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Gene Note

Comparative analysis of expression profiles of counterpart gene sets between *Brassica rapa* and *Arabidopsis thaliana* during fungal pathogen *Colletotrichum higginsianum* infection

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Abstract To perform comparative sequence and transcriptome analyses between *Brassica rapa* and *Arabidopsis thaliana*, we prepared a *B. rapa* cDNA microarray using 1,820 (ca. 2 K) cDNA clones selected from 2,166 non-redundant sequences of cDNA library of Chinese cabbage. The gene expression during infection with fungal pathogen *Colletotrichum higginsianum* and treatments with signaling molecules was analyzed using 2 K *B. rapa* and 1.2 K *Arabidopsis* cDNA microarrays. In *B. rapa*, the results suggested a large correlation coefficient between compatible pathogen *C. higginsianum*-infection and the treatment with salicylic acid, methyl jasmonate, or ethephon. The expression profiles of 145 counterpart gene sets between the *B. rapa* and *Arabidopsis* were distributed in the self-organizing map analysis. The 28% of them indicated similarities in the two species transcriptome. These expressed sequence tag (EST) and microarray data should provide a valuable resource for functional genomics on the crops.

Key words: *Arabidopsis*, *Brassica*, Chinese cabbage, counterpart gene, microarray.

Brassica includes many important vegetable crops, such as broccoli, cabbage, Chinese cabbage, cauliflower, mustard, rape, kale and turnip. Chinese cabbage (*Brassica rapa*) is an important vegetable crop in Asia. *Arabidopsis thaliana*, on the other hand, is the most widely studied plant. Sequencing of the *Arabidopsis* genomic sequence was completed in December 2000 by the *Arabidopsis* Genome Initiative (AGI) (AGI 2000). *Arabidopsis* and *Brassica* diverged 14.5–20.4 million years ago from a common ancestor (Bowers et al. 2003). Comparative genetic mapping has revealed co-linear chromosome segments (Kowalski et al. 1994; Lagercrantz et al. 1996; Paterson et al. 2000, 2001; Schmidt et al. 2001) in the family *Brassicaceae* and linkage arrangements between *Arabidopsis* and *B. oleracea* (Lukens et al. 2003). The genomes of *Brassica* species have duplicated, perhaps triplicated, counterparts of the corresponding homeologous segments of *Arabidopsis* (O'Neill and Bancroft 2000; Rana et al. 2004). *Brassica* is one of the core genera in the family *Brassicaceae*. Six *Brassica* species are cultivated worldwide; three diploids: *B. rapa* (AA, $2n=20$), *B. nigra* (BB, $2n=16$) and *B. oleracea* (CC, $2n=18$), and three amphidiploids (allotetraploids): *B. juncea* (AABB, $2n=36$), *B. napus* (AACC, $2n=38$) and *B. carinata* (BBCC, $2n=34$) (U 1935). The species *B. rapa* (syn. *campestris*) genome size is more than four times that of *Arabidopsis* and is estimated to have 529 Mb per haploid genome equivalent (Johnston et al. 2005).

In nature, although the plant interacts with many different types of microbes, only a few of them actually damage the plant. Plant diseases occur only rarely because plants have evolved sophisticated defense mechanisms against potential pathogens. Many plants defend themselves against microbial pathogens by activating both localized and systemic resistance responses. These responses include rapid localized cell death known as the hypersensitive response (Morel and Dangl 1997), cross-linking of cell wall proteins (Bradley et al. 1992), phytoalexin formation (Bailey and Mansfield 1982), and the production of other antimicrobial secondary metabolites and pathogenesis

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Abbreviations: cDNA, complementary DNA; ET, ethephon; EST, expressed sequence tag; JA, jasmonic acid; MeJA, methyl jasmonate; SA, salicylic acid.

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related-proteins (PR-proteins) (Linthorst 1991; Van Loon 1985), many of which also exert antimicrobial properties. Signaling molecules implicated in these inducible defense systems include salicylic acid (SA), jasmonic acid (JA), ethylene and reactive oxygen species (Ryals et al. 1996; Hammond-Kosack and Jones 1996). In a compatible interaction, a virulent pathogen can defeat these plant defense mechanisms, either by avoiding detection or by neutralizing host defenses. During the colonization of plant hosts, most fungal pathogens exhibit one of two modes of nutrition: biotrophy, in which nutrients are obtained from living host cells, and necrotrophy, in which nutrients are obtained from the host cells already killed by the fungus (Thrower 1966). Genetic studies with *Arabidopsis* signaling mutants have shown that an SA-dependent response is deployed against biotrophic pathogens, whereas ethylene- or JA-dependent responses are important for induced resistance to necrotrophic pathogens (Pieterse and van Loon 1999; McDowell and Dangl 2000).

Previously, we reported that inoculation of leaves of the *A. thaliana* ecotype Columbia (Col-0) with *Colletotrichum higginsianum* resulted in fungal growth, and disease symptoms reminiscent of those incited in these other cruciferous plants inoculated under the same conditions (Narusaka et al. 2004). *C. higginsianum* causes typical anthracnose lesions on the leaves, petioles and stems of turnip, mustard, and Chinese cabbage (Higgins 1917). In addition, we showed that *C. higginsianum* uses two strategies, one is biotrophy at the earliest stages of the interaction and the other is the subsequent necrotrophy (Narusaka et al. 2004). Therefore, we compared the gene expression profiles in *B. rapa* with those in *Arabidopsis* to investigate the defense strategies against the changes in the life cycle of *C. higginsianum* during the compatible interactions. These results will provide information obtained from model plants for crops.

In this study, we prepared a *B. rapa* cDNA library and cDNA microarray for the analyses of gene expression during defense responses. *B. rapa* (cultivar Kyoto No. 3; provided by Takii Seed Co. Ltd., Kyoto, Japan) seedlings were grown in soil for 16 days in a growth chamber at 22°C under a 12-h light/12-h dark cycle. A Chinese cabbage cDNA library was constructed using mRNA from the cabbage leaves treated with 5 mM SA, 1 mM ethephon (ET), 0.1 mM methyl jasmonate (MeJA), 10 mM $CuSO_4$, UV-B (0.1 kJ m^{-2}) and cold (4°C). Leaves were harvested at 5, 10 and 24 h after treatment, snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction. The cDNA library was constructed using Lambda Zap II cDNA cloning and Gigapack *in vitro* packaging (Stratagene, La Jolla, USA). The primary library size was estimated at 2.1×10^6 colony-forming units (cfu) in the library of Chinese cabbage. Consequently, 3,040 items of sequence information were obtained from the expressed sequence tag (EST) clones. The 2,166 non-redundant sequences were generated using the Sequencher ver. 4.2.2 (GeneCode, Michigan, USA). Using the BLASTX programs, with an e-value<1e-30 against the TAIR *Arabidopsis* DNA and protein databases that include coding sequences (CDS), cDNA and genomic sequences (Rhee et al. 2003), we obtained 1,235 hit sequences. In addition, using the BLASTX programs with an e-value<1e-10 or <1e-60, 1,638 or 442 sequences that correspond with *Arabidopsis* sequences, respectively. This analysis revealed that *B. rapa* cDNA showed high homology to *Arabidopsis* cDNA sequences. Then, we prepared a cDNA microarray using 1,820 (ca. 2 K) cDNA clones selected from the cDNA library of Chinese cabbage. In this study, we used an *Arabidopsis* microarray consisting of 1,200 (1.2 K) full-length cDNA clones representing putative defense-related and regulatory genes (Narusaka et al. 2006).

The *Arabidopsis* cDNA microarray analysis was carried out essentially as reported previously (Narusaka et al. 2006). The *B. rapa* cDNA microarray analysis was also carried out essentially according to the methodology. In the cDNA microarray analyses, we used ca. 2 K cDNA clones and the lambda control template DNA fragment (TX803; Takara) as an external control. The vectors used for cDNA library construction were lambda ZAP II. Inserts of cDNA clones were amplified by PCR using primers complementary to vector sequences flanking both sides of the cDNA insert, as described previously (Seki et al. 2001). PCR products were precipitated in isopropanol and the DNA was resuspended in 1×Micro Spotting Solution (Arrayit; Telechem International Inc., Sunnyvale, CA). One aliquot of the product after the reaction was electrophoresed on a 1% agarose gel to confirm amplification quality and quantity. PCR products were arrayed from 384-well microtitre plates onto a microslide glass (Super Aldehyde substrates; Telechem International Inc., Sunnyvale, CA) using a microarray stamping machine (SpotArray24; PerkinElmer, MA, USA). The blocking of printed slide glass was performed as described previously (Seki et al. 2002).

Since the life cycle of *B. rapa*, a biennial plant (winter annual), is markedly different from that of *Arabidopsis*, an annual plant, experiments were performed with *B. rapa* seedlings at the four- to five-true leaf stage and *Arabidopsis* plants at the seven- to nine-rosette leaf stage. *B. rapa* (cultivar Kyoto No. 3) was grown in soil for 16 days in a growth chamber at 22°C under a 12-h light/12-h dark cycle. *Arabidopsis* wild-type plants (Col-0) were grown in soil for 28 days in a growth chamber at 22°C under a 12-h light/12-h dark cycle. Isolate of the pathogenic fungus *C. higginsianum* (MAFF305635) was

obtained from MAFF Genebank, Japan. Plants were inoculated by placing two to four 5-μl drops of a spore suspension (5×10^5 spores ml^{-1} in distilled water) of *C. higginsianum* on each leaf. *B. rapa* was colonized by the fungus within 2–3 days, developing brown necrotic lesions surrounded by a yellow halo as well as *Arabidopsis*. Lesions spread from the inoculation site within 3–4 days, and subsequently continued to expand until they covered the entire leaf (Figure 1). These results showed that *B. rapa* (cultivar Kyoto No. 3) is susceptible to *C. higginsianum* as well as the wild-type *Arabidopsis* plant (Col-0) (Narusaka et al. 2004). For microarray analysis, plants were inoculated by spraying the leaves with a spore suspension (5×10^5 spores ml^{-1} in distilled water) of *C. higginsianum*. For other treatments, *B. rapa* and *Arabidopsis* plants were applied a foliar spray of 5 mM SA, 0.1 mM MeJA and 1 mM ET according to the methods described previously (Narusaka et al. 2006; Park et al. 2003). These treatments did not cause any chemical damage to the *B. rapa* and *Arabidopsis* plants for 24 h. Leaves were harvested at 5, 10 and 24 h after fungal inoculation and at 2, 5, 10 and 24 h after SA, MeJA or ET treatment, snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction. Total RNA was isolated as described previously (Narusaka et al. 2004). To ensure biological reproducibility of the microarray results, we replicated the experiment three to four times with nine to eighteen plants for *B. rapa* or twenty to seventy plants for *Arabidopsis* per sample. To obtain sufficient material for the experiments, we pooled samples from replicate experiments prior to RNA extraction. In addition, we conducted two to three independent microarray analyses with the same RNA for reproducibility of handling. The results demonstrated a high degree of correlation in fold change values between the different data sets.

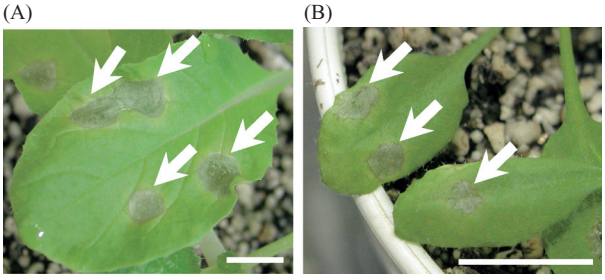

Figure 1. Infection phenotypes of *B. rapa* and *Arabidopsis* leaves inoculated with *C. higginsianum*. (A) *B. rapa* (cultivar Kyoto No.3) were grown in soil for 16 days in a growth chamber at 22°C under a 12-h light/ 12-h dark cycle. (B) *Arabidopsis* wild-type plants (Col-0) were grown in soil for 28 days in a growth chamber at 22°C under a 12-h light/ 12-h dark cycle. Plants were inoculated by placing two to four 5-μl drops of a spore suspension (5×10^5 spores ml^{-1} in distilled water) of *C. higginsianum* (MAFF305635) on each leaf. Lesion phenotypes at 6 days postinoculation are shown. Arrows indicate the inoculation sites and lesions. Each picture shows a representative of three independent experiments. Bars=1 cm.

We regarded cDNAs with expression ratios (treated/ untreated) three-fold greater than the expression ratio of the lambda control template DNA fragment (TX803; Takara, Kyoto, Japan) at one or more time points to be up-regulated, fungal infection- or compound-inducible genes. Untreated Col-0 plants grown under the same conditions as the treated plants were used for calculation of fold induction for all treatments including the water control. Image analysis and signal quantification were performed with a ScanArray Express ver. 3.0 (PerkinElmer, MA, USA). The median of 128 and 82 signal values obtained from the lambda control template DNA fragment on the *B. rapa* and *Arabidopsis* microarray, respectively, was used as an external control to equalize hybridization signals generated from different samples. Gene-clustering analysis was performed with GeneSpring ver. 6.2 (Silicon Genetics, San Carlos, CA, USA).

The gene expression during infection with *C. higginsianum* and treatments with signaling molecules was analyzed using the *B. rapa* and *Arabidopsis* cDNA microarrays. The relation between experiment pairs was analyzed by means of Pearson's correlation coefficient using the software Microsoft Excel 2004 for Mac ver. 11.2.3 (Tables 1 and 2). The quantity *r*, called the linear correlation coefficient, measures the strength and the direction of a linear relationship between two variables.

Table 1. The correlation coefficient between experiment pairs on microarray analyses in *B. rapa*.

Treatment	MeJA 2h	MeJA 5h	MeJA 10h	MeJA 24h	ET 2h	ET 5h	ET 10h	ET 24h	SA 2h	SA 5h	SA 10h	SA 24h
C. higginsianum 5h	0.434	0.705	0.584	0.322	0.568	0.794	0.693	0.416	0.467	0.520	0.683	0.452
C. higginsianum 10h	0.336	0.592	0.710	0.274	0.411	0.644	0.778	0.492	0.493	0.556	0.761	0.588
C. higginsianum 24h	0.212	0.422	0.299	0.268	0.513	0.542	0.501	0.303	0.414	0.380	0.421	0.308

Table 2. The correlation coefficient between experiment pairs on microarray analyses in *A. thaliana*.

Treatment	MeJA 2h	MeJA 5h	MeJA 10h	MeJA 24h	ET 2h	ET 5h	ET 10h	ET 24h	SA 2h	SA 5h	SA 10h	SA 24h
C. higginsianum 5h	−0.136	0.105	−0.039	−0.444	0.126	0.395	0.314	−0.084	0.399	0.453	0.544	0.347
C. higginsianum 10h	−0.297	−0.128	0.123	−0.414	−0.144	0.219	0.266	−0.244	0.288	0.394	0.482	0.298
C. higginsianum 24h	0.258	0.060	0.150	0.454	0.231	0.277	0.304	0.345	0.227	0.087	0.014	0.160

The correlation coefficient is measured on a scale that varies from +1 to −1. The correlation is +1 in the case of an increasing linear relationship, −1 in the case of a decreasing linear relationship, and some value in between in all other cases, indicating the degree of linear dependence between the variables. Complete correlation between two variables is expressed by either +1 or −1. Complete absence of correlation is represented by 0. Several authors have offered guidelines for the interpretation of the correlation coefficient. Cohen (1988), for example, has suggested the following interpretations for correlations, such as $0 \leqq |r| < 0.10$ is regarded as insubstantial, $0.10 \leqq |r| < 0.30$ as small; $0.30 \leqq |r| < 0.50$ as medium and $0.50 \leqq |r| \leqq 1.00$ as large correlation. The squared correlation, r^2, describes the proportion of variance in common between the two variables. For examples, a correlation of 0.50 shows that 25% variance is in common; and a correlation of 0.10 shows 1% in common (or 99% not in common). In *B. rapa*, the results suggest that the correlation coefficient between *C. higginsianum*-infection (CI) and SA-, MeJA-, ET-treatments was large between the two; CI 5 h and SA (5 and 10 h), CI 5 h and MeJA (5 and 10 h), CI 5 h and ET (2, 5 and 10 h), CI 10 h and SA (5, 10 and 24 h), CI 10 h and MeJA (5 and 10 h), CI 10 h and ET (5 and 10 h), CI 24 h and ET (2, 5 and 10 h), and the medium or small correlation between CI 24 h and SA- or MeJA-treatments was shown (Table 1). A combination of SA, JA and ET-dependent defenses is important at the early stage of the interaction between *B. rapa* and *C. higginsianum*, and subsequently the defense reaction may depend on the ET-dependent signaling pathway because the activation of SA- and JA-signaling decreased more rapidly than that of ET-signaling. In *Arabidopsis* plants, on the other hand, the correlation coefficient between CI (5 and 10 h) and SA-treatments indicates that two experiments are closer than other experiments (MeJA/ET) (Table 2). In contrast, the correlation coefficient between CI (5 and 10 h) and MeJA-treatments is below 0.2, which suggests that their pairs are not close or show a negative correlation. The correlation between CI 24 h and MeJA 24 h was larger than that of other experiments. These results suggest that the defense reaction relatively activates the SA-dependent signaling pathway at the early stage of the interaction between *Arabidopsis* and *C. higginsianum*, and the subsequent defense reaction may depend on the JA-dependent signaling pathway because the correlation with SA-signaling decreased rapidly and that of JA-signaling relatively increased.

To analyze the expression of the counterpart gene set between *B. rapa* and *Arabidopsis* during infection with *C. higginsianum* by cDNA microarrays, we identified the counterpart gene set between *B. rapa* and *Arabidopsis* in the lists of the genes spotted on the *B. rapa* and *Arabidopsis* cDNA microarrays. Using the 1,820 cDNA inserts spotted on the *B. rapa* microarray, the counterpart gene set between *B. rapa* and *Arabidopsis* was identified as follows: Using the BLASTX programs, with an e-value<1e-30 against the TAIR *Arabidopsis* DNA and protein databases that include coding sequences (CDS), cDNA and genomic sequences (Rhee et al. 2003), we obtained 145 sequences that correspond with *Arabidopsis* sequences in the lists of the genes spotted on the *Arabidopsis* 1.2 K cDNA microarray.

The self-organizing maps (SOMs) have a number of features that make them particularly well suited to clustering and analysis of gene expression patterns (Kohonen 1990, 2000). They are ideally suited to exploratory data analysis, allowing one to impose partial structure on the clusters and facilitating easy visualization and interpretation. SOMs have good computational properties and are easy to implement, reasonably fast, and scalable to large data sets (Tamayo et al. 1999). Using the SOM program shipped with the GeneSpring ver. 6.2, the patterns of gene expression were pursed and grouped into classes arranged in a 3×3 array according to Kohonen's methodology (1990, 2000) (Figure 2). This arrangement provided an image of gene expression for the counterpart gene set. After inoculation of *B. rapa* with *C. higginsianum*, the transcript levels of 19 counterpart genes were up-regulated, that expression ratios (infected/uninfected) are more than three-fold. On the other hand, the transcript levels of 30 counterpart genes were up-regulated during infection of *Arabidopsis* plant with *C. higginsianum*. Most gene expression profiles for the counterpart gene set between *B. rapa* and *Arabidopsis* were activated and similar during infection with *C. higginsianum*, that appeared in class (1,1). In addition, 11 counterpart gene sets were up-regulated during infection with *C. higginsianum* and appeared in class (1,1), except for one set. Most of their counterpart gene sets also were induced by SA-treatment. On the contrary, most gene expression appeared in classes (1,3), (2,1), (2,2), (2,3), (3,1), (3,2) and (3,3) was suppressed or not activated under the condition, that expression ratios (infected/uninfected) are below three-fold. For example, hevein-like protein coding gene (*PR-4*) activation is a reasonably molecular marker JA/ethylene signaling. In addition, we previously reported that *PR-4* gene expressed by the inoculation of *Arabidopsis* Col-0 plants with *C. higginsianum* (Narusaka et al. 2004). The expression of *PR-4* counterpart genes between *B. rapa* and *Arabidopsis* was induced by inoculation with *C. higginsianum*. The cluster including this gene is class (1,1). On the other hand, some of counterpart gene sets in class (1,2) appeared to be markedly different from expression patterns between *B. rapa* and *Arabidopsis*.

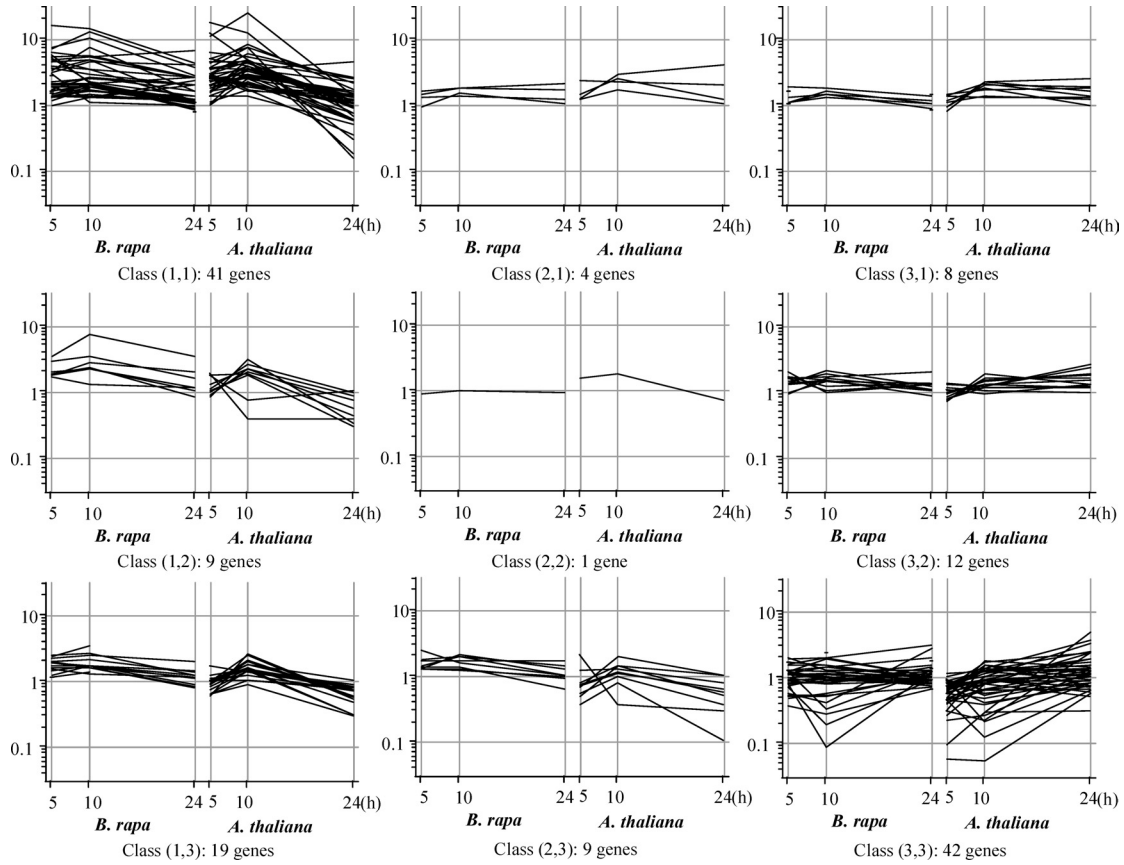

Figure 2. Self-organizing map analysis for gene expression during infection with *C. higginsianum*. Expression profiles of 145 counterpart gene sets between *B. rapa* and *A. thaliana* during infection with *C. higginsianum* arranged in a 3×3 matrix. These counterpart gene sets were selected with cut-off value of e-values＜1e-30.

This comparative analysis indicated some basic similarities and differences between the *B. rapa* and *Arabidopsis* transcriptome during the infection with the compatible fungal pathogen. In the future, we will analyze gene expression profiles in *B. rapa* during infection with *C. higginsianum* using the information obtained from *Arabidopsis*. Chinese cabbage is frequently damaged by various pathogens, which include *Peronospora brassicae*, *Plasmodiophora brassicae*, *Erwinia carotovora* subsp. *carotovora, Xanthomonas campestris* pv. *campestris*, *C. higginsianum*, *Alternaria brassicicola* and turnip mosaic virus. However, few studies on host-pathogen interactions have been performed in Chinese cabbage (Park et al. 2005). Therefore, the roles of SA, ethylene and JA on defense responses against diseases, such as basal defense, host-resistance and non host-resistance, have not been clarified in Chinese cabbage. The studies on defense mechanisms have focused on a limited number of model plants, such as *Arabidopsis*, rice and tobacco. Although the information obtained from these model plants has advanced our understanding of defense responses in plants as a whole, certain plants may have unique responsive mechanisms and regulation of gene expression. In addition, few defense-related genes in *Brassica* have been reported. Therefore, the present EST and microarray data should provide a valuable resource for a functional genomics on the crops.

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