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Induction of defense gene homologues in wheat roots during interactions with *Pseudomonas fluorescens*

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ABSTRACT

Specific strains of *Pseudomonas fluorescens* directly inhibit soilborne fungal pathogens of *Triticum aestivum* (wheat) during colonization of the wheat rhizosphere, but until now the impact of these beneficial bacteria on wheat gene expression was unknown. To test the hypothesis that *P. fluorescens* induces defense genes in wheat roots, we constructed a custom microarray of 192 oligonucleotides representing 84 wheat root *e*xpressed *s*equence *t*ags (ESTs) homologous to defense/stress genes from Arabidopsis, tomato, rice, and barley, and 11 candidate root developmental genes. The ESTs were selected from existing wheat root EST libraries. Arrays were interrogated with Alexa Fluor 546-labeled transcript (cDNA) populations from roots or coleoptiles of cultivar Finley or lines 442 or 443, near-isogenic for the cold temperature-dependent *vrn1A* flowering locus, four days after seed inoculation with the take-all-suppressive strain *P. fluorescens* Q8r1-96. Twenty-two transcripts encoding Ca^{2+}-dependent protein kinases, components of the oxidative stress, cold stress and jasmonic acid pathways, and proteins associated with the hypersensitive response were induced or repressed in wheat roots during *P. fluorescens* interactions. Transcripts encoding pathogenesis-related protein Pr-10a and hypersensitive response protein HRin1 also were induced in coleoptiles. Real-time PCR demonstrated that eleven transcripts were induced in root tissue between 2 and 6 h and remained elevated at 24 h post-inoculation. Our findings suggest that biocontrol *P. fluorescens* modulates defense/stress gene expression in wheat roots.

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1. Introduction

Interactions between host roots and certain strains of the genus *Pseudomonas* result in the suppression of diseases caused by soilborne pathogens. For example, suppressive strains of *Pseudomonas fluorescens* Migula 1895 reduce severity of take-all of wheat caused by *Gaeumannomyces graminis* (Sacc.) Arx & D. Olivier var. *tritici* Walker (Weller et al., 2002). Disease suppression by *P. fluorescens* is attributed to several mechanisms, including plant growth promotion, nutrient sequestration, production of antifungal metabolites such as 2,4-diacetylphloroglucinol (DAPG) (Haas and Keel, 2003; Iavicoli et al., 2003; Weller et al., 2002) and siderophore-dependent production of reactive oxygen species (Audenaert et al., 2002). In Arabidopsis and tomato, root-colonizing *P. fluorescens* (rhizobacteria) suppress foliar diseases through a phenomenon called induced systemic resistance (ISR) (Maurhofer et al., 1994; Park and Kloepper, 2000; Pieterse and van Loon, 1999). ISR is regulated by the salicylic acid, jasmonic acid and ethylene signal pathways, depending upon the host species, bacterial strain and pathogen (Maurhofer et al., 1998; Pieterse et al., 2000; Ton et al., 2002).

Molecular host responses to beneficial rhizobacteria have been characterized in model dicots and several crop plants. Cucumber roots undergoing colonization by *Pseudomonas* sp. displayed higher activities of phenylalanine ammonia-lyase, peroxidase and polyphenol oxidase (Chen et al., 2000). Gene expression profiling in Arabidopsis revealed that 97 genes were differentially transcribed during ISR by *P. fluorescens* strain WCS417r; none were induced in foliar tissue (Verhagen et al., 2004). *P. fluorescens* strain FPT9601-T5 modulated the expression of 200 genes (95 positively- and 105 negatively-regulated) in foliar tissues of Arabidopsis after a 3-week treatment period (Wang et al., 2005). In contrast, foliar expression of 63 host genes was altered in the Arabidopsis-*P. thivervalenensis* Ws-0-MLG25 interaction, whereas changes in root gene expression were limited to nine genes and generally negatively-regulated (Cartieux et al., 2003). Molecular responses of roots to biotic and abiotic stresses are complex and involve several signal pathways (e.g., Kreps et al., 2002).

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There remains a knowledge gap regarding molecular responses of *Triticum aestivum* L. (hexaploid wheat) to rhizosphere-colonizing pseudomonads. In this study, we test the hypothesis that *P. fluorescens* induces defense responses in wheat roots, a potential new mechanism of protection against pathogens. To this end, we mined wheat root EST libraries (Lazo et al., 2004; Chao et al., 2006) for defense- and stress-associated homologues, and assembled a wheat root microarray of 192 60-base oligonucleotides representing 95 root-expressed genes.

Three *T. aestivum* varieties were selected for their genetic potential to respond to biotic and abiotic stress in a previous study (Skinner et al., 2005). Finley, a hard red winter variety adapted to the Pacific Northwest, supported high population densities of the *P. fluorescens* biocontrol strain Q8r1-96 as early as 4 days post-inoculation (dpi) (Okubara et al., 2004). This cultivar also accumulated substantial amounts of Q8r1-96-derived 2,4-diacetylphloroglucinol (DAPG), an antifungal metabolite with activity against the take-all pathogen *Gaeumannomyces graminis* var. *tritici*, in the wheat rhizosphere (Weller et al., 2002) and rhizoplane (Okubara and Bonsall, 2008). Wheat lines 442 (winter) and 443 (spring) are near isogenic for the recessive *vernalization1* locus of chromosome 5A (Storlie et al., 1998), which confers the cold requirement for initiation of floral development. *P. fluorescens* strain Q8r1-96 was selected for the study because it is a rapid and persistent colonizer of the wheat rhizosphere (Landa et al., 2003) and strong producer of DAPG in the rhizosphere (Raaijmakers and Weller, 2001) and rhizoplane (Okubara and Bonsall, 2008).

Here, we report our data mining and annotation efforts, the construction of a custom wheat root defense gene microarray, and use of the array to monitor changes in defense gene expression in roots undergoing colonization with *P. fluorescens*.

2. Materials and methods

2.1. Root ESTs and bioinformatics

Expressed sequence tags (ESTs), single-pass sequences of the 5' portions of cDNAs, were available for nine root cDNA libraries derived from four cultivars of hexaploid wheat (Table 1). ESTs having PHRED quality scores of $\geqslant 20$ were accessed through the GrainGenes website and the GenBank dbEST database prior to publication in journals (Lazo et al., 2004; Chao et al., 2006).

To determine the degree of redundancy within each of the nine cDNA libraries, sequence identity analysis was performed using GenBank non-redundant databases 12.14.01 and Standalone Blastn (Altschul et al., 1997). The threshold for nucleotide sequence matches was 1e-100. Two sequences within a contig were considered to be redundant if all of the following criteria were met: (1) there was less than one mismatch every 120 nt; (2) there were no more than two gaps; and (3) the gaps or mismatches did not occur in tandem. Mismatches at the 5′ or 3′ ends of sequences were attributed to sequencing errors, and had less weight than mismatches in mid-sequence. The above criteria rather than match score values were used to determine redundancy, because the match score sometimes was high for a long but low-identity alignment.

Singletons and contig representatives were compared to the non-redundant GenBank databases vers. 7.14.02 using Standalone Blastx vers. 2.2.2 (Altschul et al., 1997). Matches with stringency scores of 1e-20 or lower were considered to be significant. ESTs were sorted into three groups based on the match results: (1) those matching sequences of known or assigned function (hits); (2) those matching hypothetical open reading frames or ESTs of unknown function; and (3) those having no matches. Sequences in the first group were further sorted into one of 21 function categories, listed in Supplementary Table 1. Function categories were based on gene ontogeny developed for Arabidopsis (Rhee et al., 2003), with emphasis on defense signaling and defense/stress genes. Sequences in groups 2 and 3 were re-analyzed in 2008 using the current non-redundant GenBank database and Blastx vers. 2.2.19. ESTs that were annotated by motifs or function were sorted into existing function categories. Blastx results were collated for each library using Excel (Microsoft Corp.).

To determine whether stress treatments shifted patterns of expression, frequencies of ESTs from stress and non-stress libraries were compared. The number of ESTs in five function classes (primary metabolism, secondary metabolism, defense/stress, protein synthesis and DNA replication) were compared using an algorithm for constructing 95% confidence intervals for the binomial (B. Mackey, unpublished; Zar, 1984) and SAS 9.2 (SAS Institute Inc., Cary, NC), where non-overlap of the confidence intervals for two libraries indicates a significant difference between the libraries.

2.2. Root-expressed sequences for microarrays

A total of 95 wheat homologues of Arabidopsis, tomato, rice, and barley genes involved in defense and stress, root development and housekeeping functions were selected for the microarray (Supplementary Table 2). These included signature genes of the salicylic acid, jasmonic acid and ethylene signal pathways, the hypersensitive response, oxidative stress, apoptosis and cold-stress adaptation. For ESTs that appeared to encode partial reading frames, full-length sequences were retrieved from former The Institute for Genomic Research (TIGR) wheat gene index, currently curated on line by the

Table 1
Wheat root cDNA libraries[a] and results of BlastX analysis[b].

Library	Cultivar	Treatment	Root tissue	# ESTs[c]	Hits[d]	Hypoth.[e]	Unmatched
NFT	Chinese Spring	None	Full tillering	834	0.63	0.12	0.25
DS	Chinese Spring	60–80% RWC[f]	Full tillering	942	0.65	0.14	0.21
SS	Chinese Spring	150 mM NaCl	Full tillering	1852	0.72	0.13	0.15
NE	Chinese Spring	None	Etiolated 5-day seedlings	6048	0.57	0.13	0.30
NRT	Chinese Spring	None	Seedling root tip	757	0.67	0.15	0.18
AS1	Chinese Spring	1 ppm Al	Seedling root tip	805	0.63	0.15	0.22
AS3	BH1146	3 ppm Al	Seedling root tip	879	0.62	0.12	0.27
AT	Atlas	None	8-day seedlings	949	0.54	0.03	0.44
Nov67	Novosibirskaya 67	None	Unspecified	421	0.37	0.10	0.53

[a] Described in Lazo et al. (2004) and Chao et al. (2006).
[b] Maximum score of e-20 (Altschul et al., 1997).
[c] Number of singletons plus non-overlapping contigs.
[d] Fraction of ESTs matching GenBank accessions with annotated functions.
[e] Fraction of ESTs matching hypothetical or expressed genes from GenBank.
[f] Relative water content, considered as drought stress.