

## Comparative Gene Expression Analysis of Susceptible and Resistant Near-Isogenic Lines in Common Wheat Infected by *Puccinia triticina*

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Edited by Satoshi Tabata  
(Received 2 February 2010; accepted 5 March 2010)

### Abstract

**Gene expression after leaf rust infection was compared in near-isogenic wheat lines differing in the *Lr10* leaf rust resistance gene. RNA from susceptible and resistant plants was used for cDNA library construction. In total, 55 008 ESTs were sequenced from the two libraries, then combined and assembled into 14 268 unigenes for further analysis. Of these ESTs, 89% encoded proteins similar to (*E* value of  $\leq 10^{-5}$ ) characterized or annotated proteins from the NCBI non-redundant database representing diverse molecular functions, cellular localization and biological processes based on gene ontology classification. Further, the unigenes were classified into susceptible and resistant classes based on the EST members assembled from the respective libraries. Several genes from the resistant sample (14-3-3 protein, wali5 protein, actin-depolymerization factor and ADP-ribosylation factor) and the susceptible sample (brown plant hopper resistance protein, caffeic acid *O*-methyltransferase, pathogenesis-related protein and senescence-associated protein) were selected and their differential expression in the resistant and susceptible samples collected at different time points after leaf rust infection was confirmed by RT-PCR analysis. The molecular pathogenicity of leaf rust in wheat was studied and the EST data generated made a foundation for future studies.**

**Key words:** wheat; leaf rust; ESTs; resistance; susceptible

### 1. Introduction

Leaf rust (*Puccinia triticina*) is the most common rust disease in bread wheat (*Triticum aestivum* L.), causing significant yield losses over large geographical areas.<sup>1</sup> Yield losses in wheat from this rust are usually the result of a decreased number of kernels per head and lower kernel weight. Genetic resistance is the preferred method to reduce losses from leaf rust, and 60 leaf rust resistance (*Lr*) genes have been designated in wheat.<sup>2</sup> Among the *Lr* genes, *Lr1*, *Lr10*, *Lr21* and *Lr34* were isolated and characterized through a

map-based cloning approach.<sup>3–6</sup> Most *Lr* genes confer race-specific resistance in a gene-for-gene manner. However, wheat varieties relying on race-specific resistance often lose effectiveness within a few years by imposing selection for virulent leaf rust races. High levels of virulence variation and broad adaptation of the fungus to the diverse climatic conditions where wheat is grown are characteristic of *P. triticina* and contribute to regular losses from leaf rust in global wheat production. Plant disease resistance and susceptibility are governed by the combined genotypes of host and pathogen and depend on a

complex exchange of signals and responses occurring under given environmental conditions (for review, see Yang *et al.*<sup>7</sup>). During the long process of host–pathogen co-evolution, plants have developed mechanisms to defend against pathogen attack. Whereas some of these defense mechanisms are preformed and provide physical and chemical barriers that inhibit pathogen infection, others are induced only after pathogen attack. Similar to animal immune responses, induced plant defense involves a network of signal transduction and rapid activation of gene expression following pathogen infection. A key difference between the resistant and susceptible plants is the timely recognition of the invading pathogen and the rapid and effective activation of host defense mechanisms. A resistant plant is capable of rapidly deploying a wide variety of defense responses that prevent pathogen colonization. In contrast, a susceptible plant exhibits much weaker and slower responses that fail to restrict pathogen growth and/or spread. As a result, a susceptible plant is often severely damaged or even killed by pathogen infection. The activation of defense responses in plants is initiated by the host recognition of pathogen-encoded molecules called elicitors. The interaction of pathogen elicitors with host receptors (many of which may be encoded by resistance genes) likely activates a signal transduction cascade that may involve protein phosphorylation, ion fluxes, reactive oxygen species (ROS) and other signaling events. Both primary pathogen elicitors and secondary endogenous signals may activate a diverse array of plant protectant and defense genes, whose products include glutathione *S*-transferase, peroxidases, cell wall proteins, proteinase inhibitors, hydrolytic enzymes (e.g. chitinases and  $\beta$ -1,3-glucanases), pathogenesis-related (PR) proteins and phytoalexin biosynthetic enzymes, such as phenylalanine ammonia-lyase and chalcone synthase.<sup>8</sup>

Despite the impact of leaf rust pathogenesis in wheat, very little information is available at the molecular level regarding how this pathogen regulates growth, development and pathogenesis. For this reason, the identification of differentially represented genes between near-isogenic wheat lines differing in the *Lr10* leaf rust resistance gene is essential to understand the biological events of the wheat–leaf rust interaction.

Apart from a few species for which a complete genome sequence is already available, gene discovery in most plants is primarily based on the sample sequencing of expressed sequence tags (ESTs) and the development of EST databases has been recognized as a rapid method of sampling an organism's transcriptome. In the large (16 Gb) and repetitive genome of bread wheat, the transcriptome approach is complementary to a whole genome sequencing

project. We have initiated a wheat EST project in Japan and constructed cDNA libraries from various tissues and strains of wheat after various treatments.<sup>9–11</sup> Two of the libraries were constructed to comprehensively analyze gene expression following leaf rust infection. In this study, we aimed to identify differential gene expression patterns for resistant or susceptible interactions based on the *Lr10* resistance gene.

## 2. Materials and methods

### 2.1. Plant and fungal material

The wheat cultivars Thatcher (susceptible) and its near-isogenic line (NIL) with the leaf rust resistance gene *Lr10*, ThatcherLr10 (resistant), were used for this study. Thatcher is highly susceptible to leaf rust and the resistance gene (*Lr10*) from the resistance source *cv.* AC domain was transferred to the Thatcher background (NIL).<sup>12</sup> The Swiss leaf rust race BRW 97512-19 avirulent on *Lr10* was used for infection.<sup>13</sup>

### 2.2. cDNA library construction and DNA sequencing

Total RNA was extracted 20 h after infection with leaf rust isolate BRW 97512-19 (*AvrLr10*) at the seedling stage (10 days old) of susceptible and resistant cultivars. Two cDNA libraries were constructed using the *pBluescript SK II (+)* vector in *Escherichia coli DH10B*. For libraries derived from resistant and susceptible interactions, 30 307 and 24 701 clones, respectively, were randomly selected and sequenced from both the 5' and 3' ends of the inserts.

### 2.3. Data processing and computational methods

Chromatogram files of the two libraries were separated into forward (5') and reverse (3') raw sequences, and the orientation was incorporated by a PERL script we designed. Base calling was done using the Phred program.<sup>14</sup> The vector sequence was removed by the crossmatch program (-minmatch 20, -minscore 20). Repeat and ambiguous sequences (Phred quality values <30) were eliminated using our PERL script. Poly(A) tails or poly(T) sequences (at most 10 bases) in the ESTs were processed. Subsequently, ESTs with sequences <30 bp were omitted from the final data set. The remaining high-quality sequences were used for further study and are available from the DNA Database of Japan (accession numbers CJ872808–CJ919993).

### 2.4. EST assembly

The processed ESTs from susceptible and resistant libraries were combined and assembled into contigs

using the CAP3 program<sup>15</sup> at a high stringency level of 95% homology in a 20-bp overlap. The contigs were then classified into a susceptible and a resistant group based on the total EST members assembled from the susceptible and resistant libraries. Local gene cluster (BLAST) was performed by including the *Lr10* and other published resistance genes with the parameters of -b 10, -v 10, -e 1e-40.

### 2.5. Gene annotation

Using the BLAST program (blastx with a search threshold of 1e-5), the sequences of the contigs were searched against the *T. aestivum* gene index from DFCI<sup>16</sup> and the amino acid sequence databases from NCBI (nr) and UniProt. Gene ontology (GO) terms<sup>17</sup> for contigs were derived by InterProScan software<sup>18</sup> and converted to GO Slim terms by a custom-built PERL script.

### 2.6. Reverse transcription-PCR

Susceptible and resistant seedlings were grown under 16 h light at 20°C and 8 h dark at 16°C with 70–80% humidity for 10 days. Seedlings were infected with leaf rust spores of the avirulent isolate BRW 97512-19 on *Lr10* after 10 days and maintained overnight at 16°C with 90% humidity in the dark, followed by normal growth conditions. The leaf samples were collected at time points of 0, 8, 16, 20 and 48 h after infection and RNA was extracted. cDNA from each sample was synthesized from 1 µg of total RNA and reverse transcription-PCR (RT-PCR) was carried out.

## 3. Results

### 3.1. Library construction and DNA sequencing

Two NILs of hexaploid wheat infected with leaf rust and differing in the leaf rust resistance gene *Lr10* were used for library construction. For the resistant and susceptible libraries, 30 307 and 24 701 randomly selected clones were sequenced from both the 3' and 5' ends. In all, 55 008 sequences were generated, and after trimming for low quality, shortness (<90 bp) and vector contamination, 39 928 of those sequences were used for assembly. The overall sequencing success rate, i.e. useful sequences out of the total sequenced, was 72.5%. The length of good-quality sequences varied between 40 and 860 bp, with an average of 354 bp. The good-quality ESTs from the two libraries were combined (39 928 ESTs) and assembled into 6042 contigs and 8226 singlets. The number of ESTs forming each contig varied between 2 and 556, and the average ESTs grouped into contigs varied from 2 to 5 (Fig. 1). The number of specific unigene sequences for each

library was calculated (only for contigs). The library-specific sequences can be considered an estimation of the capacity to provide new genes. The contribution of individual libraries to the total unigene set was 36% for resistant and 31% for susceptible, indicating that each library contained specific ESTs (Fig. 2).

### 3.2. Sequence annotation and analysis

cDNA libraries were constructed from susceptible and resistant cultivars after leaf rust infection with the goal of obtaining a diverse collection of ESTs representing a range of molecular functions. Unigenes were annotated using a *T. aestivum* gene index for gene searches, UniProt for protein searches and InterProScan for GO terms. To ensure the highest recovery of annotation, we submitted all unigenes derived from contigs and singletons for analysis. In total, 6004 sequences had no BLAST hit with an *E* value of  $\leq 10^{-5}$ . Out of these, 4506 sequences contained one or more predicted open reading frames of at least 75 amino acids. The sequences that have coding potential but do not share significant

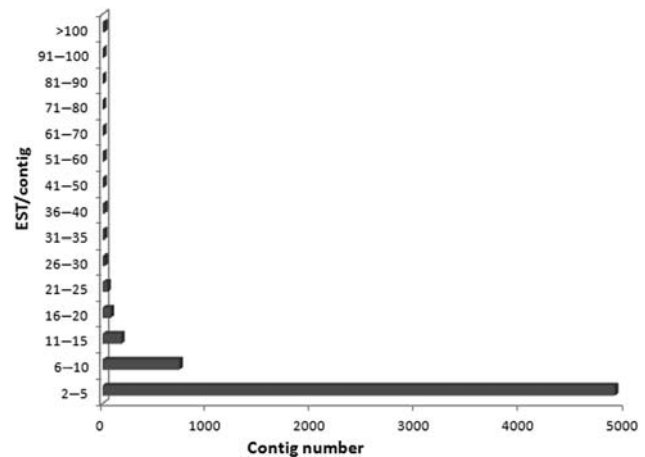


Figure 1. EST distribution in each class of contigs.

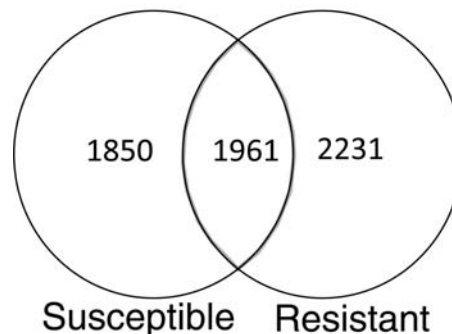


Figure 2. Venn diagram showing the number of susceptible, resistant and common genes expressed during leaf rust infection in susceptible and resistant samples.



homology to deposited sequences could represent genes unique to wheat or the leaf rust pathogen.

In the process of functional annotation of the putative-encoded proteins, unigenes were classified into biological, molecular and cellular function-based GO terms. When analyzed by biological process, the majority of annotations (31%) were involved in metabolism, followed by cellular process (25%). The remainder (44%) were distributed among several processes, including housekeeping functions, growth and the regulation of development (Fig. 3a).

The class of proteins 'catalytic and binding activities' comprised over 50% of the total number of molecular functions identified by GO analysis (Fig. 3b). In addition to this general molecular process, hydrolases, transferases and oxidoreductases were identified. Hydrolases, which utilize water molecules to break chemical bonds, perform a broad range of functions in fungi, including the extracellular digestion of complex carbon sources such as cellulose and other components of plant cell walls. Oxidoreductases catalyze the transfer of electrons between molecules and, in fungi, are involved in primary and secondary metabolism as well as the detoxification of compounds such as ROS, superoxide and hydrogen peroxide. Intriguingly, these same compounds are frequently associated with the oxidative burst component of plant defense.<sup>19</sup> Although it is reasonable to propose that oxidoreductases of fungal foliar pathogens could be involved in detoxification of ROS during pathogenesis, such a relationship has not been demonstrated in wheat.

The vast majority of annotated sequences are predicted to encode cellular and intracellular proteins (Fig. 3c). However, nearly 1% of sequences were categorized by GO annotation as comprising external encapsulating structures, defined as any constituent of a structure that lies outside the plasma membrane and surrounds the entire cell.<sup>20</sup> Unlike bacteria, filamentous fungi generally produce highly hydrophobic proteins (collectively referred to as hydrophobins) rather than polysaccharide capsules as a protective barrier against the environment.

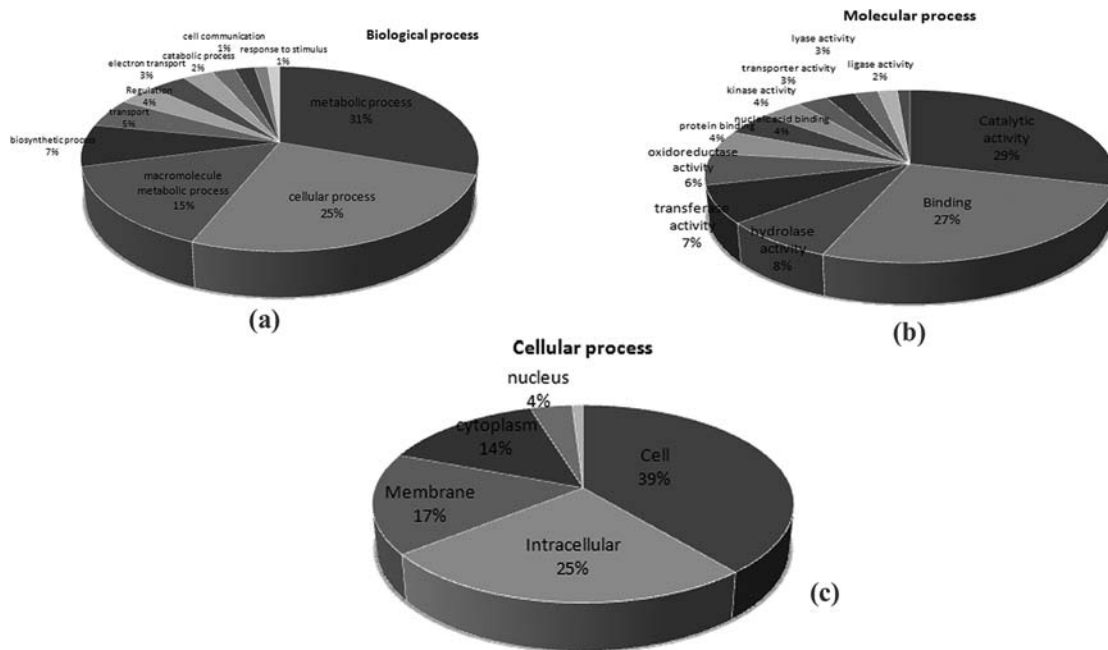
### 3.3. Genes derived from the leaf rust pathogen

To estimate the proportion of fungal ESTs in the libraries used in the present study, the unigenes were also compared with a *P. graminis* f.sp. *tritici* database ([http://www.broadinstitute.org/annotation/genome/puccinia\\_group/Multihome.html](http://www.broadinstitute.org/annotation/genome/puccinia_group/Multihome.html)). The BLAST comparison detected only a few genes from the susceptible sample. Consistent with the findings of many fungal EST projects, a substantial number of sequences could not be annotated due to either a lack of BLAST hits or hits to uncharacterized fungal

sequences.<sup>21,22</sup> Of the sequences with BLAST hits, well over half could not be annotated due to a general lack of knowledge regarding the specific molecular functions of many fungal genes. However, a recent review<sup>23</sup> and other studies<sup>24,25</sup> have reported a comprehensive view of genes from leaf rust and related pathogens. Among these genes, chitinase, catalase,  $\beta$ -tubulin, thiazole and 40S ribosomal sequences were found in this study and were derived mainly from the susceptible sample. Twenty-eight unigenes encode fungal ribosomal protein, presumably indicative of active protein synthesis during the infection process. A cDNA-AFLP approach at several time points after wheat inoculation with leaf rust identified the chitinase gene.<sup>24</sup> The chitinase gene had homology to the same gene from the bean rust *Uromyces fabae*, which is induced in haustoria.<sup>26</sup> Recently, an EST database was developed representing each of several life-cycle stages of *P. triticina*.<sup>24</sup> The presumed fungal cDNAs encoded proteins involved in general metabolism, protein synthesis and transport and stress- or virulence-related proteins (e.g. chitinase, catalase,  $\beta$ -tubulin and thiazole). In addition, a few genes were homologous to gene encoding virulence factors in other fungi or that are induced during infection with other fungal pathogens.

### 3.4. Highly differentially expressed sequences

Consensus sequences consisting of ESTs found exclusively or predominantly in either susceptible or resistant libraries could reveal molecular mechanisms involved in regulating fungal development or the resistance reaction, and the library-to-library distribution of ESTs corresponding to a single cluster offers at least a qualitative measure of gene expression. The consensus sequences that consisted of at least 10 ESTs that were substantially enriched in either the susceptible or resistant library were defined as highly differentially expressed sequences (HDEs). Twenty-five and 24 consensus sequences from resistant and susceptible libraries, respectively, met those criteria (Tables 1 and 2). Three of the resistant-specific HDEs returned no hits after a BLAST search, indicating that these sequences may be unique resistance genes in wheat. The GO terms for 12 resistant genes were not identified, showing that these sequences may be specific to leaf rust infection in wheat. The further study on the molecular function of individual genes will give the clear result. The others, *wali5*, thioredoxin, barley 14-3-3 protein, *wrsi5-1* protein, peroxisomal membrane protein, glutamate dehydrogenase, ADP-ribosylation protein, Tubby-like F-box protein genes and other cell wall-specific genes, were found in resistant samples. Three unknown function HDEs were



**Figure 3.** Functional annotation of contig sequences based on GO categorization. Sequences were evaluated for their predicted involvement in a biological process (a), molecular function (b), and cellular localization (c).

identified in susceptible samples, indicating these sequences may be unique to the fungal pathogen or to the self-defense mechanism in wheat. In contrast to the resistant genes, only four susceptible genes were resulted without GO terms. But the study of those genes will give further understanding of susceptible mechanism in plants. In addition to multiple genes of chlorophyll *a/b*-binding protein, brown plant hopper (BPH)-induced resistance protein, wheat ribulose-1,5-biphosphate carboxylase, caffeic acid *O*-methyltransferase (OMT), senescence-associated protein, *O*-acetylserine lyase, calreticulin-like protein and PR protein were found in susceptible samples. When comparing the compatible (susceptible) and incompatible (resistant) genes in terms of its molecular function showed the catalytic and binding activities in common, but the action of oxidative burst was specific to the resistant sample. The striking event occurred early after pathogen infection is the rapid production and accumulation of ROS, such as  $O_2^-$  and  $H_2O_2$ , known as the oxidative burst.<sup>19</sup> Elicitation of the oxidative burst by pathogen elicitors appears to be mediated by multiple signal cascades in addition to oxalate oxidase and cell wall peroxidase genes.<sup>27</sup> The enrichment of ESTs corresponding to a specific cluster sequence in one set of libraries over the other could reflect differences in reaction mechanism against the leaf rust pathogen. To correlate the occurrence and distribution of ESTs with levels and patterns of gene expression, RT-PCR was performed. Four resistant and four susceptible group

genes were selected and their expression was studied in resistant and susceptible samples collected in the same time course after infection (Fig. 4). The genes were chosen based on their role in plant defense against biotic or abiotic stress. The 14-3-3 protein gene is found in all eukaryotes and functions by interacting directly with numerous target proteins, thereby altering their activity.<sup>28</sup> The *wali5* gene has been identified for its role in aluminum tolerance in wheat.<sup>29</sup> Actin-depolymerization factor and ADP-ribosylation factor genes have been reported as resistance genes in other crops.<sup>30</sup> BPH resistance protein, identified in susceptible samples, contains a CBS domain. In rice, a CBS domain containing gene (*OsBi1*) is induced in response to herbivore feeding.<sup>31</sup> Another gene, caffeic acid OMT, responded differently in susceptible samples in different time period. The induction of an OMT gene by wounding or pathogen invasion suggests the deposition of lignin, which provides a barrier for protection of tissues from damage. Two other susceptible genes are PR and senescence-associated DIN1 protein.<sup>32,33</sup>

### 3.5. Characterization of ESTs potentially associated with plant-pathogen interaction

ESTs corresponding to genes encoding proteins potentially related to the plant-pathogen interaction were characterized from wheat-leaf rust unigene set collections (30 genes, Table 3). Resistant and susceptible libraries were sequenced to obtain information

**Table 1.** Functional annotation of unigenes expressed in resistant sample

| Unigene | Number of ESTs in libraries |     | Functional annotation                                 | E value   |
|---------|-----------------------------|-----|---|-----------|
|         | Res                         | Sus |   |           |
| Ct286   | 33                          | 2   | wali5 protein ( <i>Triticum aestivum</i> )            | 9.00E-41  |
| Ct1828  | 27                          | 0   | Thioredoxin M ( <i>Triticum aestivum</i> )            | 1.00E-97  |
| Ct1043  | 20                          | 0   | HvB12D ( <i>Hordeum vulgare</i> )                     | 2.00E-43  |
| Ct3233  | 19                          | 0   | Hv14-3-3b ( <i>Hordeum vulgare</i> )                  | 2.00E-142 |
| Ct1593  | 18                          | 0   | wrsi5-1 protein ( <i>Triticum aestivum</i> )          | 8.00E-42  |
| Ct1619  | 18                          | 0   | Photosystem II reaction center Z protein              | 1.00E-25  |
| Ct546   | 17                          | 0   | Hypothetical protein                                  | 4.00E-123 |
| Ct2571  | 17                          | 0   | Protein translation factor SU11 ( <i>Zea mays</i> )   | 5.00E-57  |
| Ct791   | 16                          | 0   | WAP1 aspartic proteinase ( <i>Triticum aestivum</i> ) | 0         |
| Ct14    | 15                          | 1   | hblt14.2 protein ( <i>Hordeum vulgare</i> )           | 4.00E-20  |
| Ct1154  | 15                          | 1   | ATP-dependent Clp protease ( <i>Oryza sativa</i> )    | 3.00E-81  |
| Ct1546  | 15                          | 0   | Peroxisomal membrane protein PEX11-1                  | 6.00E-111 |
| Ct2311  | 13                          | 0   | Protein nifU  | 2.00E-114 |
| Ct1104  | 12                          | 0   | Molybdenum cofactor biosynthesis protein Cnx1 Barley  | 1.00E-30  |
| Ct1565  | 12                          | 1   | Polyubiquitin containing 7 ubiquitin monomers-Maize   | 3.00E-124 |
| Ct2654  | 12                          | 0   | Ubiquitin-conjugating enzyme E2-17 kDa                | 1.00E-84  |
| Ct3047  | 12                          | 1   | Glutamate dehydrogenase 2                             | 4.00E-104 |
| Ct2025  | 14                          | 1   | Actin-depolymerization factor                         | 7.60E-84  |
| Ct635   | 11                          | 0   | Cell wall-associated hydrolase                        | 1.00E-42  |
| Ct1287  | 11                          | 1   | Hypothetical protein                                  | 6.00E-10  |
| Ct1839  | 11                          | 0   | Putative MtN19 ( <i>Oryza sativa</i> )                | 0         |
| Ct1870  | 11                          | 0   | Hypothetical protein                                  | 8.00E-14  |
| Ct2301  | 10                          | 0   | RGH1A ( <i>Hordeum vulgare</i> )                      | 4.00E-37  |
| Ct2982  | 10                          | 1   | Full = Tubby-like F-box protein 12                    | 5.00E-66  |
| Ct5783  | 18                          | 6   | ADP-ribosylation factor                               | 1.30E-71  |

Ct, contig; Res, resistant; Sus, susceptible.

on genes whose expression is potentially related to the plant-pathogen interaction. We took advantage of these two libraries not being normalized to compare their gene expression. Owing to the relatively thorough sampling approach, the frequency of ESTs in a given library can be used to obtain information on relative gene expression levels in the tissues from which the library was constructed.<sup>34</sup> Table 3 lists the potential wheat-leaf rust interaction genes, showing differences in the EST members assembled from the susceptible and resistant libraries. On the basis of the number of ESTs, the genes were further classified as resistance, susceptible and common genes. In the resistance group, the most abundant contig members were found in proton ATPase followed by calcium-binding protein. Various fungal elicitors have been reported to trigger fluxes of protons across the plasma membrane.<sup>35</sup> Sequences related to resistance to pathogens (protease inhibitors and the specific Bowman-Birk type wound-induced proteinase inhibitors) and to

detoxification (ABC transporter), which play a major role in plant-pathogen defense, were encountered. Genes related to cell wall biosynthesis and metabolism, such as cysteine proteinase, phenylalanine ammonia-lyase, plasma membrane ATPase and chalcone synthase, were also found and may be related to resistance mediated by alteration of plant cell wall composition. CDP and MAP kinases were the signal transduction genes found in the resistance reaction. In susceptible reaction, mainly genes with functions related to the oxidative burst (glutathione S-transferase, peroxidase and oxalate oxidase) were found. Different groups of transcription factors (Myb, WRKY, EREB/AP2, bZIP) and sequences related to responses against pathogens (receptor-like kinase, GTP-binding protein, RING finger protein, cytochrome P450, LRR, PR protein, G-box binding protein, STAR-related lipid transfer protein and starch synthase) were involved in both the susceptible and resistant samples infected with the leaf rust pathogen. Among these, STAR-related lipid transfer and RING finger

**Table 2.** Functional annotation of unigenes expressed in susceptible sample

| Unigene | Number of ESTs in libraries |     | Functional annotation  | E value   |
|---------|-----------------------------|-----|--|-----------|
|         | Res                         | Sus |  |           |
| Ct4160  | 0                           | 36  | Putative brown planthopper-induced resistance protein 1              | 2.00E-67  |
| Ct5199  | 1                           | 34  | Wheat ribulose-1,5-bisphosphate carboxylase                          | 7.00E-84  |
| Ct4748  | 0                           | 28  | Caffeic acid O-methyltransferase; LPOMT2 ( <i>Lolium</i> sp.)        | 5.00E-149 |
| Ct5424  | 2                           | 22  | Chlorophyll <i>a/b</i> -binding protein WCAB precursor               | 2.00E-141 |
| Ct4440  | 0                           | 19  | Photosystem I hydrophobic protein ( <i>Hordeum vulgare</i> )         | 1.00E-106 |
| Ct5191  | 0                           | 19  | Membrane protein-like ( <i>Triticum aestivum</i> )                   | 1.00E-78  |
| Ct3844  | 0                           | 17  | Type III LHCII CAB precursor protein ( <i>Hordeum vulgare</i> )      | 5.00E-121 |
| Ct3524  | 0                           | 16  | Full = autophagy-related protein                                     | 2.00E-57  |
| Ct4370  | 0                           | 15  | Chlorophyll <i>a/b</i> -binding protein ( <i>Triticum turgidum</i> ) | 5.00E-133 |
| Ct4331  | 1                           | 12  | Senescence-associated protein DIN1 ( <i>Zea mays</i> )               | 1.00E-40  |
| Ct4898  | 1                           | 12  | O-acetylserine(thiol)lyase ( <i>Triticum aestivum</i> )              | 1.00E-164 |
| Ct4039  | 0                           | 11  | Hypothetical protein   | 2.00E-57  |
| Ct4730  | 0                           | 11  | Ubiquitin-conjugating enzyme E2-17 kDa 9 ( <i>Zea mays</i> )         | 1.00E-82  |
| Ct5852  | 0                           | 11  | Zinc finger A20 domain containing stress-associated protein          | 5.00E-76  |
| Ct5624  | 1                           | 11  | Calreticulin-like protein ( <i>Triticum aestivum</i> )               | 1.00E-109 |
| Ct3689  | 0                           | 10  | Chlorophyll <i>a/b</i> -binding protein LHCb1.2                      | 5.00E-133 |
| Ct4194  | 0                           | 10  | Chlorophyll <i>a/b</i> -binding protein WCAB precursor               | 4.00E-142 |
| Ct4611  | 0                           | 10  | Full = 60S ribosomal protein L5-1                                    | 1.00E-118 |
| Ct4769  | 0                           | 10  | Pathogenesis-related protein 1.2 ( <i>Triticum aestivum</i> )        | 2.00E-98  |
| Ct4911  | 0                           | 10  | Hypothetical protein   | 2.00E-116 |
| Ct5495  | 0                           | 10  | Hypothetical protein   | 9.00E-24  |
| Ct1862  | 1                           | 10  | Senescence-associated protein DIN1 ( <i>Zea mays</i> )               | 1.00E-116 |
| Ct3270  | 1                           | 10  | Ran-related GTP-binding protein ( <i>Festuca arundinacea</i> )       | 7.00E-61  |

Ct, contig; Res, resistant; Sus, susceptible.

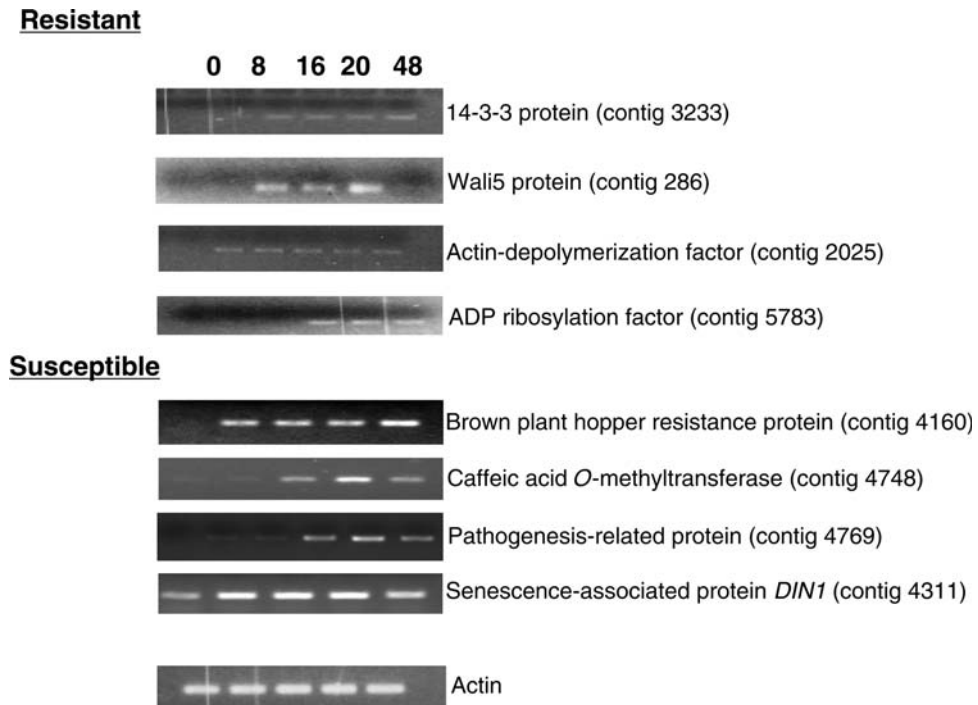
protein have been reported as candidate genes for the *Lr34* locus.<sup>6</sup>

#### 4. Discussion

Genetic studies of leaf rust resistance in wheat have been conducted by wheat researchers worldwide. In this study, we aimed to identify host–pathogen relationships between susceptible vs. resistant wheat and leaf rust (*P. triticina*). For this purpose, EST libraries were constructed from the leaf rust susceptible (Thatcher) and the resistant NIL for Thatcher containing the *Lr10* gene. This represents an ideal situation for comparative transcriptome studies in hexaploid wheat.<sup>23</sup> The EST sequencing approach is of particular interest in organisms for which very little sequence data are available. As far as is known, the wheat–leaf rust interaction data presented here are therefore the first effort in sequencing of the expressed genome aimed at understanding leaf rust disease. An overview for the unigene development methodology is presented in Fig. 5. A unigene set of 17 268 sequences was

generated from 39 928 high-quality sequences and showed a redundancy of ~35%, a value corresponding to the expected level compared with other published works. Equivalent or lower redundancy levels have been reported in wheat ([http://wheat.pw.usda.gov/NSF/library\\_redundancy.html](http://wheat.pw.usda.gov/NSF/library_redundancy.html)), whereas other published libraries showed higher levels of redundancy, such as 72.5% in *Lotus japonicas*.<sup>36</sup> To reach a high quality of annotation and to avoid error propagation,<sup>37</sup> the EST clusters were annotated systematically using a semi-automated approach, in which a functional annotation is assigned after human examination of the results of various automated analyses, as described by Journet *et al.*<sup>38</sup> Using this classification scheme, a putative functional category could be assigned to ~89% of the unique sequences. The most prevalent categories were the metabolic and cellular processes in biological function and catalytic and binding activities in molecular processes. This result could be correlated with the higher level of sequences related to plant reaction to the pathogen. But the difference between the susceptible and resistant samples against the pathogen was the generation of ROS in later case. The generation of





**Figure 4.** Gene expression studies of candidate genes. Representative contigs were selected and analyzed by RT-PCR. Susceptible (Thatcher) and resistant (ThatcherLr10) samples were collected 0, 8, 16, 20 and 48 h after leaf rust infection. Resistant and susceptible samples are shown for their respective genes. Actin was used as the internal control.

ROS is likely dependent on the cultivation of a plasma membrane NADPH oxidase similar to that present in mammalian phagocytes. Two distinct phases of ROS production have been observed during plant–pathogen interaction. The first burst occurs within minutes in both susceptible and resistant interaction, whereas the second, sustained burst occurs within a few hours of infection and only in a resistant interaction.<sup>39</sup>

For the libraries constructed from plants infected by leaf rust, in particular for the susceptible library, pathogen tissue was not separated from host tissue. Therefore, it could be expected that a portion of the sequences derived from this library was of fungal, not wheat, origin. Therefore, the proportion of fungal ESTs in the present libraries was estimated. Only a few sequences from the susceptible library demonstrated a high probability of corresponding to leaf rust genes, which represents only 0.05% of the susceptible sequence set, less than the proportion of ESTs of fungal origin observed in a similar analysis from citrus-*Phytophthora* and cacao-*Moniliophthora perniciosa* interaction.<sup>40,41</sup> These data suggest that only a small fraction of the sequences obtained from the pathogen-challenged libraries were derived from the pathogen.

By examining sequences to the total unigene, it was possible to identify ~25 HDEs each related to the susceptible and resistant plant–pathogen interactions (Fig. 6). Out of 25 unigenes in the resistant

interaction, three were new and four were previously identified wheat genes. *wali5*, *wrsi5-1*, *WAP1* and actin-depolymerization factor were identified as abiotic stress responsive genes in wheat.<sup>29,30,42</sup> This observation suggests the different stress-signaling pathways are also cross-talking with each other, which can lead plants to cross-tolerance. Cross-tolerance is defined as a biological phenomenon by virtue of which a plant that is resistant to one stress is able to develop tolerance to another form of stress.<sup>43</sup> Capiati *et al.*<sup>44</sup> reported that wounding increases salinity tolerance in tomato plants, suggesting cross-talk between these stresses. The resistance-specific expression of *wali5* and the actin-depolymerization factor gene was confirmed by RT-PCR. Actin-depolymerization factor family members can be described as stimulus-responsive modulators of actin cytoskeleton dynamics. Several cellular processes are associated with the reorganization of the actin cytoskeleton in plants. Actin filaments are tightly linked to the plasma membrane and are believed to be involved in signal transduction events in plants.<sup>45</sup> 14-3-3 protein was another gene confirmed through the expression study. This protein comprises a family of highly conserved proteins with central roles in many eukaryotic signaling networks<sup>28</sup> (Fig. 6). In barley, 14-3-3 transcripts accumulate in the epidermis when leaves are inoculated with the fungus causing powdery mildew (*Erysiphe graminis*



**Table 3.** Wheat–leaf rust interaction genes

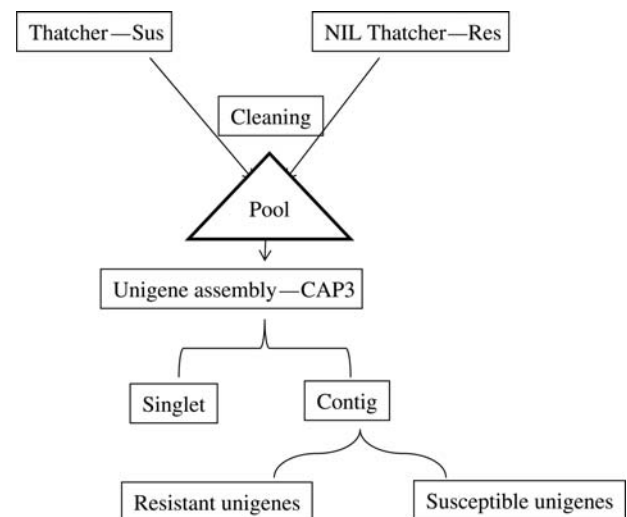
| Plant pathogen interaction gene     | Number of contigs | Res EST | Sus EST |
|-------------------------------------|-------------------|---------|---------|
| <b>Resistant genes</b>              |                   |         |         |
| Proton ATPase                       | 30                | 62      | 28      |
| Ca-binding protein                  | 27                | 62      | 28      |
| PI                                  | 20                | 221     | 99      |
| Cysteine proteinase                 | 19                | 92      | 37      |
| ABC transporter                     | 14                | 42      | 20      |
| Phenylalanine ammonia-lyase         | 11                | 30      | 15      |
| Bowman–Birk type wound-induced PI   | 10                | 169     | 63      |
| Plasm ATPase                        | 8                 | 40      | 27      |
| 14-3-3 protein                      | 12                | 82      | 55      |
| CDPK                                | 10                | 27      | 6       |
| MAPK                                | 3                 | 10      | 2       |
| Leucine zippers                     | 3                 | 7       | 2       |
| Chalcone synthase                   | 1                 | 4       | 0       |
| <b>Susceptible genes</b>            |                   |         |         |
| GST <sup>a</sup>                    | 30                | 160     | 223     |
| Peroxidase                          | 53                | 159     | 204     |
| Oxalate oxidase <sup>a</sup>        | 10                | 18      | 38      |
| <b>Common genes</b>                 |                   |         |         |
| Transcription factor                | 36                | 57      | 45      |
| Receptor-like kinase                | 32                | 51      | 46      |
| GTP-binding protein                 | 30                | 70      | 65      |
| RING finger protein                 | 28                | 52      | 45      |
| CYP                                 | 21                | 58      | 45      |
| LRR                                 | 9                 | 12      | 9       |
| PR protein                          | 13                | 41      | 39      |
| NBS                                 | 8                 | 11      | 11      |
| Myb-like prot and tran factor       | 7                 | 8       | 7       |
| Ethylene res trans factor           | 5                 | 6       | 4       |
| NADPH                               | 3                 | 4       | 2       |
| G-box-binding factor                | 3                 | 5       | 7       |
| STAR-related lipid transfer protein | 2                 | 2       | 8       |
| Starch synthase                     | 1                 | 0       | 2       |

The contigs were grouped based on their function related to plant pathogenesis. The order is based on the number of contigs in each gene. GST, glutathione *S*-transferase; CYP, cytochrome P450; PR, pathogenesis-related; LRR, leucine-rich repeats; NBS, nucleotide binding site.

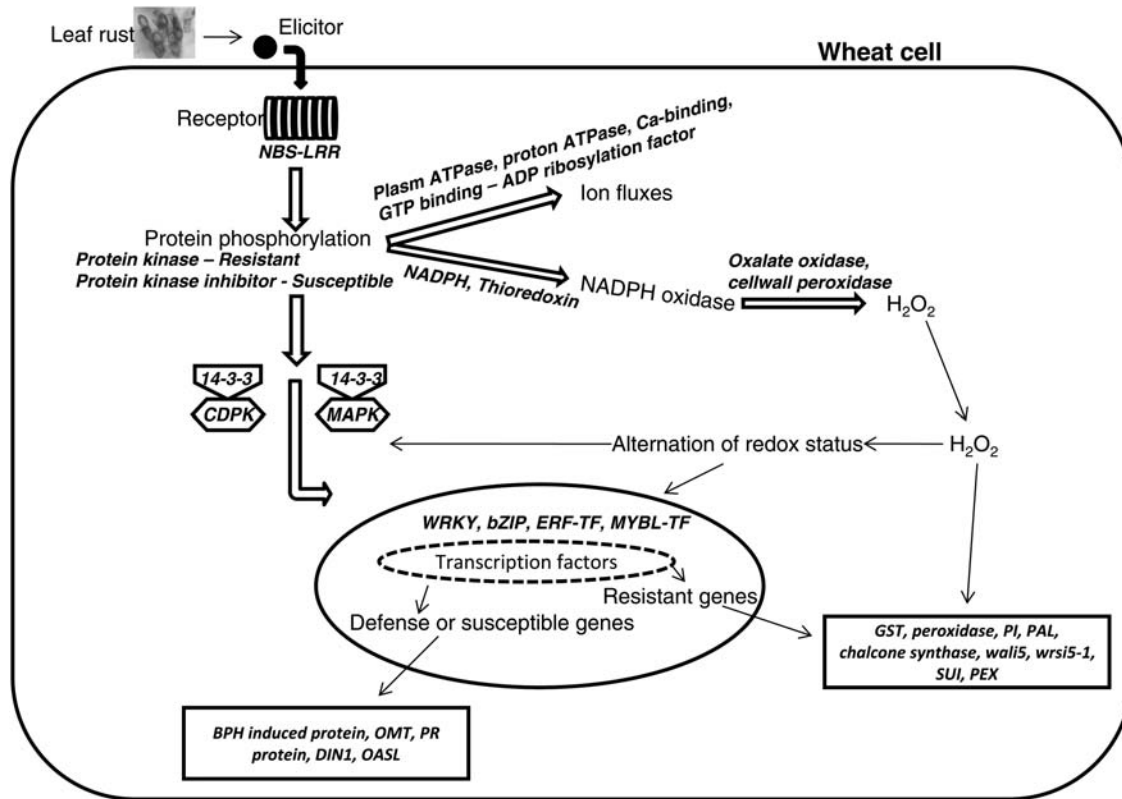
<sup>a</sup>Genes derived only from wheat.

f.sp. *tritici*); they bind to and activate plasma membrane proton ATPase, and they create a binding site for the phytotoxin fusicoccin.<sup>46</sup>

In the susceptible interaction, multiple genes of chlorophyll *a/b*-binding protein were identified. Recent work to identify ESTs involved in a rice–rice

**Figure 5.** Flow chart for EST assembly and analysis.

blast fungus interaction found that transcription of photosynthetic genes, such as ribulose 1,5-diphosphate carboxylase and chlorophyll *a/b*-binding genes, was suppressed in both the susceptible and resistant interaction.<sup>47</sup> Here, we observed the more expression only in the susceptible sample that showed the unique interaction in wheat. Among the HDEs, the expression of four unigenes in susceptible samples was confirmed by RT–PCR. The CBS domain containing BPH-induced protein contained the highest numbers of ESTs from the susceptible library. Many unknown protein members in rice that correlate with abiotic stress tolerance contain the CBS domain and are differentially regulated in contrasting genotypes of rice, indicating their probable role in both biotic and abiotic stress responses.<sup>48</sup> A gene involved in the synthesis of molecules such as alkaloids (caffeic acid 3-OMT) was found in the susceptible unigene, which may be correlated with the higher content of caffeine and tannins in infected plants than in uninfected ones observed by Scarpari *et al.*<sup>49</sup> In studies of plant–pathogen interactions, special attention has focused on OMT, which is involved either in formation of precursors of lignin<sup>50</sup> or in synthesis of the diverse group of antimicrobial secondary metabolites called phytoalexins.<sup>51</sup> The enzymatic activity of both classes of OMTs has been shown to increase as a response to infection attempts by pathogens or to elicitor treatments, and, in some cases, accumulation of the corresponding gene transcripts in reacting plant tissues has been reported.<sup>52</sup> Thus, the activity of specific OMTs apparently plays an important role in plant responses to pathogens. In addition, the OMT identified in this study is a new class of OMT in wheat differed from other reported OMTs (data not shown). Senescence-associated and PR proteins expressed in response to pathogen



**Figure 6.** A simple pathway for leaf rust interaction in wheat. The genes identified in the present study were indicated in bold and italic letter. NBS-LRR, nucleolar binding site-leucine-rich repeats; GST, glutathione *S*-transferase; PI, proteinase inhibitors; PAL, phenylalanine ammonia-lyase; OASL, *O*-acetylserine(thiol)lyase; OMT, *O*-methyltransferase.

attack were encountered and confirmed by RT-PCR. Similar observation of PR gene expression was observed in wheat seedlings infected with *E. graminis* f.sp. *tritici*. (<http://www.ncbi.nlm.nih.gov/UniGene/library.cgi?ORG=Ta&LID=12158>). The accumulation of PR protein is one of the best-characterized plant defense responses. In monocots, several homologs of the dicot PR genes have been identified. In particular, PR1 and PR2 homologs have been characterized in wheat and found to be induced in both incompatible and compatible interactions with *E. graminis*.<sup>32</sup> Here, we observed PR1.2 protein expression only in susceptible or compatible reaction showing activation of the systemic acquired response. Finally, it is important to note that some other genes—with functions not related to plant-pathogen interaction, genes of unknown function or genes without homology—may also be differentially represented and may play a role in the wheat-leaf rust interaction, although they were not analyzed here.<sup>41</sup> A simple pathway was illustrated in Fig. 6 to show the molecular pathogenesis reaction of susceptible and resistant wheat lines against the leaf rust. It indicated that incompatible and compatible interactions share signaling pathways but should be induced at different times. A resistant plant is capable of rapidly deploying a

wide variety of defense responses that prevent pathogen colonization. In contrast, a susceptible plant exhibits much weaker and slower responses that fail to restrict pathogen growth and/or spread. To determine if this is the case between wheat and leaf rust as well, additional expression profiling experiments over a broader time span should need to be performed.

On the basis of the great number of genes identified in the present study, a large-scale analysis of the expression of candidate genes has been investigated. The uniformity of the genetic background used in this study to compare susceptible and resistant reaction mechanisms against leaf rust has resulted in data with a level of detail comparable to microarray studies.

In conclusion, the ESTs generated in this study provide a good tool for more studies to understand the resistant and susceptible interactions of wheat and leaf rust. Functional annotation and expression profiling implicate subsets of genes in compatibility and incompatibility of leaf rust in wheat. Genes involved in signal transduction, scavenging of ROS and other abiotic stress-related genes are strongly implicated as key determinants in wheat against incompatible pathogenic fungi. Especially, the future study of signal transduction role played by

the 14-3-3 gene by interacting with other signal cascade proteins will give new insight from this research. In compatible reaction, the role of new class of OMT, which is involved in lignin biosynthesis and cell wall fortification, is important for future research. Extensive studies on other related genes have helped to understand their role in leaf rust infection in wheat. Many new genes have been identified that can be useful for future studies. The sequences may also be a source of single-nucleotide polymorphisms or simple sequence repeats for molecular marker development.

## Funding

This work was supported by Grants-in-Aid for Scientific Research on priority areas 'Comparative Genomics' and the National Bio-resource Project from the Ministry of Education, Culture, Sports, Science and Technology of Japan. This is Contribution no. 1003 from the Kihara Institute for Biological Research, Yokohama City University.

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