (NC_007886), and the chloroplast genomes of *A. thaliana* (NC_000932), *Cucumis sativus* (NC_007144), *Glycine max* (NC_007942), *Lotus japonicus* (NC_002694), *Morus indica* (NC_008359), *O. sativa* (NC_008155) and *Populus alba* (NC_008235); and (4) blastn comparison with the Genome Database for the Rosaceae (GDR) EST *Malus* Unigene Assembly (version 2, December 19, 2005; Genome Database for Rosaceae 2006).

Some of the SSH sequences were then edited by deleting sequences similar to ribosomal RNA when blastn *e* values were less than e^{-10}, by splitting potential sequence chimeras and by restoring sequence gaps resulting from crossmatch screening (see "Results"); ESTs with similarity to organelle coding regions, organelle non-coding spacer regions, or introns were retained. EST sequences within individual SSH libraries were then assembled into contigs using the PHRAP algorithm (CodonCode Aligner v1.5.2), and ESTs completely duplicated within the sequence of other ESTs were deleted. The edited sequences were then deposited in GenBank and BLAST comparisons were repeated for the edited sequences and within-library EST contigs using BLAST2GO (BLAST2GO 2006; Conesa et al. 2005). The best GDR *Malus* unigene matches to the EST with a threshold value of e^{-20}, and the ESTs with no match above the threshold were then assigned to functional categories using BLAST2GO and manual annotation.

Table 1 Suppression subtractive hybridization (SSH) libraries obtained from *E. amylovora*-challenged 'Gale Gala' apple leaf tissue

SSH code[a]	Expected response[b]	Tester[c]	Driver[c]	Number SSH clone sequences[d]	Number unique ESTs[e]	Efficiency of EST recovery (%)	Number new apple ESTs[f]
F	Early ↑	Ea 0.25 hpi+1 hpi+2 hpi+ 6 hpi+12 hpi+24 hpi	Mock 0.25 hpi+1 hpi+2 hpi+6 hpi+12 hpi+24 hpi	125	6	5	0
G	Late ↑	Ea 48 hpi+72 hpi	Mock 48 hpi+72 hpi	79	56	71	4
H	↓ at 2 hpi	Mock 2 hpi	Ea 2 hpi	113	100	92	2
J	↓ at 12 hpi	Mock 12 hpi	Ea 12 hpi	14	9	64	1
K	↓ at 24 hpi	Mock 24 hpi	Ea 24 hpi	75	51	69	0
M	↓ at 48 hpi	Mock 48 hpi	Ea 48 hpi	50	41	82	2
NF	↑ at 1 hpi	Ea 1 hpi	Mock 1 hpi	226	106	42	2
NR	↓ at 1 hpi	Mock hpi	Ea 1 hpi	78	59	76	2
O	↑ at 2 hpi	Ea 2 hpi	Mock 2 hpi	30	21	70	6
P	↑ at 12 hpi	Ea 12 hpi	Mock 12 hpi	23	19	83	3
Q	↑ at 48 hpi	Ea 48 hpi	Mock 48 hpi	99	5[g]	NA	NA
R	↓ at 48 hpi	Mock 48 hpi	Ea 48 hpi	84	4[g]	NA	NA

The SSH 'Tester' is the cDNA in which changes in gene expression are being investigated in comparison to a reference treatment cDNA, termed 'Driver'
NA Not applicable
[a] SSH codes used in Genbank Accession descriptions
[b] ↑ = up- and ↓ = down-regulated genes following *Erwinia amylovora* (fire blight) challenge, *hpi* = hours post-challenge inoculation, *early* = within 24 hpi, *late* = 48 and 72 hpi
[c] *Ea* = amount of time after *E. amylovora* challenge that leaf tissue was collected, *mock* = amount of time after mock challenge with phosphate buffer that leaf tissue was collected, *hpi* = hours post-challenge inoculation
[d] Number of sequences with a PHRED quality value greater or equal to 200 that were not deleted by vector-screening (see 'EST sequence editing and analysis' in "Materials and methods").
[e] Number of SSH clones unique to the treatment (see 'EST sequence editing and analysis' in "Materials and methods").
[f] SSH-EST that appeared unique in comparison to other Malus ESTs at Genbank. In general, an EST was considered unique to *Malus* when blastn comparison to other *Malus* ESTs resulted in an e score ≥20; however, an EST was not considered unique if it had >20 bp end-to-end match to another *Malus* EST with >95% similarity.
[g] Sequences removed from further analysis due to high level of ribosomal RNA contamination within the library

To compare the SSH ESTs with *A. thaliana* genes regulated in response to bacterial pathogen inoculation, a non-redundant list of 2,800 differentially regulated *A. thaliana* genes was obtained from Table S1 of Thilmony et al. (2006); the corresponding gene sequences were bulk-downloaded from The Arabidopsis Information Resource website (The Arabidopsis Information Resource 2006), and tblastx comparisons were run locally using BioEdit (v7.05.3; Hall 1999).

Confirmation of suppression subtractive hybridization results

Portions of the cDNAs synthesized as described above were used as templates in touchdown PCR reactions as per Bassett et al. (2006). The primer pairs for each gene analyzed are provided in Supplementary Table S1. Serial dilutions of each cDNA template were made so that the resulting PCR product mass did not exceed image saturation density, thereby enabling quantification with digital image analysis software (ImageQuant, GE Healthcare). This strategy ensured that the semi-quantitative characteristics of this assay were preserved.

Results

Suppression subtractive hybridization indicated rapid transcriptional response following *E. amylovora* challenge

Symptoms of fire blight (water-soaked tissue along leaf mid-rib at inoculation site) were first observed on some plants 96 h post-challenge inoculation (hpi). A group of three non-sampled *E. amylovora*-challenged plants developed fire blight symptoms as did most of the sampled *E. amylovora*-challenged plants. None of the mock-inoculated plants developed fire blight symptoms. cDNA libraries were constructed from *E. amylovora*- and mock-challenged apple leaf tissue at various time intervals after challenge treatment and utilized in different combinations in SSH to identify up- and down-regulated genes responding to *E. amylovora* challenge (Table 1). Successful SSH was indicated by gel electrophoresis of PCR-amplified cDNAs that showed a reduction in the quantity and size diversity of SSH samples in comparison to unsubtracted controls (Fig. 1). Additionally, SSH procedural controls that consisted of *Malus* cDNA spiked with ϕX174 DNA/*Hae* III-digested size markers, resulted in amplification of the predicted size fragments of the ϕX174 markers ("Controls" in Fig. 1).

Gel electrophoresis of PCR-amplified SSH cDNAs also indicated a greater quantity and size diversity in reverse SSH samples (down-regulated genes) collected at 1 and 2 hpi in comparison to forward SSH samples (up-regulated; 1 hpi in Fig. 1, 2 hpi data not shown) or in comparison to reverse SSH samples (down-regulated) at 12, 24, and 48 hpi (data not shown). Because the cloned PCR-amplified SSH cDNAs (ESTs) were not verified by molecular methods prior to sequencing and sequencing efficiency varied over time, the estimated efficiency of EST recovery among treatments was based on the percent of unique sequences obtained per sequence with a PHRED quality value ≥200 that were not deleted by vector screening (Table 1). The greater diversity of ESTs down-regulated 1 and 2 hpi is also reflected in the greater efficiency of EST recovery observed in reverse versus forward SSH treatments at these times, 76% versus 42% at 1 hpi (NR and NF in Table 1) and 92% versus 70% at 2 hpi (H and O in Table 1). Although the efficiency of EST recovery was lower in up-regulated SSH libraries at 1 hpi (NF) and 2 hpi (O), a considerable number of ESTs for genes up-regulated early after *E. amylovora* challenge were identified (Table 1).

A total of 468 non-redundant *Malus* ESTs were identified by SSH and submitted to the Genbank database (Tables 1 and S2). Twenty-two of the ESTs appeared unique in comparison to other *Malus* ESTs at Genbank

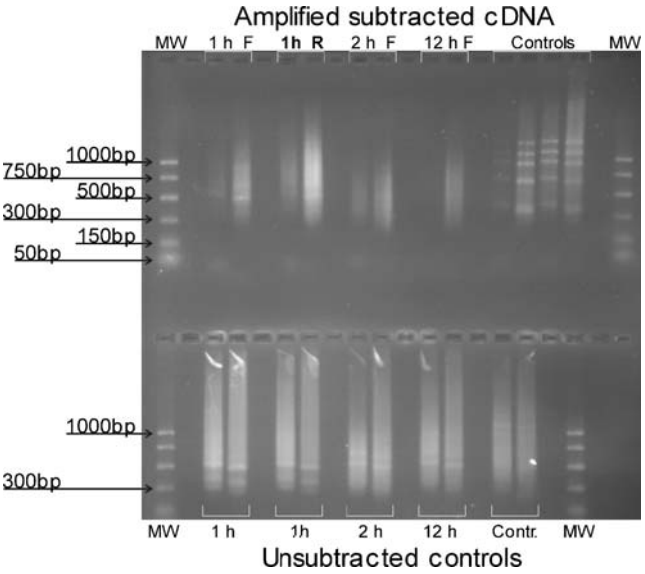

Fig. 1 PCR-amplified suppression subtractive hybridization (SSH) cDNAs and unsubtracted cDNA controls derived from apple RNA isolated from leaf tissue inoculated with *Erwinia amylovora*. PCR products were separated in 2% agarose gels and visualized by EtBr staining. All treatments are in pairs; first lane is the primary PCR amplification, second lane is the secondary PCR amplification. *F* = forward SSH (up-regulated) samples, *R* = reverse SSH (down-regulated) samples collected at various times following challenge with the fire blight pathogen. Controls = first and second lanes are control subtractions of *Malus* cDNA spiked with φX174 DNA/*Hae* III-digested size markers that were subsequently subjected to SSH procedure; third and fourth lanes are manufacturer provided φX174 DNA control subtractions for comparison. Molecular mass markers (*MW*) are indicated in base pairs to the *left* of the gel

(Tables 1 and S2) and approximately 60% of the ESTs did not have significant matches to other *Malus* ESTs at Genbank that were previously recovered from *E. amylovora*-challenged tissue. Because the majority of the SSH clone sequences obtained from libraries Q (up-regulated 48 hpi) and R (down-regulated 48 hpi) were ribosomal contaminants, the few remaining sequences from these libraries were removed from further analysis and were not submitted to the Genbank database. The low efficiency of ESTs recovery from library F (pooled tester and driver, Table 1) was primarily due to multiple clones of a few ESTs and partially due to ribosomal contaminants. The low recovery of ESTs from libraries J, O, and P was the result of poor quality sequence data with PHRED values <200 and/or the removal of sequences during vector screening (Table 1).

Confirmation of the efficacy of SSH was obtained by semi-quantitative RT-PCR analysis of pre-SSH (unsubtracted) cDNAs using primer pairs designed to amplify ESTs randomly selected from both early (1 and 2 hpi) and late (48 and 72 hpi) subtractions that were predicted by SSH to be either up- or down-regulated in response to fire blight challenge (Fig. 2 and, Tables 2 and S1). Eighty-seven percent of 15 ESTs evaluated by semi-quantitative RT-PCR analysis appeared to respond as predicted by SSH (Table 2). Two ESTs, EH009494 (unclassified, hypothetical protein) and EH009593 (oxysterol-binding protein containing a PLAC8 family domain), were both predicted by SSH to be up-regulated 1 hpi and were found to be down-regulated 1 hpi by semi-quantitative RT-PCR (Table 2). Although the observed response of several genes was less than a two-fold change in expression, the differences were quantifiable, consistent and distinguishable from no response. For example, the protein-binding protein gene containing tetra-tricopeptide-like helical and PDZ/DHR/GLGF domains (EH009522) predicted by SSH to be up-regulated 1 hpi was found by RT-PCR to be up-regulated 1 hpi (1.3-fold difference) and to not respond to challenge 48 hpi (Fig. 2). Other ESTs identified at a single hpi by SSH were found to be similarly regulated at other hpi. For example, the protein kinase (ATP binding, protein serine–threonine kinase, EH034623) and an unidentified protein (EH034616) both predicted by SSH to be down-regulated 2 hpi were both found by RT-PCR to be down-regulated 2 and 48 hpi (data not shown). These data, while supporting the highly efficient nature of SSH for the global analysis of gene expression, also demonstrate the importance of this type of post-subtraction confirmation in verifying the expression profile of specific ESTs.

EST sequence editing

During the SSH procedure, cDNA is prepared from experimental RNA samples and partially digested with the restriction endonuclease *Rsa* I to create blunt-end fragments. The tester cDNA, in which changes in gene expression are being investigated, is then ligated separately to two different adapters. Although the molar ratios of cDNAs and adaptor are adjusted to greatly favor ligation of a single cDNA molecule to an adaptor, it is possible for coding region chimeras to occur. To correct for possible EST sequence artifacts resulting from the SSH procedure, EST sequences were screened for potential adaptors or vector contamination using crossmatch (Phred, Phrap, Consed 2007) and subjected to BLAST analysis. Crossmatch screening masked internal blocks of sequence within

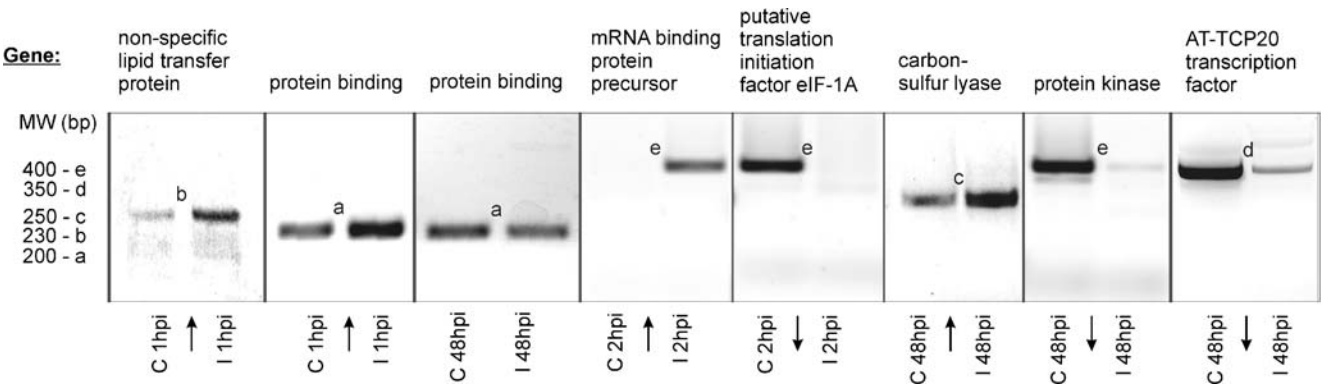

Fig. 2 Semi-quantitative RT-PCR confirmation of SSH method. The results from SSH treatment were confirmed using cDNAs synthesized from RNA isolated from apple leaf tissue samples at various hours post-inoculation (hpi) with *E. amylovora* (*I*) or mock-inoculated (*C*). See Table S1 for specific primer pairs used in PCR. PCR products were separated in 2% agarose gels and visualized by SYBR safe staining. This figure is a composite from several gels; *superscript letters* indicate the approximate fragment sizes in base pairs (*MW*) estimated from molecular mass markers on each gel. *Arrows* indicate if SSH predicted response was up- or down-regulated: non-specific lipid transfer protein precursor = Genbank Accession EH009497/EH009552, protein binding = EH009522, mRNA binding protein precursor = EH090762, putative translation initiation factor eIF-1A = EH034532, carbon–sulfur lyase = EG974770/EG974814, protein kinase = EH034623 and AT-TCP20 transcription factor = EH034602

Table 2 Quantification of changes (fold difference) in the abundance of randomly selected ESTs used in the confirmation analysis of up- and down-regulated genes identified by SSH

EST[a]	Gene identification	SSH library of origin (expected response)	Tissue sampled (hpi)	Fold difference[b]
EG974767/EG974808[c]	Polyphenol oxidase	G (48 hpi and/or 72 hpi up-regulated)	48	1.9
EG974770/EG974814[c]	At1g34060 carbon–sulfur lyase	G (48 hpi and/or 72 hpi up-regulated)	48	2.2
EH009489	Receptor-like protein kinase	NF (1 hpi up-regulated)	1	1.4
EH009494	Unclassified[d]	NF (1 hpi up-regulated)	1	−2.0
EH009497/EH009552[c]	Non-specific lipid-transfer protein precursor (Allergen Mal d 3)	NF (1 hpi up-regulated)	1	3.1
EH009522	Protein binding	NF (1 hpi up-regulated)	1	1.3
EH009583	Oxysterol binding	NF (1 hpi up-regulated)	1	−30
EH009593	Unidentified[e]	NF (1 hpi up-regulated)	1	2.4
EH034508	Photosystem I P700 apoprotein A2	H (2 hpi down-regulated)	2	−1.2
EH034532	Putative translation initiation factor eIF-1A	H (2 hpi down-regulated)	2	−67
EH034586	Cinnamyl alcohol dehydrogenase	H (24 hpi down-regulated)	1	−500
EH034602	AT-TCP20 transcription factor	M (48 hpi down-regulated)	4	−6.4
EH034616	Unidentified[e]	M (48 hpi down-regulated)	48	−2.0
EH034623	Protein kinase	M (48 hpi down-regulated)	48	−7.0
EH090762	mRNA-binding protein precursor	O (2 hpi up-regulated)	2	29

[a] ESTs identified in apple leaf tissue following inoculation with the fire blight pathogen, *E. amylovora*
[b] Ten-fold dilutions of the various cDNA templates were used to determine optimal PCR conditions for quantitation. Bands of PCR products from reactions in the linear phase of the amplification curve were quantified with digital imaging analysis software to compute fold change in expression (Bassett et al. 2006).
[c] Contig formed by more than one EST
[d] Not enough information to assign to a specific functional category
[e] No hits by blastx analysis above an e^{-3} threshold

57 ESTs (potential EST–adaptor–EST chimeras); however, subsequent BLAST analysis suggested that 74% of masked internal blocks of sequence were not vector or artifact, and the original sequence was restored. Potential vector or adaptor sequences deleted at the 5′ and 3′ ends of EST sequence following crossmatch screening were not verified by BLAST analysis. Blast analysis also suggested that two distinct genomic regions were ligated at *Rsa* I restriction endonuclease sites during SSH in 19 ESTs. In total, 7% of the ESTs seemed to contain some sequence artifact resulting from the SSH procedure that was corrected by sequence editing.

Biological processes associated with ESTs

In determining the probable biological processes associated with the ESTs, annotations were done in two steps. First, we identified potential unigenes from a *Malus* EST assembly (Genome Database for Rosaceae 2006) using blastn and then conducted a blastx search against the nr database at NCBI using the putative *Malus* unigenes (contigs or singletons) or if there was no unigene match above a predetermined threshold (e^{-20}), using the original EST sequence. Due to a large number of ESTs (55%) with sequences less than 500 bp in length, the reliability of blastx comparisons was improved by this procedure. In general, most ESTs had unigene matches above the threshold (Table 3). Using the *Malus* unigene most similar to the EST versus the original shorter EST sequence for blastx analysis decreased the number of blastx comparisons below an e^{-3} cut-off threshold by more than 50% and improved the reliability of the sequence comparisons (Tables 3 and S2).

The blastx analyses for both the EST and the *Malus* unigenes were then used to assign the probable biological process associated with the ESTs. Because sequences for the highly abundant proteins ribulose 1,5-bisphosphate carboxylase small subunit (RUBISCO) and metallothionein-like protein type 3 (MT3) were identified in both forward and reverse libraries at the same hpi, these sequences were removed from the analysis and not assigned to functional categories. Many of the ESTs were assigned to the functional categories of ‘general metabolism’ (13% over all hpi), ‘photosynthesis’ (12%), ‘protein metabolism’ (10%) and ‘defense/stress’ (9%; Tables 4 and S2). Although fewer ESTs were assigned to the functional categories of ‘nucleic acid metabolism’ (5%) and ‘signaling’ (9%), several of these ESTs were for genes of interest with

Table 3 Reliability measures of blast comparisons used to annotate the probable biological function of ESTs identified by SSH following *E. amylovora* challenge of 'Gale Gala' apple leaf tissue

Comparison	Type of blast	Percent of no hits	75th percentile	50th percentile	25th percentile
ESTs against *Malus* Unigene Assembly[a]	blastn	7[b]	$2.5e^{-85}$	$1e^{-125}$	0
ESTs against NCBI nr database[c]	blastx	37[d]	$1e^{-22}$	$1e^{-33}$	$1e^{-56}$
Malus unigene most similar to EST against NCBI nr database[e]	blastx	16[d]	$3.25e^{-35}$	$1e^{-65}$	$7.8e^{-108}$

Measures include percent of blast comparisons with expected values (*e*) below a predetermined threshold (percent of no hits), and the 3rd, 2nd, and 1st quartile of *e* values (75th, 50th and 25th percentiles, respectively).
[a] Genome Database for the Rosaceae, EST *Malus* Unigene Assembly, version 2, December 19, 2005 (Genome Database for Rosaceae 2006)
[b] Cut-off threshold for blastn comparisons = e^{-20}
[c] National Center of Biotechnology Information, non-redundant database
[d] Cut-off threshold for blastx comparisons = e^{-3}
[e] Includes both putative *Malus* unigenes (contigs or singletons) and in cases where there was no unigene match above e^{-20}, the original EST sequence

Table 4 Summary of probable biological function of ESTs identified by SSH following *E. amylovora* challenge of 'Gale Gala' apple leaf tissue

Functional category	Percent of ESTs identified within specific SSH libraries								
	↑ 1 hpi	↓ 1 hpi	↑ 2 hpi	↓ 2 hpi	↑ 12 hpi	↓ 12 hpi	↓ 24 hpi	↑ 48 and/or 74 hpi	↓ 48 hpi
General metabolism[a]	16	11	5	13	24	14	15	22	3
Energy[b]	3	5	5	3	0	0	8	10	3
Photosynthesis[c]	17	7	5	15	12	0	15	4	14
Cell growth/development[d]	0	0	5	0	0	0	2	0	0
Nucleic acid metabolism[e]	6	5	0	5	0	0	8	4	5
Protein metabolism[f]	4	18	5	11	18	29	10	10	16
Transport[g]	3	0	5	3	0	0	4	0	0
Cellular trafficking[h]	0	0	0	0	0	14	2	0	8
Signaling[i]	6	7	5	8	0	0	6	6	16
Defense/stress[j]	5	11	10	7	18	14	10	20	0
Unclassified[k]	26	23	20	20	18	0	17	16	11
Unidentified[l]	13	16	35	15	12	29	2	8	24
Total number of ESTs[m]	95	56	20	96	17	7	48	50	37

[a] Includes amino acid, nucleotide, nitrogen/sulfur, phosphate, sugar/polysaccharide, and lipid/sterol metabolism; secondary metabolites; generic hydroxylases, oxidoreductases, esterases, transferases, and 'other' kinases; and solute-binding proteins
[b] Includes respiration, glycolysis, TCA, electron transport, and ATP synthesis
[c] Includes light reaction, dark reaction, chloroplast structure, chloroplast metabolism, and chloroplast protein synthesis
[d] Includes cell division, organ/tissue/cell development, movement, senescence-related (unless documented as stress responsive), and cell adhesion
[e] Includes transcription and RNA metabolism, DNA synthesis, DNA modification, chromatin modification, and proteins with a SET domain
[f] Includes protein synthesis, protein degradation, protein localization, and protein modification; generic protein–protein-interacting polypeptides including ankyrin repeats, tetra- and penta-tricopeptide repeats (TPR, PPR), Kelch repeats, ARM, WWE, WD40 repeats, and F-box domains; and chaperones
[g] Includes ions, sugars, lipids, ABC-type, other small molecules, macromolecules, carrier proteins, proton translocators, transporters/symporters/antiporters, permeases, voltage-gated channels, and exchangers
[h] Includes nuclear, organellar, cell wall/membrane proteins (including: plasma and tonoplast intrinsic membrane proteins), cytoskeleton, ER/golgi, and secreted proteins
[i] Includes hormone metabolism (except where hormone component is associated with stress), receptors, receptor kinases, two component systems, protein kinases, protein phosphatases, and transducers, second messenger metabolism, pleckstrin homology domain-containing proteins, and proteins with 14-3-3 or Ras/Rho/Rac/Rab/Sar/Ran homology
[j] Includes wounding, environmental extremes, and salt/chemical/heavy metal tolerance
[k] Not enough information to assign to a specific functional category. Includes metal-binding or zinc finger proteins with no catalytic/biological function information, proteins containing leucine-rich repeats (LRR) or glycine-rich proteins of unknown function, hypothetical and expressed proteins of unknown function, and proteins of unknown function
[l] No hits by blastx analysis above an e^{-3} threshold
[m] EST contigs were counted as a single EST

similarity to transcription factors (ESTs Genbank Accn. EG974811, EH009550, EH009562, EH034557, EH034565, EH034641, EH034689), similarity to an elongation factor (EH034476), and similarity to a high mobility group B8 protein (EH009496) that is involved in chromatin assembly and disassembly in *A. thaliana* (Stemmer et al. 2003). Fourteen percent of the ESTs had no blastx matches with *e* values below e^{-3} and were designated 'unknown'. The largest group of ESTs (21%) were assigned to an 'unclassified' functional category because there was insufficient information available to assign them to a specific functional category. As expected, most of the new *Malus* ESTs were assigned to the 'unknown' functional category (Table S2). The remaining four new *Malus* ESTs were most similar to a late embryogenesis abundant (LEA)-like protein (EST's Genbank Accn. EH034457; Mogami and Tanaka 2002), a catalytic hydrolase (EH090779), an At-TCP20 transcription factor (EH034602; Li et al. 2005), and a CBS domain-containing protein similar to At4g27460 (EH009521).

Discussion

Currently, there are approximately 261,000 ESTs of apple in the Genbank database with approximately 3,900 of those ESTs identified from *E. amylovora*-challenged tissues. Although these ESTs are a useful resource, most of these fire blight-associated ESTs were identified from whole leaf samples collected 12 to 96 h after challenge (pooled samples) using methods that were non-selective for fire blight-specific ESTs. Because early gene regulation events could be important in determining the outcome of host–pathogen interactions, our goal was to gain insight into the temporal dynamics of gene expression during fire blight development. We used SSH to identify fire blight-specific sequences in a side-by-side comparison of mock- and *E. amylovora*-challenged tissue shortly after challenge treatment of a susceptible host. Genes regulated in response to specific treatments are selected in SSH by sequential nucleic acid hybridizations in which the reference treatment cDNA, designated as the "driver", is present in a molar excess compared to "tester" cDNA, in which changes in gene expression are being investigated. Therefore, optimal SSH results are obtained when non-treatment variability between samples used for isolation of tester and driver cDNAs are controlled by experimental design. Because the apple–*E. amylovora* host–pathogen system facilitated experimental methods that reduced variability between tester and driver treatment plants, SSH methods were well suited to the analysis of gene expression over time. The use of clonally propagated trees in this study virtually eliminated genetic variability between tester and driver samples. To minimize environmental differences between treatment samples, one group of 60 trees were grown together in a single plant growth chamber, and *E. amylovora* and mock inoculation treatments were applied at the same time. The effect of plant growth vigor on fire blight susceptibility was controlled by sorting trees into similar growth vigor blocks immediately prior to challenge treatment. Because *E. amylovora* moves systemically through the plant after inoculation (Keil and van der Zwet 1972; Lewis and Goodman 1965; Momol et al. 1998; Vanneste and Eden-Green 2000), the temporal response of apple to *E. amylovora* challenge can be obscured by using whole leaves or shoots containing cells exposed to the bacteria for varying amounts of time. Temporal synchrony was facilitated in this study by limiting the sample tissue to a 3–6-mm wide strip of leaf tissue cut parallel to the original inoculation cut.

The lack of redundancy in ESTs isolated from libraries of similar hpi and the relatively low level of EST duplication in most libraries (Table 1) suggest that the ESTs identified by SSH in this study represent an incomplete catalogue of all genes regulated in *Malus* in response to *E. amylovora* challenge. Only two sequences were shared between various forward SSH libraries and only 11 sequences were shared between reverse libraries. The observation that ESTs identified at a single hpi by SSH were sometimes found to be similarly regulated at other hpi by RT-PCR also supports the conclusion that not all genes regulated at a specific hpi were identified.

Partial digestion of cDNAs with restriction endonuclease during SSH often results in EST sequences that do not represent the entire gene transcript. Using the *Malus* unigene most similar to the shorter EST for blastx comparisons was useful in improving the reliability of BLAST analysis and expanding the amount of biological information derived from the SSH ESTs. In general, using the *Malus* unigene most similar to the EST for blastx comparisons was most informative when the EST contained primarily 3′-untranslated region sequence. When cDNA sequence was available, blastn comparisons to the NCBI nr database usually produced equivalent results to blastx comparisons using the *Malus* unigene most similar to the EST. However, for species which lack extensive cDNA and genomic sequence data, such as apple, the utility of blastn comparisons is limited. Despite the utility of using the *Malus* unigene most similar to the EST for blastx comparisons, caution is needed in interpreting these BLAST results.

The mechanics of SSH also favors the normalization of up- and down-regulated sequences, regardless of the relative abundance of the original mRNA in the cell. Although this favors the identification of rare transcripts, the method cannot be used to make quantitative conclu-

sions regarding the relative abundance of specific transcripts. Therefore, quantitative conclusions should not be drawn from Table 4 regarding the number of ESTs within different functional categories at various hpi; Table 4 is meant only to provide a summary of the types of genes represented among the ESTs. However, some qualitative changes in EST recovery were evident over time. For example, within the 'defense/stress' functional category (Table 4), ESTs representing several different PR proteins were first detected 48 hpi (up-regulated), whereas earlier defense/stress ESTs were primarily associated with oxidative and osmotic stress. These included the initial up-regulation 1 hpi of peroxidase, catalase, and superoxide dismutase, followed by the down-regulation of peroxidase at 2 and 24 hpi; the initial down-regulation 1 and 2 hpi of an aquaporin and two dehydration-responsive proteins, RD11 and ERD15; and the up-regulation 2 hpi of a type II SK2 dehydrin which is also up-regulated in cold-treated *Prunus persica* (peach) bark (Bassett et al. 2006) and in water-limited apple (C. Bassett, personal communication). In a study exploring the effects of HrpN treatment on *A. thaliana*, Dong et al. (2005) observed that the *E. amylovora* HrpN effector activates an ABI2-dependent pathway that induces drought tolerance. Analysis of HrpN or ABA treatment effects on resistance to *P. syringae* pv. *tomato* or induction of PR-1 in an *abi2* mutant suggested that the ABI2 pathway does not mediate induced resistance. By extrapolation, the results also suggest that the effects of HrpN on water status and SAR are mediated by different signaling pathways.

Down-regulation of ERD15 (EARLY RESPONSIVE TO DEHYDRATION) is predicted to increase sensitivity to ABA and enhance the effects of HrpN, since Kariola et al. (2006) have shown that RNAi silencing of ERD15 results in *A. thaliana* plants that are more drought-/cold-tolerant and hypersensitive to exogenous ABA application. Furthermore in the same study, modulation of ERD15 by epigenetic overexpression resulted in plants presumably having lowered sensitivity to ABA, but with better resistance to *E. carotovora carotovora* and enhanced induction of genes associated with SAR. These observations support the hypothesis that pathogen effects on drought tolerance and pathogen resistance reflect different signaling pathways. Along these same lines, the early down-regulation of ERD15 and the up-regulation of a cold-/drought-responsive dehydrin that we observe in response to *E. amylovora* infection is consistent with a pathogen-mediated increase in ABA sensitivity; although up-regulation of the abiotic stress dehydrin may not relate directly to *E. amylovora* infection, down-regulation of ERD15 may be critical to the infection process in apple. In contrast, both the Kariola et al. (2006) study and a study by Timmusk and Wagner (1999) reported an increase in ERD15 transcript abundance in *A. thaliana* plants treated with either *E. carotovora carotovora* or the plant-growth-promoting *Rhizobacterium paenibacillus polymyxa*. In the former study, ERD15 increased in abundance in control plants up to 24 hpi, but was reduced to basal levels by 48 hpi (Kariola et al. 2006). The differences in ERD15 expression in our study versus those of Kariola et al. (2006) and Timmusk and Wagner (1999) could result from differences in the host–pathogen experimental systems investigated, differences in infection mechanisms, or differences in timing of ERD15 modulation, all of which could reflect diversity in the host–pathogen interactions.

Aquaporins constitute a large family of major intrinsic proteins, and different functions are associated with specific members (Hachez et al. 2006; Kaldenhoff and Fischer 2006). Although aquaporins show a rather complicated pattern of expression in response to different stresses, Alexandersson et al. (2005) demonstrated that several members of the PIP class were down-regulated in *A. thaliana* in response to drought. A study monitoring gene expression profiles in cotton infected with *Fusarium oxysporum* f. sp. *vasinfectum* found that PIP2-2 was down-regulated, as were several other classes of aquaporins (Dowd et al. 2004). The apple aquaporin down-regulated at 1 h post-infection appears to be a member of the plasma membrane intrinsic (PIP) class, specifically a PIP1 homologue. Interestingly, an aquaporin identified as being down-regulated early in response to *E. amylovora* in a resistant cultivar is a PIP2-type aquaporin (unpublished observation).

The ESTs identified in this work were generally consistent with previous studies on the transcriptional response of apple to fire blight disease. In agreement with the work of Venisse et al. (2002), ESTs for chitinase, β-1,3-glucanase and phenylalanine ammonia lyase were up-regulated in response to *E. amylovora* challenge, and in agreement with the work of Bonasera et al. (2006b), an up-regulated EST for *PR-8* was identified 48/72 hpi. In disagreement with Bonasera et al. (2006b), an EST for *PR-1a* was detected 48/72 hpi; however, expression of this EST has not yet been confirmed. Consistent with the work of Malnoy et al. (2006) demonstrating a glutathione *S*-transferase (GST) promoter of potato is induced in apple following *E. amylovora* challenge, up-regulated ESTs for an inducible GST were identified 2 and 48/72 hpi. Surprisingly, there was limited overlap between the SSH-ESTs identified in response to fire blight challenge in this work and those identified by Degenhardt et al. (2005) as differentially expressed in apple scab-resistant and -susceptible *Malus* cultivars in response to challenge by *Venturia inaequalis* (apple scab). In addition to the metallothionein-like protein type 3, which was eliminated from analysis in this work because they were isolated from both forward and reserve subtractions, the only shared ESTs were for *Mal*

d1 1.03F (annotated as Ribonuclease-like PR10b by Dagenhardt et al.) and cytochrome b6, both of which were identified from the scab-resistant cultivar Remo. Blastn analysis identified 11 other fire blight SSH-ESTs with some similarity (Expected value ≤3) to the Dagenhardt SSH-ESTs; however, they appeared to represent distinct family members.

The ESTs identified following *E. amylovora* challenge of apple had clear similarity to genes known to respond to bacterial challenge in the *A. thaliana–P. syringae* pv. *tomato* DC3000 host–pathogen system. A tblastx comparison of the ESTs identified in this work to the 2,800 *A. thaliana* genes that are regulated in responce to bacterial challenge, identified similar matches for 50% of the ESTs at an e^{-3} threshold and similar matches to 33% of the ESTs at an e^{-20} threshold (Table S3). Two unique *Malus* ESTs annotated as unknown proteins had tblastx matches to the 2,800 *A. thaliana–P. syringae* pv. *tomato* genes; EG974781 matching (e^{-31}) AT4G22530.1, an *S*-adenosylmethionine-dependent methyltransferase (SAM), and EH0095210 matching ($4e^{-19}$) AT5G53750.1, an unknown protein containing a cystathionine beta-synthase (CBS)-conserved domain. SAM transferases contain a highly conserved structural fold; however, the substrate-binding region of the fold varies enormously making it impossible to predict function from primary sequence alone (Martin and McMillan 2002). In humans, SAM enzymes may play a role in cell apoptosis by influencing decisions between different phenotypic forms of cell death (Schwerk and Schulze-Osthoff 2006). It has been proposed that CBS domains may play a regulatory role; however, their exact function is unknown (Marchler-Bauer et al. 2005; NCBI Structure Database 2006).

Among the apple ESTs assigned to the transport functional category, two had significant similarity to monosaccharide transporter (-like) genes that respond to bacterial challenge in *A. thaliana* (Arabidopsis Gene Family Information 2007; Thilmony et al. 2006). This included apple EST EH090759 that aligned to the 3′-UTR of *M.* x *domestica* sorbitol transporter 5 (*MdSOT5*, AB125648) and had significant similarity ($2e^{-107}$) to the *A. thaliana* polyol transporter 6 gene (*AtPLT6*, At4g36670) that responds to bacterial challenge (Arabidopsis sugar transporter homepage 2007). *AtPLT6* is regulated in response to pathogen-associated molecular patterns (PAMPs), suggesting it is associated with the basal resistance response to non-pathogens (Thilmony et al. 2006). The detection of *MdSOT5* by SSH in apple 2 hpi with *E. amylovora* is consistent with the expected initial basal resistance response following pathogen challenge (Chisholm et al. 2006). Because most plant-associated bacteria are not intracellular parasites, they are dependent upon the availability of nutrients and water in the plant apoplast for their growth. In the Rosaceae, sorbitol is a major photosynthetic product and the major phloem-translocated photoassimilate (Klages et al. 2001). *MdSOT5* is normally expressed in dormant buds, sink leaves, source leaves, and flowers and has been implicated in sorbitol unloading from the apoplast (Watari et al. 2004). Enhanced expression of *MdSOT5* following bacterial challenge might be responsible for sorbitol accumulation in cells resulting in reduced nutrient and water availability in the apoplast. In *Prunus*, a sorbitol transporter gene is linked to a disease resistance locus for powdery mildew (Derick et al. 2007).

Similar to the rapid transcriptional response of apple to *E. amylovora* challenge observed in this work, *A. thaliana* also responds to bacterial challenge within 1 hpi (de Torres et al. 2003). The initial transcriptional response observed in *A. thaliana* during the first 2 hpi is the result of basal resistance (aka innate immunity) reflecting host responses common to both pathogens and non-pathogenic bacteria (de Torres et al. 2003; Thilmony et al. 2006; Truman et al. 2006). Because the practical limitations of SSH did not allow inclusion of challenge treatments with both a wild-type *E. amylovora* strain and either an Hrp-TTSS mutant or a non-pathogenic species, it was not possible to conclude if the initial transcriptional response observed in this work was due to basal resistance responses or pathogenesis; future studies will address the nature of apple's initial response to bacterial challenge. In *A. thaliana*, approximately 325 genes are involved in the initial basal resistance response with approximately three times as many genes induced as repressed; major R-gene resistance response occurs approximately 4 hpi and involves a total of 1,100 genes that are equally induced and repressed; the largest response to the wild-type pathogen *P. syringae* pv. *tomato* DC3000 occurs 12 hpi and involves approximately 2,700 genes, 57% being repressed (de Torres et al. 2003; Truman et al. 2006). Although SSH is not quantitative, the greater quantity and size diversity of PCR-amplified SSH cDNAs observed by gel electrophoresis with down-regulated samples 1 and 2 hpi compared to up-regulated samples (Fig. 1) suggest that the largest response of apple to infection by *E. amylovora* was an initial down-regulation of many genes within the first 2 hpi. This possible difference between the *A. thaliana–P. syringae* pv. *tomato* and *Malus–E. amylovora* host–pathogen systems could be due to the atypical oxidative burst observed during the development of fire blight (Venisse et al. 2001).

In summary, the similarities between the ESTs identified in this work and genes expressed in the *A. thaliana–P. syringae* pv. *tomato* DC3000 host–pathogen system, genes expressed in response to abiotic stress and genes previously associated with fire blight infection suggests that SSH was effective in identifying genes in apple that respond during

fire blight infection. Although gene expression assays will be necessary to confirm the response of specific ESTs to fire blight challenge, 87% of the ESTs evaluated by semi-quantitative RT-PCR responded to challenge as predicted by SSH. The recovery of many ESTs within 2 hpi indicates that apple quickly responds to *E. amylovora* challenge and suggests that early transcriptional events could play an important role in determining whether apple succumbs to or resists pathogen infection. The ESTs identified following *E. amylovora* challenge in this work should provide a useful genomic resource for biologists, horticulturalists, and plant breeders developing new strategies for improving plant resistance to fire blight disease.

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