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Gene Expression Profiling of *Arabidopsis thaliana* in Compatible Plant-Aphid Interactions

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Phloem feeding involves unique biological interactions between the herbivore and its host plant. The economic importance of aphids, whiteflies, and other phloem-feeding insects as pests has prompted research to isolate sources of resistance to piercing-sucking insects in crops. However, little information exists about the molecular nature of plant sensitivity to phloem feeding. Recent discoveries involving elicitation by plant pathogens and chewing insects and limited studies on phloem feeders suggest that aphids are capable of inducing responses in plants broadly similar to those associated with pathogen infection and wounding. Our past work showed that compatible aphid feeding on leaves of *Arabidopsis thaliana* induces localized changes in levels of transcripts of genes that are also associated with infection, mechanical damage, chewing herbivory, or resource allocation shifts. We used microarray and macroarray gene expression analyses of infested plants to better define the response profile of *A. thaliana* to *M. persicae* feeding. The results suggest that genes involved in oxidative stress, calcium-dependent signaling, pathogenesis-related responses, and signaling are key components of this profile in plants infested for 72 or 96 h. The use of plant resistance to aphids in crops will benefit from a better understanding of induced responses. The establishment of links between insect elicitation, plant signaling associated with phloem feeding, and proximal resistance mechanisms is critical to further research progress in this area. Arch. Insect Biochem. Physiol. 51:182–203, 2002.

KEYWORDS: plant-insect; aphid; phloem; DNA microarray; gene expression profiling

INTRODUCTION

Studies of plant-insect interactions have typically incorporated ecology, evolution, behavior, physiology, and biochemistry. These disciplines have been the foundation for the development of phytocentric, or plant-based (Mattson, 1980; Coley et al., 1985; Krischik, 1991; Karban and Baldwin, 1997), hypotheses to explain the diverse and complex outcomes of herbivory. Induced responses and resistance in plants may result from insect feeding or physical or chemical simulation of attack by an herbivore (Karban and Baldwin, 1997). In studies of plant-pathogen interactions, elicitation and in-

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Abbreviations used: BGL2, β-1,3-glucanase gene; BTH, benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester; EST, expressed sequence tag DNA clone; JA, jasmonic acid; LOX2, lipoxygenase gene; PDF1.2, defensin gene; PAL1, phenylalanine-ammonia lyase gene; PR, pathogenesis related; SA, salicylic acid; SAR, systemic acquired resistance; STP4, sugar transport protein gene.

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duction have been central concepts in defining defensive barriers and resistance (Chester, 1933; Ross, 1961; Ryals et al., 1992; Glazebrook, 2001). Clear ecological and physiological links exist between herbivorous insects and plant pathogens, in the context of both primary plant metabolism (nutrition and resource allocation) and defense (Krischik, 1991; de Nooij et al., 1992). The feeding, growth, and reproduction of insects that feed on the phloem sap of plants, such as aphids (Homoptera: Aphididae) and whiteflies (Homoptera: Aleyrodidae), are often affected by plant pathogen infection (Barbosa, 1991; Purcell and Nault, 1991). Changes in the nutritive and physical attributes of tissues commonly accompany these infections. Phloem feeding can in turn influence the distributions of both plant pathogens (via vectoring) and other insects, including conspecifics (Jiang and Miles, 1993; McElhany et al., 1995; Prado and Tjallingii, 1997; Nault, 1997; Stout and Bostock, 1999; Gianoli, 2000; Sauge et al., 2002). Phloem feeding has received relatively little attention from the point of view of plant defensive induction as compared to wounding, leaf-chewing, and mining herbivory or pathogen infection (Walling, 2000).

This review highlights our studies on the effects of feeding by aphids on *Arabidopsis thaliana*, a host plant in the Brassicaceae (mustard) family and a model in plant molecular biology. Over the past decade, the use of molecular biological tools has helped to define the roles of numerous regulatory and defense genes in plant-pathogen interactions (Glazebrook, 2001, Jones, 2001). Recent studies have identified similar and distinctive patterns of gene expression following chewing herbivory by insects (Arimura et al., 2000; Reymond et al., 2000; Stotz et al., 2000; Kessler and Baldwin, 2002). Gene induction patterns produced by phloem feeding on *A. thaliana* can be compared to plant response pathways and resistance mechanisms uncovered in other plant-aphid systems, and in more general plant-pathogen and plant-insect interactions.

APHIDS AS ELICITORS

Phloem feeding insects comprise a diverse and economically destructive (Dixon, 1998) group of herbivores. Aphids (Aphididae), whiteflies (Aleyrodidae), and other phloem feeders employ unique morphological adaptations, physiological food perception, digestion and excretion systems, and feeding behaviors. These traits allow them to use an equally unique plant resource, the sometimes nutritionally limiting (Sandström and Moran, 1999) phloem sap. Reviews of the insect morphology and physiology of phloem feeding (Pollard, 1973; Tjallingii, 1995; Miles, 1999) suggest a crucial role for saliva in eliciting changes in plant gene expression. When an aphid feeds, stylet sheaths are formed from a gelling saliva secreted by the insect and are left behind after penetration and feeding. Aphid stylet pathways are largely intercellular, but in some cases they follow an "intramural" pathway involving cell wall disturbance and damage to the plasma membranes of mesophyll and parenchyma cells (Pollard, 1973; Tjallingii and Hogen Esch, 1993). The gelling saliva contains free amino acids as well as multiple oxidative and 1,4-glucosidase enzyme activities that can polymerize both insect- and plant-derived proteins and phenolics (Miles, 1990, 1999). Salivary sheaths may suppress wound-triggered phenolic accumulation by the host plant by sequestering oxidized forms into the sheath (Miles and Oertli, 1993). A non-gelling watery saliva is secreted by aphids during cellular punctures along the stylet pathway, and while feeding on phloem sieve elements. The composition of watery saliva secretions into artificial diets has been studied with chromatographic separations and enzyme activity assays (Madhusudhan and Miles, 1998). Reviews (Campbell and Dreyer 1990; Miles, 1990, 1999) have suggested the presence of pectinase, cellulase, polyphenoloxidase, peroxidase, and lipase activities. The enzymes may perform critical roles in feeding, including lubrication of the stylets, maintenance of favorable oxidative-reduction (redox) conditions and detoxification of phenolics (Miles and Oertli, 1993), and prevention of sieve element blockage by callose or polymerized P-proteins (Evert, 1990; Miles, 1999). Salivary secretions can be translocated in the phloem (Madhusudhan and Miles, 1998), but are mostly reingested during uptake of phloem assimilates by aphids.

In contrast to chewing arthropods such as beet armyworm (Turlings et al., 2000), *Manduca* spp. larvae (Halitschke et al., 2001), European corn borer (*Helicoverpa zea*) (Felton and Eichenseer, 1999), and *Pieris brassicae* (Mattiaci et al., 1995), elicitors have not been isolated from aphid saliva or regurgitant. However, the activities of aphid salivary enzymes and the presence of aromatic compounds in the saliva suggest roles for several types of elicitors. Oxidative conditions around the stylet sheath and in the phloem could lead to the formation of reactive oxygen species (Miles, 1999; Walling, 2000). These molecules participate in induction of defenses following plant pathogen infection (Bollwell and Wojtaszek, 1997; Nurnberger and Scheel, 2001) and wounding (Bi and Felton, 1995; Stout and Bostock, 1999). Limited evidence suggests that oligosaccharides may be released from plant cell walls and intercellular spaces during aphid penetration (Campbell and Dreyer, 1990) and feeding (Madhusudhan and Miles, 1998). Oligosaccharides have elicitation roles in pathogen infection (Hahn, 1996), wounding (Howe et al., 1996), and chewing herbivory (Stout and Bostock, 1999). In the case of aphids, they may act as nonsense signals to suppress host responses (Miles, 1999). Minute changes in turgor pressure and electrical potential in plant tissues can stimulate defense signaling in plants (Yahraus et al., 1995; Rhodes et al., 1996). Aphid behavioral studies detect electrical signals through the use of the EPG technique (Tjallingii and Hogen Esch, 1993). Endosymbiotic microbes play roles in the biosynthesis and action of elicitors related to chewing herbivory (Spiteller et al., 2000). The intimate associations between aphids and symbiotic bacteria (Douglas, 1998) may include a similar cooperative role.

PLANT DEFENSE RESPONSES AND APHID FEEDING

Based largely on information about elicitation mechanisms, recent reviews have hypothesized that aphids induce profiles of genes that bear strong similarities to pathogen-inducible gene profiles (Felton and Eichenseer, 1999; Stout and Bostock, 1999; Walling, 2000). The limited number of studies that have examined the responses of defense-related metabolites to infestation (Table 1) support this assertion. Phenolics, their amino acid precursors, biosynthetic enzymes associated with aromatic compounds, and oxidative enzymes are important facets of the plant response profile in cereal crops (Table 1). Aphid feeding sometimes results in localized or systemic necrosis in leaf tissues of these plants (Miles, 1990, Ryan et al., 1990). Some responses are idiosyncratic to specific plants and aphids. For example, in one set of wheat genotypes, peroxidase activity increased only in resistant genotypes of wheat and barley infested with *D. noxia*, and *R. padi* feeding had no effect on activity in any genotype (Forslund et al., 2000; Ni et al., 2001). *R. padi* increases peroxidase activity in other resistant wheat lines (Leszczynski, 1985). Increases in lipid peroxidation and glutathione reduction-associated enzymes (Table 1) could represent stimulation by wounding (Bi and Felton, 1995) or elicitors mimicking pathogen infection (Bollwell and Wojtaszek, 1997). Oxidative and reductive responses are induced by other phloem-feeding insects, including three-cornered alfalfa hopper (*Spissistilus festinus* (Say)) on alfalfa (Felton et al., 1994) and multiple whitefly species on squash and tomato (Walling, 2000).

Induction of pathogenesis-related (PR) genes and proteins, including those with unknown functions (e.g., *PR-1* in *Arabidopsis thaliana*, *P4* in tomato) and chitinase and glucanase enzymes, are locally and possibly systemically associated with aphid feeding in diverse plants (Table 1). PR genes and proteins are common plant responses to pathogen infection (Van Loon and Van Strien, 1999), although resistance can occur without *PR* gene induction (Glazebrook, 2001). In cereal crops, induction by aphids involves mostly apoplastic proteins with both basic and acidic pI values, and is often stronger and more rapid in resistant plant genotypes (Botha et al., 1998; van der Westhuizen et al., 1998a, b; Forslund et al., 2000; but see Krishnaveni et al., 1999). Work in barley (Forslund et al., 2000), tomato (Fidantsef et al., 1999), and *A. thaliana* (Moran and Thompson, 2001) has dem-

TABLE 1. Plant Responses Induced by Aphid Feeding That Have Established Defensive/Signaling Roles

Aphid	Plant	Enxyme activities, protein accumulation, or other defense[a]	Local or systemic[b]	Correlated with resistance[c]	Reference
Russian wheat aphid (*Diuraphis noxia* (Mordvilko))	Sorghum	Collapsed autofluorescent material in cells (phenolics)	Local	Yes	Belefant-Miller et al. (1994)
	Wheat	Peroxidase	Systemic	Yes	Van der Westhuizen et al. (1998b); Ni et al. (2001)
	Barley	Peroxidase	ND	Yes[d]	Ni et al. (2001)
Birdcherry-oat aphid (*Rhopalosiphum padi* L.)	Wheat	Peroxidase, polyphenol oxidase, phenolic biosynthesis enzymes, phenolics	ND	Yes[d]	Leszczynski (1985); Havíicková et al. (1998); Gianoli and Niemeyer (1998)
Spotted alfalfa aphid (*Therioaphis maculata*)	Alfalfa, lucerne	Lipid peroxidation, soluble phenolics, peroxidase	ND	ND	Dillwith et al. (1991); Jiang and Miles (1993)
Grain aphid (*Sitobion avenae* L.)	Wheat, barley	Glutathione reductase, NADPH-supplying enzymes	ND	ND	Argandoña (1994)
Potato aphid, (*Macrosiphum euphorbiae* (Thomas))	Tomato	*P4* (pathogenesis-related protein), peroxidase	ND	ND	Stout and Bostock (1999); Fidantsef et al. (1999)
Greenbug (*Schizaphis graminum* (Rondani))	Barley, sorghum	Peroxidase, chitinase, glucanase	ND	No	Krishnaveni et al. (1999); Chaman et al. (2001)
Russian wheat aphid	Wheat	Chitinase, glucanase	Systemic	Yes	Botha et al. (1998); van der Westhuizen et al. (1998a)
Birdcherry-oat aphid	Barley	Chitinase, glucanase	Local	Yes	Forslund et al. (2000)
Green peach aphid (*Myzus persicae* (Sulzer))	Tomato[e]	*P4* (pathogenesis-related protein) lipoxygenase	ND	No	Fidantsef et al. (1999)
	Arabidopsis thaliana[e]	*PR-1, BGL2, PDF1.2, LOX2, STP4, PALI*	Local	No	Moran and Thompson (2001)
Spotted alfalfa aphid	Alfalfa	Phytoalexin (coumesterol)	Local	Yes[f]	Loper (1968)
Cotton-melon aphid (*Aphis gossypii* Glover); Pecan aphids; Spirea aphid (*Aphis spiraecola* Patch)	Melon, pecan, apple	Callose deposition in phloem sieve elements	Local	No	Wood et al. (1985); Kaakeh et al. (1992); Shinoda (1993)

[a]Activities or concentrations are higher in infested compared to control plants.
[b]Induction is restricted to a leaf or other distinct structure on which aphids are feeding (local) or occurs in leaves or other structures on which aphids are not feeding (systemic). ND, not determined (only locally infested examined).
[c]mRNA, protein, or metabolite concentrations or enzyme activities are induced more rapidly and/or to a higher level in aphid-resistant than in aphid-susceptible plant genotypes. ND, not determined (only susceptible plants examined).
[d]In some cases, peroxidase, polyphenol oxidase activities, and phenolics are elevated to a greater degree in susceptible plant genotype.
[e]Responses measured as levels of mRNA transcripts.
[f]Greater induction in susceptible plant genotypes.

onstrated that PR protein responses and some oxidative factors are induced in aphid-plant interactions that do not involve necrosis or other symptom development. Along similar lines, infestation by both silverleaf whitefly (*Bemesia argentifolii* Bellows and Perring) and asymptomatic greenhouse whitefly (*Trialeuroides vaporariorum* [Westwood]) nymphs both induce PR proteins in tomato and squash (Mayer et al., 1996; Walling, 2000), with stronger induction of basic forms.

Omitted from Table 1 are cases of induction of "indirect defenses" in the form of volatile compounds that attract aphid parasitoids (Du et al., 1998; Guerrieri et al., 1999; Bradbourne and Mithen, 2000). The actual defensive roles of induced responses related to oxidation, phenolics, PR proteins, phytoalexins, and callose against phloem feeding are uncertain (Walling, 2000). For example, *R. padi* feeding on wheat reduces the reproduction of *S. avenae* and influences tissue choices of *R. padi*, but these effects are not clearly associated with hydroxamic acid induction (Gianoli and Niemeyer, 1998). Some responses could be coupled to changes in photosynthesis and protein and amino acid composition (Dillwith et al., 1991; Haile et al., 1999; Telang et al., 1999) that facilitate aphid feeding. Integration of studies on plant performance, responses, and insect behavior, coupled with a molecular approach will better define the defensive capacities of plants against phloem feeding.

PLANT-APHID INTERACTIONS IN THE BRASSICACEAE

Crop plants in the Brassicaceae, such as *Brassica napus* (canola or rapeseed), *B. oleracea* (cabbage, Brussels sprouts, and others) and *B. rapa* (turnips, mustards), and their wild relatives have been intensively studied from insect behavior and plant performance perspectives for resistance to aphids (Ellis et al., 2000). The model insects have been *Myzus persicae* Sulzer (green peach aphid), a generalist with hosts in over 40 plant families (Pollard, 1973) and *Brevicoryne brassicae* L. (cabbage aphid), which feeds only on brassicaceous hosts. The two aphids show different feeding patterns on the same host (Cole, 1997a). Behavioral studies suggest constitutive resistance factors in both phloem and non-phloem tissues (Cole, 1994) and differential sensitivity to glucosinolates (Cole, 1997b). However, constitutive (Ellis et al., 2000) and inducible (Cole, 1996) variation in glucosinolates is not consistently correlated to resistance phenotypes. Little is thus known about plant response or resistance mechanisms in brassicaceous crop species. Gene expression and mutant analysis research in the annual plant *Arabidopsis thaliana* in the same plant family has defined at least four distinct but partially overlapping molecular plant response pathways involved in pathogen infection (Glazebrook, 2001). Plant-insect and wounding studies are now refining these pathways and uncovering new responses (Rojo et al., 1999; Reymond et al., 2000; Stotz et al., 2000).

Our previous work (Moran and Thompson, 2001) examined compatible plant-aphid interactions on *A. thaliana*. Infestations of 24–96 h with *Myzus persicae* (green peach) aphids on rosette leaves led to strong increases in mRNA levels of two pathogen- and SA-inducible (Thomma et al., 1998) *PR* genes: *PR-1*, of unknown function, and *BGL2*, which encodes an acidic, apoplastic β-1,3-glucanase. *PAL1*, encoding a phenolic biosynthetic enzyme (Wanner et al., 1995) was more modestly induced. These three responses are consistent with those found in both compatible and resistant plant-aphid interactions in other plants (Table 1). Increases also occurred in transcript levels of *PDF1.2*, encoding a low molecular weight antimicrobial defensin (Penninckx et al., 1998), *LOX2*, encoding a key enzyme in the JA biosynthetic pathway (Bell and Mullet, 1993), and *STP4*, encoding a protein that mobilizes carbohydrates to areas of stress (Buttner et al., 2000). *PDF1.2* and *LOX2* expression are controlled by different signaling pathways than are *PR* gene responses (Glazebrook, 2001). The aphid induction profile, thus, appears to involve multiple signaling factors. Consistent with this conclusion, the full profile was not induced by either mechanical wounding or treatment with BTH, a commercial SA analog. In addition, induction was dependent on both the key regulatory gene *NPR1* (Moran and Thompson, 2001), which binds proteins associated with SA regulation of *PR-1* and other genes (Zhang et al., 1999), and *COI1*, which encodes a ubiquitination factor that regulates repressors of JA perception (Xie et al., 1998). Responses were localized to infested leaves after one week of infestation (Moran and Thompson, unreported data). In contrast, systemic long-term responses occur after infection by most compatible fungi and bacteria in *A. thaliana* (Thomma et al., 1998).

The use of microarrays and other genomic profiling tools in plant-insect interactions have broadened plant response profiles to chewing insect feeding and wounding, bringing to light the complex integration of signaling pathways (Arimura et al., 2000; Reymond et al., 2000; Schenk et al., 2000; Stotz et al., 2000; Kessler and Baldwin, 2002). We used microarray and macroarray techniques to evaluate the hypothesis that plant responses to phloem feeding by *M. persicae* on *A. thaliana* involve a similar complexity. To compare responses across aphid species differing in behavior and host range, we examined the induction of a limited set of genes by *B. brassicae* aphids with RNA blotting.

MATERIALS AND METHODS

Plant and Insect Cultures

Arabidopsis thaliana Heynh. (ecotype Columbia) and *Brassica napus* (L.) cv "Ceres" were grown in 2.5-cm-wide square pots at 20°C, 50% relative hu-

midity and a 12-h photoperiod (150 mmol m^{-2} s^{-1}) under incandescent and fluorescent lighting. Plants were watered with dilute fertilizer solution (0.5 g L^{-1} 20:20:20, 0.125 g $L^{-1}Ca(NO_3)_2 \cdot 4H_2O$). Plants 20–25 days old were used for aphid infestations. Colonies of *Myzus persicae* Sulzer and *Brevicoryne brassicae* (L.) aphids were maintained in large sealed cages on *Brassica napus* cv "Ceres" plants at 25°C under a 12-h photoperiod.

Aphid Infestations

Expanded, non-senescent *A. thaliana* rosette leaves (3–5 leaves per plant) on three to eight plants per infested or control treatment, per experiment were individually infested with 10 apterous *M. persicae* aphids (5 adults, 5 nymphs). Aphids were confined to leaves by applying Tanglefoot (Tanglefoot Co., Grand Rapids, MI) to the petiole. Adults were allowed to reproduce asexually during the experiments (20–30 aphids per leaf by 72 h; 30–50 by 96 h). Plants were placed in clear Plexiglas cages to contain the insects. Aphids were removed after 72 h (microarray) or 96 h (macroarray) of feeding by spraying plants with a 1% (v/v) sodium dodecyl sulfate (SDS) solution that caused aphids to remove their mouthparts from plant tissues. Control plants received Tanglefoot and SDS treatment and were brushed with a paintbrush without adding aphids. For microarray and macroarray experiments with *M. persicae*, tissues from replicate plants were pooled. For infestations with *B. brassicae* aphids, conducted as above, replicate plants were harvested separately. Tissues were frozen in liquid nitrogen and stored at –80°C.

Construction of a cDNA Microarray

A collection of 105 sequenced EST clones was obtained from the *Arabidopsis* Biological Stock Center (ABRC, University of Ohio, Columbus, OH) based on information generated by Reymond et al. (2000) on induction by wounding or chewing herbivory by *Pieris rapae* L. caterpillars on *A. thaliana*. Clones were assigned to broad functional groups of genes consistent with the supplemental information provided (http://www.unil.ch/ibpv). The groups were: non-inducible expression controls (8 clones), such as histone H4 (*HIST*) and ubiquitin (*UBQ4*); *PR* genes (17 clones), such as hevein-like protein (*HEL*), hydroxyproline-rich glycoprotein (*ELI9*), and acidic apoplastic chitinase (*PR3AIII*); defense compound biosynthesis genes (24 clones), such as myrosinase (*TGG1*), anthranilate synthase β-subunit (*ASB*), and chalcone synthase (*CHS*); oxidative stress genes (10 clones), such as glutathione-S-transferase (*GST1*, *GST11*) and superoxide dismutase (*SODCU*, *SODFE*); genes involved in fatty acid signaling and metabolism (14 clones), such as acyl-CoA oxidase (*ACX1*) and allene oxide synthase (*AOS*); genes associated with general signaling and regulation (27 clones), such as aminocyclopropane-1-carboxylic acid synthase and oxidase (*ACC2*, *ACO1*), touch-sensitive genes and calmodulin (*TCH1*, *TCH2*, *TCH3*, *TCH4*) and jasmonate-responsive proteins (*JIP*, *JR3*); and drought-inducible genes (5 clones) including a sugar transporter (*ERD6*) and proline dehydrogenase (*PRODH*). Clones of four genes were used as positive controls for aphid induction based on results from Moran and Thompson (2001) (*PR-1*, *BGL2*, *PDF1.2*, and *LOX2*). The array included four plant-derived control ESTs representing genes that were not expected to be altered by aphid feeding (*βTUB4*, *βTUB6*, *ACT2*, *ACT8*) and two human-derived negative hybridization controls (*Iga-2*, *Pbp1*) (see Table 2). All clones were maintained as plasmid inserts in *Escherichia coli* bacteria. Inserts were PCR-amplified from 1-µl culture in duplicate 50-µl reactions using 5′ amino-modified T7 (5′-AATACGACTCACTATAGGG-3′) and SP6 (5′-ATTTAGGTGACACTATAG-3′) primers (94°C for 2 min; 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; 72°C for 5 min). Reactions were checked on Tris-acetic-acid-EDTA gels and the products purified using a vacuum manifold and 96-well filter (Millipore, Bedford, MA). Eluted products were dried under reduced pressure and resuspended in 3 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M $C_6H_5Na_3O_7$) in 96-well format. Arrays were printed onto aminoalkylsilane-coated slides (Sigma, St Louis, MO) using an Omnigrid spotter (Gene Machines, San

TABLE 2. Expression, Induction, and Background Signal Control Elements Used in Microarray

Gene abbreviation	Genbank accession no.[a]	Function	Cy3 Signal (±S.E.)[b]	Cy5 signal (±S.E.)
βTUB4	H76557	β-tubulin subunit	9,371 (7,239)	7,911 (4,692)
βTUB6	T21508	β-tubulin subunit	7,160 (6,027)	7,452 (2,932)
ACT2	N65512	Actin	10,854 (8,669)	11,707 (5,501)
ACT8	N38701	Actin	7,391 (6,728)	7,858 (4,382)
PR-1	M90508	Pathogenesis-related protein	7,463 (5,049)	11,148 (4,721)
BGL2	M90509	Acidic β-1,3-glucanase	1,616 (543)	12,289 (3,190)
PDF1.2	T04323	Defensin	2,294 (916)	8,295 (3,742)
LOX2	L23968	Lipoxygenase	7,360 (4,000)	8,092 (3,412)
Iga-2	H28469	*Homo sapiens Iga-2* chain C	381 (106)	746 (307)
Pbp1	AA456109	*H. sapiens* scaffold protein	428 (154)	918 (291)
Blank wells	—	—	361 (122)	477 (247)

[a]Accession number for full-length cDNA.
[b]Unstandardized signal intensities averaged across three hybridizations.

Carlos, CA) equipped with quill-tip pins (Majer Engineering, Phoenix, AZ). Along with the clones mentioned above, the array contained spots from blank PCR reactions run with plasmids containing no inserts, and with no DNA. All samples were printed in triplicate sets of adjacent spots. Wells containing EST amplicons were printed twice (total of six spots per EST). *A. thaliana*- and human-derived control clones were printed redundantly across the array. The array also contained several thousand spots derived from clones from a subtractive cDNA library; results from these clones are not reported here. The EST and control gene portion of the array comprised 1,404 spots representing 486 source wells. Slides were stored at room temperature. Additional information about slide preparation and other protocols can be found at http://latin.arizona.edu/galbraith/labprotocols.

Preparation of Fluorescently Labeled Targets

Total RNA was extracted from *A. thaliana* leaves as described previously (Moran and Thompson, 2001). In some experiments, poly-A messenger RNA (mRNA) was isolated (Oligotex, Qiagen, Valencia, CA). Total RNA (75–100 μg) or poly-A RNA (1–2 μg) was labeled via incorporation of fluorescent nucleotide analogs during reverse-transcription reactions (30 μl) as in Kawasaki et al. (2001) using Superscript II RT enzyme (Invitrogen, Carlsbad, CA) and 2 nmol Cy5-dUTP (infested) or Cy3-dUTP (control) fluors (Amersham, Arlington Heights, IL). The reaction was stopped by adding 25 mM EDTA and 1M NaOH and heating at 65°C for 10 min. 1M HCl, 1M TRIS-HCl, pH 6.8, and 500 μl TE (10 mM TRIS, 1 mM EDTA) were added to neutralize and buffer the reaction. Labeled cDNA targets were combined, purified in Microcon-20 columns (Amicon, Beverly, MA), partially dried under vacuum, and reconstituted in hybridization buffer (containing 1.9 μl 20 × SSC, 1.25 μl 0.25 mg/ml yeast tRNA blocking agent, and 0.5 μl 10% [w/v] SDS) to a final volume of 13.0 μl.

Microarray Hybridizations

Spots on printed slides were immobilized by briefly re-humidifying slides at 40°C, snap-drying on a heat block, UV-crosslinking at 650 mJ (Fisher, Pittsburgh, PA), washing in 1% SDS, denaturing in water at 95°C, and rinsing in absolute ethanol. Slides were dried with a brief centrifugation. The Cy5/Cy3 labeled cDNA hybridization mixture was denatured at 95°C for 2 min, briefly quenched, loaded onto slides, and covered with a plastic slip (Hybri-Slip, Sigma). Slides were incubated 12–14 h at 62°C in humid chambers containing 2 × SSC. Hybridized arrays were washed (5 min each) in 2 × SSC/0.1% SDS at 62°C, 0.5 × SSC/0.1%SDS, and 0.2 × SSC/0.1% SDS (latter two at room temperature) and centrifuge-dried. Slides were laser-scanned with a ScanArray 3000 (GSI Lumonics, Billerica, MA). Signal and background pixel intensity data were extracted with Imagene 4.0 software

(BioDiscovery, Los Angeles, CA). Spots with visibly high background caused by dust particles and streaking were excluded from analysis.

Data Analyses

The microarray data presented here represent four hybridizations from three independent aphid infestation and RNA extraction experiments. Mean signal intensities for Cy5 and Cy3 across each spot were corrected by subtracting the median local background. Data for the two fluors were normalized by examining the ratio of Cy5 to Cy3 signal for all EST and control spots on the array (except human clones and blank spots) and multiplying the weaker fluor by this ratio (Kawasaki et al., 2001). Spots of positive induction and expression controls with intensities below a low signal cutoff (signal for blank spots + 2 S.D.) were excluded from the analysis. The *ACT2* expression control gene (see Table 2) had the strongest spot signal in both Cy5 and Cy3 in all experiments. Data for this gene were used to standardize signal intensities (all intensities for each fluor were divided by the average intensity of that fluor across all *ACT2* spots in the array). Ratios for the two non-independent hybridizations were averaged prior to interpretation. Standardized Cy5/Cy3 ratios for each spot were examined for differential expression in infested or control tissues with a cutoff determined by the average ratio across the four *A. thaliana*–derived expression control genes ± 2 S.D., or with an arbitrary cutoff of 1.5 or greater Cy5 / Cy3 ratios for clones showing up-regulation and 0.5 or lower ratio for down-regulated clones. Whichever method produced the more stringent cutoffs was used to distinguish induction and repression. In examining data for the EST clones, at least half of the six spots of a clone had to meet this threshold in each of two or more independent hybridizations to designate the clone as being representative of a differentially expressed gene. The average ratios for spots meeting the induction or repression threshold within each experiment were averaged across experiments (see Table 3).

TABLE 3. Genes Showing Replicated Differential Expression After 72 H of *M. persicae* Feeding in Microarray Analysis and Results at 96 H in Macroarray Experiments

EST abbreviation	Stock number/ Genbank accession no.[a]	Functional group	Function	Infested/uninfested signal (± S.E.)[b]	Induction at 96 h[c]
Upregulated in infested tissues					
GST1	ATTS1553/Z26426	Oxidative stress	Glutathione-S-transferase	2.92 (0.20)	Up-R
GST11	134B20T7/Y14251		Glutathione-S-transferase	4.78 (0.31)	Up-NR
Cu/ZnSOD	247O9T7/X60935		Cu/Zn-superoxide dismutase (cytosolic)	1.68 (0.12)	ND
HEL	245P5T7/U01880	Pathogenesis-related protein	Hevein-like protein	2.43 (0.11)	ND
ASB	241P6T7/L22585	Tryptophan biosynthesis	Anthranilate synthase beta subunit	1.61 (0.02)	Up-R
ACO1	187C14T7/X66719	Signaling/regulatory	ACC oxidase	2.05 (0.25)	Up-R
TCH2	92I17T7/AF026473		Calmodulin-related	1.96 (0.09)	ND
TCH3	221D18T7/L34546		Calmodulin-related	1.94 (0.10)	Up-R
Downregulated in infested tissues					
FeSOD	166F23T7/M55910	Oxidative stress	Fe-superoxide dismutase	0.198 (0.019)	—
PRX7	119F5T7/X98316		Peroxidase	0.267 (0.069)	—
EL15	193C4T7/AJ011048	Aromatic metabolism	Tyrosine decarboxylase	0.249 (0.057)	Up-NR
CHS	187C23T7/M20308		Chalcone synthase	0.288 (0.025)	—
PAL2	CD3-122/L33678		Phenylalanine-ammonia lyase	0.218 (0.12)	ND
PIOX	218B16T7/AF334402	Fatty acid signaling/metabolism	Alpha-dioxygenase	0.178 (0.055)	—
TCH4	146L14T7/AJ011048	Signaling/regulatory	Endo-transglycosylase (signaling/regulation)	0.292 (0.057)	Up-NR

[a]Arabidopsis Biological Resource Center (ABRC) stock number for the EST clone/Genbank number for full-length cDNA.
[b]Standardized ratio of Cy5/Cy3, averaged across three independent hybridizations.
[c]Up = infested/control signal met induction threshold; ND = signal did not meet induction threshold; R = induction replicated in two independent macroarray experiments; NR = induction in only one experiment; — = EST not included in macroarray.

Macroarray Construction and Analysis

EST clones showing differential expression in *A. thaliana* tissues in at least one of the three 72-h experiments, selected clones in the same functional groups not meeting thresholds (total of 38 EST clones, spotted in duplicate), positive induction controls (*PR-1*, *BGL2*), negative induction controls (*ACT2*, *ACT8*), negative human-derived expression controls (*Iga-2*, *Pbp1*), and wells from blank reactions were arrayed in 96-well format on positively-charged nylon membranes (Hybond N+, Amersham) using the method of Zhang et al. (1996). Two *M. persicae*–derived cDNA fragments (16S rRNA [Genbank accession no. U36742] and an E4 esterase [accession no. X74554]), cloned in our subtractive hybridization cDNA library, were spotted as internal controls. Approximately 250 ng PCR-amplified DNA of each EST was denatured in 2M NaOH, boiled, and neutralized with 3 M sodium acetate. Blots were generated by loading samples into a vacuum manifold (Hybri-Dot, Invitrogen). Blots were dried and UV-crosslinked. Total RNA (10 μg) from infested (96 h) and uninfested plants was DNAse-treated (DNAse I Amp grade, Stratagene) and reverse transcribed (50 μl reaction volume) with CDSIII/3′ primer (Clontech) and Superscript II enzyme. 0.5 mM dATP, dGTP, and dTTP were used along with 5 μl [α-p^{32}] dCTP (3,000 Ci/mmol) (New England Nuclear/DuPont, Boston, MA). Probes were purified in Microcon-20 columns. Hybridization buffer containing 5 × SSC, 5 × Denhardt's solution (5× = 1 mg/ml ficoll, 1 mg/ml polyvinylpyrrolidone, 1 mg/ml bovine serum albumin) and 0.1% SDS was combined with 5×10^6 cpm probe/ml buffer. Duplicate blots were hybridized at 65°C for 16 h. Blots were washed according to the membrane manufacturer's instructions and exposed to autoradiogram film (Kodak Blue XB-1, Eastman-Kodak, Rochester, NY) for 24 h. The integrated signal intensity for each spot was determined with a camera imaging system (UVP, Upland, CA). Background correction, standardization, ratio calculation, and induction threshold establishment methods were as with microarray data, except that *ACT8* rather than *ACT2* was used for standardization.

Gene Induction by *B. brassicae* Feeding

RNA extraction, blotting (5–10 μg total RNA per sample) and hybridization involved methods described previously for *M. persicae* (Moran and Thompson, 2001). Template clones of *PR-1*, *BGL2*, *PDF1.2* (see Table 2) and *STP4* (Genbank accession no. X66857) were [α-p^{32}] dATP-labeled via random-priming (Invitrogen) and used as probes. Estimates of integrated signal intensity on autoradiograms were obtained with a camera imaging system (Chemi-Doc, BioRad, Hercules, CA) and normalized according to signals obtained from hybridization to maize (*Zea mays*) 18S ribosomal RNA.

RESULTS

Performance of Microarray

Hybridizations of a microarray containing EST and control clones with Cy5 (72 h infested)- and Cy3 (uninfested)-labeled cDNA revealed variable signal intensities and colors (Fig. 1A). Signals and ratios for the positive and negative controls mostly agreed with expectation. Spot intensities of the two human negative expression control genes and blank spots were usually an order of magnitude or more lower than the intensities of plant-derived β-tubulin and actin EST clones (negative induction controls) and *PR-1*, *BGL2*, *PDF1.2*, and *LOX2* clones (positive induction controls) (Table 2). Across three independent experiments, β-tubulin and actin spots had standardized Cy5/Cy3 signal ratios close to 1, while ratios for *PR-1*, *BGL2*, and *PDF1.2* all exceeded 2.0, a level greater than the average mean ratio + 2 S. D. for all tubulin and actin spots (Fig. 1B). *LOX2* spots did not show ratios of induction. Other than *LOX2*, the microarray results for the positive induction controls are consistent with RNA blot data (Moran and Thompson, 2001) although the latter approach detected substantially greater (10–20-fold) induction of *PR*

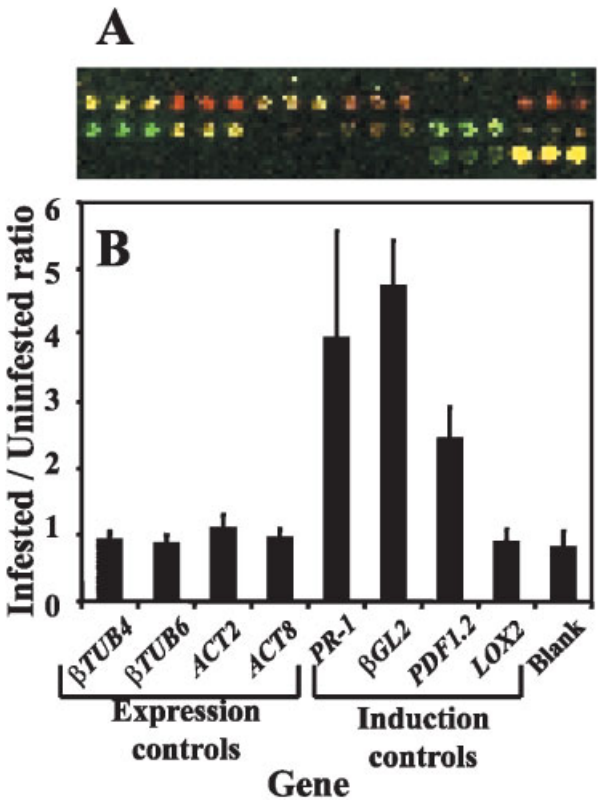


Fig. 1. **A:** Close-up of part of a false-color image of Cy5 and Cy3 intensities resulting from hybridization of labeled cDNA from infested (72 h) and control plants to a microarray containing EST clones and controls. Variation in color indicates greater competitive hybridization by Cy3-labeled (uninfested plants, red) or Cy5-labeled (infested plants, green) cDNA. Yellow color indicates roughly equal hybridization of the two fluors. **B:** Cy5/Cy3 ratios (mean ± S.E. from three hybridizations) for clones representing genes not inducible by aphid feeding (Expression control) genes, inducible (Induction control) genes, and spots containing no DNA (Blank).

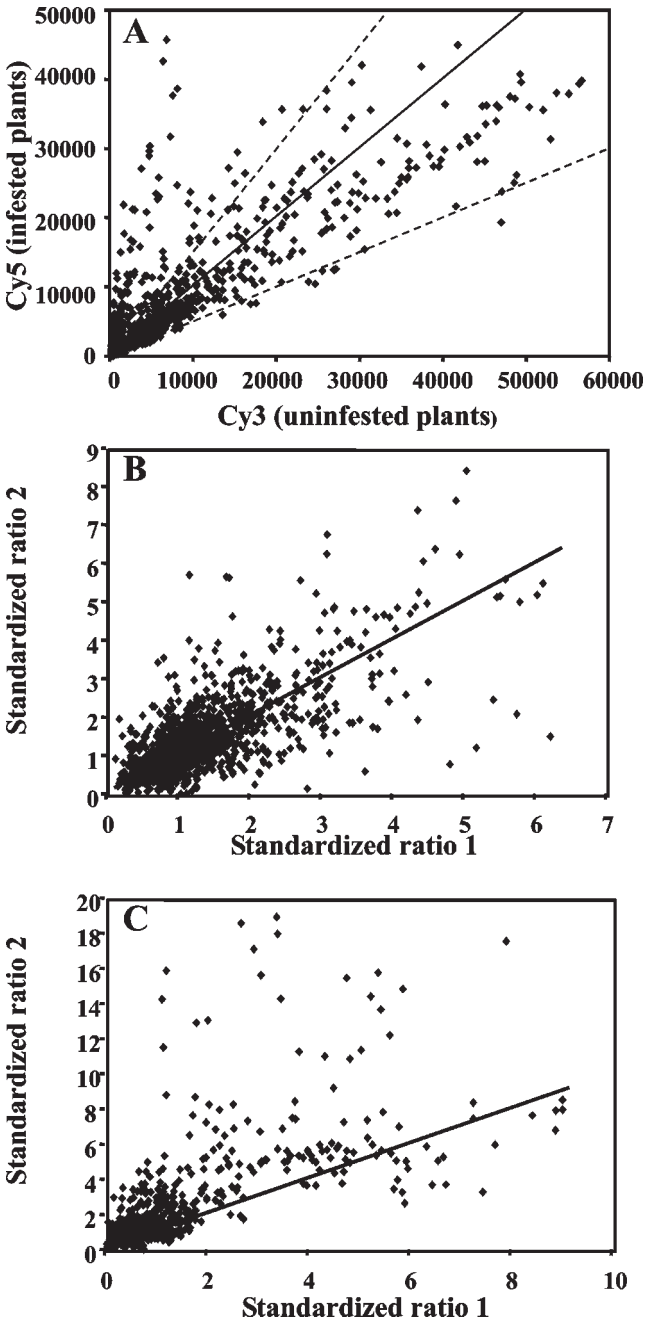
genes than did array hybridization. *PDF1.2*, on the other hand, showed similar induction ratios in RNA blots as in the array.

Data from a representative hybridization for the EST clones and positive and negative controls, plotted as Cy5 vs. Cy3 signal, are shown in Figure 2A. An average of about 20% of the 105 EST clones were found to represent induced or repressed genes in each hybridization, substantially more than would be expected randomly (Chi-square test, χ = 5.532, $P < 0.0001$, df = 4). Threshold ratios for induction in individual experiments ranged from 1.5 to 2.0 (always equal to or greater than the mean +2 S.D. of the average Cy5/Cy3 across all four tubulin and actin genes) while repression thresholds ranged from 0.5 to 0.30 (equal or less than the control average –2 S.D.). After expressing ratios in $\log_{10}$ terms (Kawasaki et al., 2001), 29.7% of the ratios for ESTs and induction controls varied by 0.2 log units or more (= >1.6-fold variation) between two non-independent hybridizations derived from the same plant infestation (Fig. 2B). This figure increased to 58.2% when comparing two independent hybridizations. In the latter case, a skew towards higher ratios in one of the two experiments was evident (Fig. 2C). Because of ratio variation among experiments, the data were screened for cDNA spots that showed replicated induction or repression.

Induction Profiles at 72 H

Eight EST clones showed Cy5/Cy3 ratios reproducibly above thresholds in microarray experiments, suggesting induction by 72 h of *M. persicae* feeding on *A. thaliana*. Seven other ESTs yielded ratios suggestive of gene repression (Table 3). Oxidative stress genes were the most commonly represented functional group. Interestingly, members of this group were both positively and negatively affected by aphid infestation. Levels of transcripts of both glutathione-S-transferase genes on the microarray (*GST1*, *GST11*) increased, as did one cytosolic form of superoxide dismutase (*Cu/ZnSOD*, or *CSD1*), while another form (*FeSOD*) and a peroxidase gene (*PRX7*) decreased. Transcript levels of an additional GST (*GST5*) and several other glutathione-associated genes were not altered. Similar dual induction/repression of expression occurred in a group of Ca^{2+}/calmodulin-related signaling genes: *TCH2* and *TCH3* mRNAs increased as a result of aphid feeding, while *TCH4* decreased. *PR* genes were a group of particular interest because of strong evidence of induction of these genes by aphids on many plants (see Table 1). Other than the *PR-1* and *BGL2* controls, a gene encoding hevein-like protein (*HEL1)* was the only *PR* gene repeatedly induced by 72 h of feeding.

Aphid feeding increased mRNA levels of one tryptophan biosynthesis pathway gene (anthranilate synthase β-subunit [*ASB*]) but not several others. Induction occurred of a gene encoding an ethylene biosynthesis gene, 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (*ACO1)*, while a gene encoding an ACC synthase (*ACC2*) showed no change. Three aromatic biosynthesis genes (*ELI5*, *CHS*, and *PAL2*) were repressed by aphid feeding, while levels of 10 other genes in this functional group were not altered.



Induction Profiles at 96 H

Infested and control RNA derived from two 96-h infestation experiments was hybridized to filter arrays spotted with EST clones showing unreplicated or replicated induction at 72 h. Induction thresholds for the macroarray (Fig. 3) were higher than those used for microarray analysis because of greater *ACT2* and *ACT8* control signals in infested plants. *PR-1* but not *BGL2* positive induction control spots yielded clear evidence of induction (Fig. 3). Of the eight genes induced after 72 h of aphid feeding, only *GST1*, *ASB*, *ACO*, and *TCH3* showed infested/control signals consistently indicative of induction at 96 h, while *GST11* met the induction threshold in one experiment (Table 3, Fig. 3). Two genes showing non-replicated induction at 72 h were reproducibly induced at 96 h (a tryptophan biosynthesis gene, *TSA*, and *MPK3*, encoding a MAP kinase) and two genes with no difference at 72 h showed robust induction at 96 h (*PR3AIII*, an acidic apoplastic chitinase *PR* gene, and *LECRK*, a lectin receptor kinase) (Fig. 3). Interestingly, *ELI5* and *TCH4*, repressed at 72 h, had ratios indicative of induction in one of the two 96-h experiments (Fig. 3).

Defense Gene Expression by *B. brassicae* Feeding

Feeding by *B. brassicae* cabbage aphids on *A. thaliana* rosette leaves for 72 h increased mRNA

Fig. 2. **A:** Cy5 (infested) vs. Cy3 (uninfested) signal intensities from a representative microarray hybridization. Solid line, 1:1 ratio of signal intensity. Dashed lines,1.5 and 0.5 Cy5/Cy3 signal ratio cutoffs applied in this hybridization to standardized signal ratios as thresholds of increased or decreased expression in infested relative to uninfested plants. **B:** Standardized intensity ratios from two hybridizations involving the same RNA pools from infested and uninfested plants as template for reverse transcription. Solid line, equal ratios in both hybridizations. **C:** Standardized intensity ratios from two hybridizations involving independent RNA sources as template. Solid line as in B.

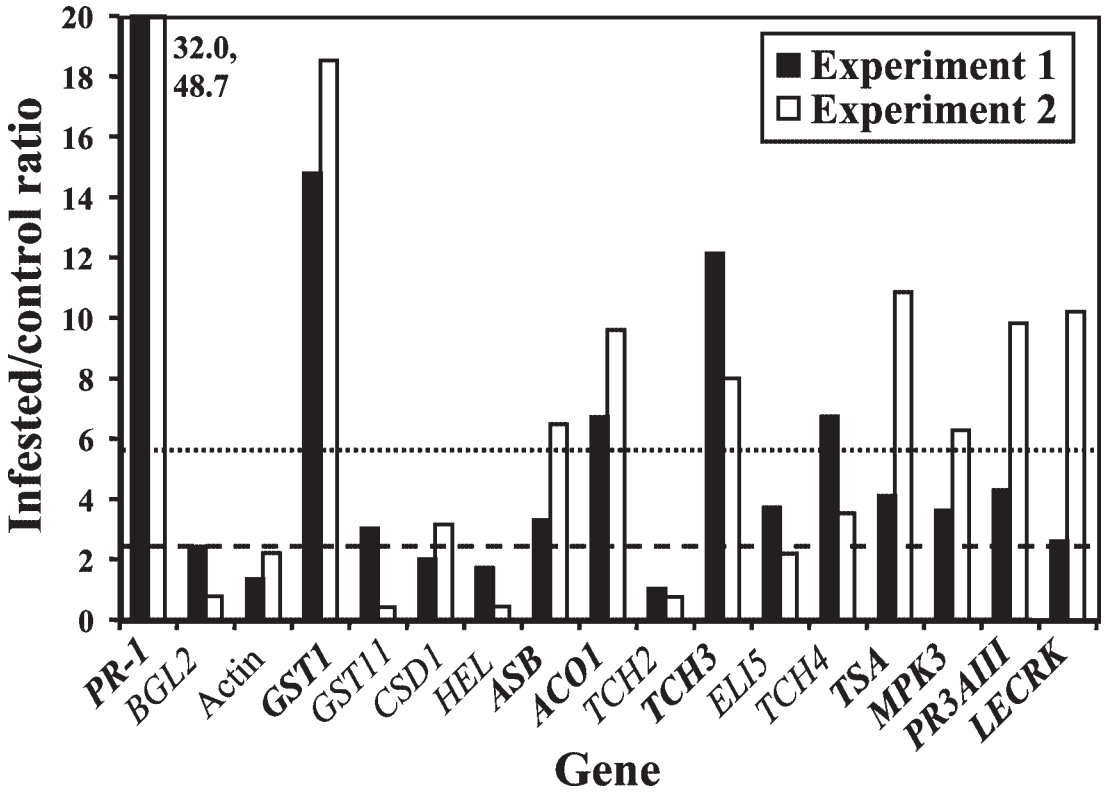


Fig. 3. Infested (96 h)/uninfested signal ratios for selected clones on the macroarray. Pairs of bars for each gene represent ratios obtained from two experiments involving independent sets of RNA. Numbers next to bars for *PR-1* indicate off-scale ratios. Dashed line, the threshold ratio for up-regulation in Experiment 1. Dotted line, the threshold for Experiment 2. Boldface gene abbreviations, genes that met the threshold in both experiments.

levels of *PR-1* (23-fold), *BGL2* (6-fold), *PDF1.2* (48-fold), and *STP4* (11-fold) genes (Fig. 4). Levels of mRNAs of these four genes did not increase in apical (younger) non-infested leaves harvested from infested plants after 72 h of feeding (Moran and Thompson, unreported data).

DISCUSSION

Plant Responses to Aphids in a Functional Context

Our previous work suggested that 72 to 96 h of *M. persicae* infestation of *A. thaliana* led to induction of multiple response pathways (Moran and Thompson, 2001). The microarray and macroarray data are focused on the expression of a small (relative to the entire estimated *A. thaliana* transcriptome) set of selected genes over a short time period defined by our previous experiments. During infestations, asexually reproducing adults and increasing numbers of nymphs of *M. persicae* engaged in continual removal and insertion of feeding stylets over time. The biology and behavior of the aphids may have obscured linear sequences of elicitation, signaling, and response development, in comparison to mechanical wounding or chemical elicitor treatment. However, the array results suggest that several groups of genes with functions in responses to these and other stresses are involved in compatible plant-aphid interactions. Contrasts between the response profile at 72 and 96 h could reflect the substantial differences in labeling and hybridization techniques used to examine microarrays and macroarrays, as well the biological dynamics of aphid feeding and plant responses.

Expression levels of a small profile of oxidative stress responses were influenced by 72 h of *M.*

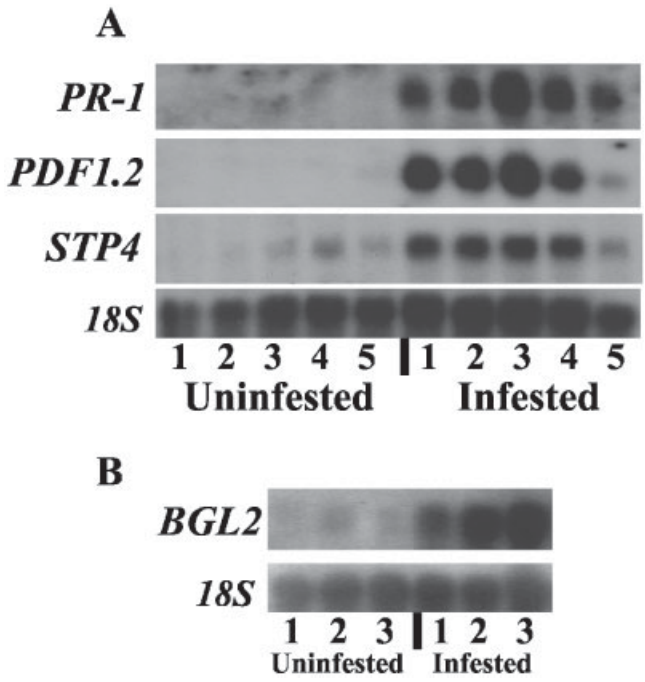


Fig. 4. RNA blots of plants infested for 72 h with *B. brevicoryne* aphids and control (uninfested) plants. **A:** *PR-1*, *PDF1.2*, and *STP4* probes. **B:** *BGL2* probe. Numbers below lanes indicate replicate plants.

persicae feeding, but not 96 h with the exception of *GST1*. Aphid salivary secretions can alter plant oxidative conditions (Dillwith et al., 1991; Jiang and Miles, 1993; Miles and Oertli, 1993; Miles, 1999; Walling, 2000) and plant oxidative stress can be triggered by oligosaccharides and cell wall fragments (Hahn, 1996). Superoxide dismutases (SODs) deactivate oxygen radicals, converting them to hydrogen peroxide (Raychaudhuri, 2000). GSTs conjugate glutathione to a wide range of exogenous and endogenous toxins, including phenolics and lipid-derived molecules containing oxygen radicals, and can also act as glutathione oxidases to directly detoxify radicals (Marrs, 1996). Structural features of *GST1* and *GST11* in *A. thaliana* suggest that they carry out both functions (Edwards et al., 2000). Many types of abiotic and biotic stress can induce GSTs and SOD proteins, and their corresponding genes (Bolwell and Wojtaszek, 1997) including chewing herbivory (Stout and Bostock, 1999). In *A. thaliana*, *GST1* and *GST11* expression increases after drought stress or wounding (McConn et al., 1997; Reymond et al., 2000). *GST1* and *Cu/ZnSOD* increase after infection by *Alternaria brassicicola* fungus (Schenk et al., 2000) and SA treatment (Kliebenstein et al, 1999). The repression at 72 h of *FeSOD* (and a peroxidase gene, *PRX7*) suggests different regulatory mechanisms and functions for some oxidative stress genes as compared to *Cu/ZnSOD*. Cluster analysis of mRNA wound responses (Reymond et al., 2000) and the lack of induction of *FeSOD* by SA, BTH, and pathogen infection (Kliebenstein et al., 1999) support this conclusion. The reduced responsiveness of oxidative stress genes at 96 h could be the result of increased aphid populations overwhelming plant anti-stress systems. However, even one week of infestation of *A. thaliana* did not result in necrosis or other symptoms indicative of a breakdown in regulation of oxidation (Moran and Thompson, unreported data).

Three calmodulin-associated signaling genes (*TCH2*, *TCH3*, *TCH4*) showed altered expression patterns in *M. persicae*–infested plants. In contrast to wounding (Reymond et al., 2000) and mechanical stress (Johnson et al., 1998), aphid feeding did not coordinately increase expression of the *TCH* genes at either 72 or 96 h. *TCH4* expression was actually repressed at 72 h and only *TCH3* was robustly induced at 96 h. Calmodulin binds calcium and plant proteins that play key roles in developmental and defensive responses (León et al., 1998; Bergey and Ryan, 1999). Calcium is important in initiating oxidative stress cascades similar to that possibly stimulated by *M. persicae* in *A. thaliana* leaves (Bollwell and Wojtaszek, 1997) and also promotes induction of *PR* genes (Nurnberger and Scheel, 2001). *TCH2* and *TCH3* could participate in early signaling following aphid feeding. Stimulation could occur via either specific elicitors or more general wounding stress (Bergey and Ryan, 1999). Tactile stimulation is also possible, but in our studies control plants were brushed without adding aphids. *TCH4* encodes a xyloglucan endotransglycosylase that removes and re-attaches oligosaccharides, leading to cell wall strengthening (Campbell and Braam, 1999). *M. persicae* stylet penetration of the spaces between cell walls and plasma membranes (Tjall-

ingii and Hogen Esch, 1993) and cellular punctures could have been aided by repression of this gene. The reduced induction of *TCH* genes after 96 h could be a reflection of diminished expression of oxidative stress and PR genes.

Among a group of 17 diverse *PR* genes, only *HEL*, along with *PR-1* and *BGL2*, were robustly induced by green peach aphids after 72 h of feeding. Interestingly, *HEL* was the only gene induced by chewing insect feeding but not by wounding among 150 ESTs examined in *A. thaliana* (Reymond et al., 2000). This gene encodes a protein with possible antifungal, lectin-binding properties (Potter et al., 1993). Its role in defense or facilitation of herbivory is unknown. As with most of the *GST/SOD* and *TCH* gene responses, induction was not evident at 96 h. An increase occurred at this time point in mRNAs of an acidic chitinase similar to those found in cereal crop-aphid interactions (Botha et al., 1998; Forslund et al., 2000). *PR3AIII* does not respond within 24 h to wounding or chewing herbivory and *PR-1* responds only ephemerally (Reymond et al., 2000). Aphid feeding as a stimulus thus appears to be distinct from both wounding and pathogen infection even though some responses are shared.

Defense compound synthesis genes were variably influenced by aphid feeding. The repression of transcripts of three aromatic biosynthesis genes after 72 h (*CHS*, *PAL2*, *ELI5*) contradicts evidence of increases in phenolic enzymes and their products in compatible and resistant plants (Table 1) and induction of *A. thaliana PAL1* by *M. persicae* (Moran and Thompson, 2001). As with the *SOD* genes, different isoforms of *PAL* are differentially expressed and regulated. *PAL1* is two to three times more abundant than *PAL2* in leaves and roots (Wanner et al., 1995), induction is at least partially independent of JA (McConn et al., 1997), and caterpillar feeding does not affect expression. *PAL2* induction by wounding is JA-dependent and expression is moderately repressed by chewing herbivory (Reymond et al., 2000). Anthranilate synthase-β subunit (*ASB*), a part of the tryptophan biosynthetic pathway, was the only chemical synthesis gene reproducibly induced at 72 and 96 h. Another gene in the same pathway, encoding tryptophan synthase (*TSA*), showed increases at 96 h. Both *ASB* and *TSA* can be induced by wounding, pathogen infection, and oxidative stress (Zhao et al., 1998). Production of a tryptophan-derived phytoalexin, camalexin, is dependent on the product of the *PAD3* gene (Zhou et al., 1999). A partial cDNA of *PAD3* present on the microarray did not show induction (Moran and Thompson, unreported data), suggesting that camalexin production does not increase during aphid feeding. Typtophan enhancement may provide substrates for amino acid synthesis to facilitate the aphid-plant interaction. However, tryptophan is not among the few amino acids that are essential to *M. persicae* when equipped with its usual endosymbiotic bacteria (Mittler, 1971).

Plant Regulation of Responses to Aphids

M. persicae feeding led to only partial induction of groups of genes functioning in oxidative stress, calcium-dependent signaling, PR responses, and defense compound synthesis. The majority of the oxidative responses (*GST1*, *GST11*, *Cu/ZnSOD*) and the *TCH* genes have been experimentally associated with a SA-inducible, JA- and ethylene-independent response pathway to wounding and pathogen infection (McConn et al., 1997; Johnson et al., 1998; Reymond et al., 2000; Schenk et al., 2000). Our array analysis may have captured the portion of the aphid response that was elicited by wounding. However, the *HEL* and *PAL2* genes respond to chewing herbivory in a manner different from wounding (Reymond et al., 2000) and they responded in the same ways to feeding by *M. persicae* as to chewing. *HEL* and *PAL2* are dependent on JA and ethylene for expression, although HEL is strongly induced by ethylene alone and moderately induced by SA analogs (Potter et al., 1993). *M. persicae* feeding triggers changes in both JA-dependent and SA-dependent genes, consistent with other studies of phloem feeding (Walling, 2000). Work in tomato and barley support our conclusion that aphid feeding is not the same as wounding even though some responses are shared

(Fidantsef et al., 1999; Forslund et al., 2000). Feeding by caterpillars on *A. thaliana* only ephemerally or weakly induces SA-regulated acidic *PR* genes and others, like *GST1* and *GST2* (Reymond et al., 2000; Stotz et al., 2000). The set of stimuli associated with *M. persicae* feeding on *A. thaliana*, while eliciting multiple response pathways, is still selective; large groups of functionally-related genes are not co-induced. Selectivity may be derived from the unique feeding biology of phloem feeding. However, Arimura et al. (2000) found a similar breadth of induction by spider mite volatiles in lima bean. Our studies were limited to 72 to 96 h of feeding. Early induction could involve JA and/or ethylene-dependent responses that are later suppressed by stronger SA induction (van Wees et al., 1999). Mutant analyses of the roles of signaling factors like *MPK3* (a mitogen-activated protein kinase) (Asai et al., 2002) and *LECRK* (a lectin receptor kinase) (Hervé et al., 1999), both induced at 96 h by *M. persicae*, would provide clues about elicitation mechanisms.

To our knowledge, local and systemic fluxes in SA and JA have not been evaluated in relation to aphid feeding. Our RNA blotting and microarray studies included a number of genes known to be involved in fatty acid signaling and biosynthesis of JA and SA. One fatty acid gene showed evidence of repression (*PIOX*) while a form of lipoxygenase (*LOX2)* was not distinguishable from negative induction control genes in array experiments. *PAL1* was the only potential SA biosynthetic gene that showed increased expression. Isochorismate mutase, rather than PAL, is believed to be the key enzyme involved in SA production in *A. thaliana* (Wildermuth et al., 2001). SA and JA could, therefore, be static in concentration during aphid feeding, but this does not preclude a role in facilitating plant responses (van Wees et al., 1999). In our studies, a wound- and ACC-inducible ACC oxidase (*ACO1*) (Gomez-Lin et al., 1993) was reproducibly induced by *M. persicae* at 72 and 96 h, although expression of an ACC synthase gene was not altered at 72 h and was not analysed at 96 h. Ethylene concentrations could increase during phloem feeding on *A. thaliana*. Feeding by two aphid species on barley stimulates ethylene production after less than a day of infestation (Argandoña et al., 2001). Evolution was higher in a resistant line, suggesting a defensive role. However, ethylene promotes senescence (Gan and Amasino, 1997), which may facilitate nutrient assimilation by aphids on plants.

Cabbage Aphid Feeding on Compatible *A. thaliana*

Feeding by *B. brassicae* aphids on leaves of *A. thaliana* for 72 stimulated responses similar to those resulting from infestation by *M. persicae*, an aphid with a much broader host range. The magnitude of increases in expression of SA-sensitive *PR-1* and *BGL2* was not stronger than the levels associated with a JA/ethylene-regulated defensin gene (*PDF1.2*) and a carbohydrate resource allocation response gene (*STP4*). *M. persicae*, in contrast, more strongly induced SA-inducible transcripts (Moran and Thompson, 2001). Elicitation factors involved in feeding by *B. brassicae* may differ from those associated with *M. persicae*, perhaps due to known differences in probing behavior (Cole, 1997a). *M. persicae* takes longer to reach the phloem and spends more time penetrating xylem elements, while *B. brassicae* penetrates more cells along the stylet pathway, perhaps leading to more wound-related stimulation. Variation in salivary components may also mediate differences in induction (Miles, 1999). Aphid species-specific responses occur on cereal host plants (Ni et al., 2001) although on tomato responses to two species were similar (Fidantsef et al., 1999). The upregulation of *STP4*, a monosaccharide symporter (Buttner et al., 2000) in *A. thaliana*, by both aphid species may contribute to the creation of nutrient sinks at feeding sites, a process likely to involve other genes and proteins. *ERD6* (a drought-inducible sugar transporter) was not induced at 72 or 96 h in array experiments (unreported data).

Compatible Plant-Aphid Interactions and Resistance

Plant resistance to aphids has been defined from a mostly constitutive point of view, both within

the Brassicaceae and in other plants. Resistance characteristics on uninfested plants are used to infer the cause of resistance in infested plants. Barriers to aphid feeding may involve ecological/phenological factors (Pilson and Rausher, 1995), physical structures like trichomes (Lapointe and Tingey, 1984), structural variation in plant host tissues (Campbell and Dreyer, 1990), and genetic variation in the concentrations of plant metabolites such as phenolics, alkaloids, glucosinolates, and lectins (Harrewijn, 1990; Montllor, 1991; Cole, 1994; Ciepiela et al., 1994; Sauvion et al., 1996; Tran et al., 1997; Tosh et al., 2001). Aphid probing behavior varies according to plant resistance in different genotypes of lupins (Zehnder et al., 2001), melons (Klingler et al., 1998), wheat (Ryan et al., 1990), and other crops (Montllor, 1991). Differences usually involve a specific tissue type or probing stage. Almost no studies have determined whether resistance is truly constitutive or relies on an unknown stimulus provided by the aphid. Recent findings indicate that aphid infestation can reduce reproduction in a subsequent infestation by conspecifics (Sauge et al., 2002) or other species (Gianoli, 2000). The new molecular perspective in plant-insect interactions is enhancing the possibility that inducible resistance can be identified and developed in plant-aphid interactions.

Certain types of responses, like chitinases, may have direct toxicity (Kramer and Muthukrishnan, 1997). Genetic alteration of signaling factors or exogenous signal treatment is another approach. Transgenic suppression of an enzyme (hydroperoxide lyase) involved in inducible traumatin production increases aphid fecundity on potato plants (Vancanneyt et al., 2001). Pretreatment of *A. thaliana* with BTH, an SA analog, increases callose deposition responses to pathogen infection or wounding (Kohler et al., 2002) and callose blockage of sieve elements is a possible defense against aphids (see Table 1). However, efforts to induce resistance to aphids and other phloem feeders via pretreatment with general elicitors like BTH as well as SA, JA, or mechanical stress have yielded mostly negative or equivocal results (Ellis et al., 1998; Inbar et al., 2001; Moran and Thompson, 2001; Thaler et al., 2001). The most compelling case for induction in resistance to aphids involves a class of genes that rely on highly specific elicitors. *R* genes encode membrane-bound or cytoplasmic R proteins that can bind avr proteins secreted by plant pathogens in a genotype-specific manner as pathogens invade host tissues (reviewed in Jones, 2001). *R* gene resistance leads to strong and rapid increases in mRNAs of *PR* genes and proteins also associated, at a slower pace, with compatible plant-pathogen interactions (Glazebrook, 2001). Induction patterns of PR proteins in resistant vs. susceptible genotypes of aphid-infested cereal plants generally fit this pattern (van der Westhuizen et al., 1998a,b; Forslund et al., 2000). The first aphid resistance gene to be cloned, *Mi* from tomato, is an *R* gene (Rossi et al., 1998). Strong candidates are being isolated from melon (Brotman et al., 2002) and other crops (Walling, 2000). In many plants, comparisons of induction on resistant vs. susceptible plant genotypes could identify elicitor-related specificities. Calcium-dependent signaling events, similar to those suggested by responses to *M. persicae* on compatible *A. thaliana*, are required for at least one R-avr interaction involving a plant pathogen (Jones, 2001). *R* genes do not encode toxins or deterrents. The relationship between these genes and proximal resistance traits is thus unclear.

CONCLUSIONS

Elicitation and signaling usually occur in both compatible and incompatible interactions between plants and their parasites, and aphids appear to be no exception. The induction patterns associated with phloem feeding on *A. thaliana* are not indicative of generalized stress responses, but are likely a combination of abortive plant defense, plant protection possibly associated with tolerance, and, most interestingly, facilitation of the host plant by the aphid. The partial response profile identified here suggests areas of overlap in induction with other forms of biotic and abiotic stress. However, there is a clear idiosyncratic component of plant sensitivity to aphids that could be exploited for

resistance. The main obstacle is the current critical lack of information about elicitation mechanisms associated with phloem feeding. A better understanding of elicitation and induction will lend insight to studies of the roles of aphids as members of ecological communities, and will lead to the development of novel and durable resistance traits for crops.

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