Abstract  Heterosis or hybrid vigor describes a phenomenon that superior phenotypes compared to the two parents are observed in the heterozygous F₁-hybrid plants. Identification and characterization of heterosis-related genes (HRGs) will facilitate hybrid breeding in crops. To identify HRGs in *Brassica rapa*, we analyzed transcriptome profiling using a Br300K microarray in non-heading Chinese cabbage at three developmental stages. A large number of genes were differentially expressed in F₁ hybrids and non-additive expression was prominent. Genes that are expressed specifically for F₁ hybrid at all three stages were *Brassica*-specific uncharacterized genes and several defense-related genes. Expression of several photosynthesis- and stress-related genes were also F₁ hybrid-specific. Thirteen NBS-LRR class genes showed high and specific expression in F₁ hybrid Shulu: some of them were characterized as defense genes in *Arabidopsis*, but most have not been. Further characterization of these defense-related genes in *Brassica* species and its application will be helpful for understanding the role of defense responses in heterosis. In addition, results obtained in this study will be valuable to develop molecular markers for heterosis and disease resistance in *B. rapa*.

Keywords  Heterosis, F₁ hybrid, Non-heading Chinese cabbage, NBS-LRR, microarray

Introduction  Hybrid vigor or heterosis is a phenomenon in which the heterozygous F₁-hybrid plants exhibit superior phenotypes in biomass, growth, size and stress resistance, over their homozygous parent inbred lines. Due to superior phenotypes of F₁ hybrids, the heterosis has been widely used in the commercial seed production of crops, like maize and rice, and vegetable cultivar like Chinese cabbage (Basunanda et al. 2010; Schnable and Springer 2013; Fu et al. 2015; Kawamura et al. 2016; Jeong et al. 2017). Elucidation and application of the heterosis mechanism will help to develop breeding strategies to enhance crop productivity.

The genetic analyses of F₁ hybrids in maize and rice to understand heterosis mechanism revealed that a large number of QTLs contribute to superior phenotypes of the hybrids in complex manners (Baranwal et al. 2012; Chen 2013; Groszmann et al. 2013; Schnable and Springer 2013). Gene interactions, such as dominance, overdominance, pseudo-overdominance, and epistasis, have therefore been suggested to explain heterosis phenotypes (Lippman and Zamir 2007; Charlesworth et al. 2009). Recent molecular analyses of transcriptomes, proteomes, and metabolomes with two parents and hybrids have led to the appreciation of new aspects on the establishment of hybrid vigor, such as epigenetic effects (Groszmann et al. 2011 and 2013; Baranwal et al. 2012; Schnable and Springer 2013; Li et al. 2016; Saeki et al. 2016)].

Transcriptome-wide gene expression study is one of methods to dissect the heterosis at the gene expression level. The initial studies insisted that the increased gene expression level in the hybrids may contribute to heterosis (Romagnoli et al. 1990; Tsafaris 1995). More recent studies have suggested that heterosis is due to the relative frequencies of genes showing additive and non-additive expression in hybrids (Auger et al. 2005; Guo et al. 2006; Stupar et al. 2008). Additive expression occurs when the hybrid expression
Epigenetic change in protein coding genes and rDNA genes by small RNAs, DNA methylation and chromatin remodeling results in the alteration of gene expression by reprogramming of interacting genomes in hybrids (Chen 2013; Greaves et al. 2015). In *Arabidopsis*, the role of DNA methylation in hybrid vigor, maintained by METHYLTRANSFERASE 1 (MET1) or DNA METHYLATION 1 (DDM1), has been reported (Shen et al. 2012; Kawanabe et al. 2016). In addition, alteration of circadian rhythms affected growth vigor and biomass increase via change in expression timing of *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELNGATED HYPOCOTYL* (*LHY*) in *Arabidopsis* and maize (Ni et al. 2009; Miller et al. 2015; Ko et al. 2016). Particularly, the activation of morning-phased genes in hybrids by circadian genes promoted photosynthesis and growth vigor. The early shift of *CCA1*-binding to photosynthesis-and metabolism-associated genes in the morning leads to additive and non-additive expressions, which in turn establish and maintain heterosis (Ko et al. 2016). A transcription factor *PHYTOCHROME-INTERACTING FACTOR 4* (*PIF4*) also plays an important role in hybrid vigor by altering auxin biosynthesis and auxin responsive genes (Wang et al. 2017).

*Brassica rapa* ssp. *chinensis* F1 cultivar, Shulu, was bred by cross between Aijiaohung self-incompatible line as female parent and Suzhouqing inbred line as male parent (Hou et al. 2005). The Shulu exhibited improved quality in leaf morphology, disease resistance and yield (Hou et al. 2005), providing a good material to identify growth promoting genes and disease-resistant genes in heterosis. In this study, we applied genome-wide transcript profiling to obtain gene expression information during heterosis in this non-heading Chinese cabbage. Key observations include differential expression of known heterosis-genes in other plants and disease-resistant genes in hybrids.

### Materials and Methods

#### Plant materials

Seeds for *Brassica rapa* ssp. *chinensis* F1 cultivar, Shulu (25081), female parent Aijiaohung (25080) and male parent Suzhouqing (25083) were obtained from Korea *Brassica rapa* Genome Resource Bank (KBGRB), Chungnam National University, Republic of Korea. Seeds were sown in greenhouse at Chungnam National University, growing from April to May and sampled at indicated time. Shoots from 5 plants for each line were sampled at 15 and 30 DAGs, and frozen in liquid nitrogen until use. For 60 DAG plants, one young and mature leaf from one individual plant were taken from 5 independent plants.

RNA isolation and hybridization to the Br300K microarray GeneChip

Total RNA was isolated from samples using TRIzol reagent (Invitrogen, USA), and further purified with a NucleoSpin RNA Clean-up Kit (Macherey-Nagel GmbH & Co., Germany). For biological repeats, RNA extracted from two independent samples was used in microarray experiments. Microarray experiments and subsequent analyses were performed as described previously (Dong et al. 2013; Song et al. 2017).

*Brassica rapa* 300K Microarray (Br300K microarray), version 2.0, was composed of 47,548 unigenes as follows: seven 60-nt long probes were designed from each gene, covering 150 bp in the 3' region of the gene starting from 60 bp upstream of the stop codon with 15 bp shifting. After the hybridization with MAUI chamber (BioMicro, USA), the microarray was scanned with GenePix 4000 B (Axon, USA) preset with a 5 µm resolution for Cy3 signal. Signals were digitized and analyzed by NimbleScan (Nimblegen, USA). The normal distribution of Cy3 intensities (prove intensity, PI) was tested with qqline. The data was normalized and processed with cubic spline normalization using quantiles to adjust signal variations between chips, and Rubust Multi-Chip Analysis (RMA) using a median polish algorithm implemented in NimbleScan (Workman et al. 2002; Irizarry et al. 2003).

#### RT-PCR analysis

Total RNA (1 µg) from each sample was used in cDNA synthesis with the Ace-α kit and the Oligo (dT) primers (Toyobo, Japan). cDNA was diluted 10-fold, and 1 µl of diluted cDNA was used in a 20 µl PCR reaction. RT-PCR primers are listed in Supplementary Table 1; primer sequences for *BrACT2*, used as a control, were 5′-GAAACCGGGTG CTCCCTCAGGA-3′ (forward) and 5′-ATGGTACCGGAATG GTCAAGGC-3′ (reverse). A standard PCR was performed with a 5 min denaturation at 94°C, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s. PCR
products were analyzed by electrophoresis through 1.2% agarose gels.

Results and Discussion

Description of non-heading Chinese cabbage samples

As mentioned by Hou et al. 2005, growth of F1 cultivar Shulu is faster than its parents (Fig. 1). Particularly, self-incompatible female parent Aijiaohung grew slower than male parent Suzhouqing inbred line even at 60 DAG. Significant difference in growth phenotype between two parents, as well as between F1 hybrids and either parent, implies that a large number of genes are differentially expressed.

Analysis of Br300K microarray

To identify heterosis-related genes (HRGs), we performed Br300K microarray experiment twice with 9 samples collected at 3 stages (15 DAG, 30 DAG and 60 DAG) and results were summarized in Supplementary Table 1. As expected, a large number of genes were differently expressed (Table 1). Regarding to DEGs, the largest number was detected at 15 DAG samples or between two parents. These results indicated that the early stage of growth may determine the heterosis phenotypes in F1 cultivar Shulu. The highest difference between two parents also expected that the genetic difference on the gene expression regulation will be different from those obtained from heading-type Chinese cabbage, in which they have used similar parents (Saeki et al. 2016).

Table 1 Summary of Br300K microarray results. DEGs represent differentially expressed genes over two-fold between two Chinese cabbage lines. For non-additive expression, genes showing over 1.5-fold difference between F1 hybrid and mid-parent values were counted. 80, 81 and 83 represented abbreviation of 25080, 25081 and 25083, respectively

| Classification       | 15 DAG  | 30 DAG  | 60 DAG  | 81/80 | 81/83 | 80/83 | 81/80 | 81/83 | 80/83 | 81/80 | 81/83 | 80/83 |
|----------------------|---------|---------|---------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| DEGs                 | Up      | 1,714   | 2,060   | 2,882 | 2,462 | 1,165 | 2,415 | 1,412 | 1,730 | 2,749 |
|                      | Down    | 1,557   | 3,523   | 4,295 | 1,736 | 1,239 | 3,423 | 1,528 | 1,638 | 2,404 |
| Additive expression  | 815     | 952     | 808     | 81/80 | 81/83 | 80/83 |       |       |       |       |       |       |
| Non-additive expression | 13,695 | 14,358  | 12,692  | 81/80 | 81/83 | 80/83 |       |       |       |       |       |       |

( ): number of genes showing over 1,000 in PI values, which has been considered as significant levels of transcripts.
Expression pattern analysis revealed that non-additive expression was prominent compared with additive expression in non-heading type (Table 1), different from the previous results for heading type Chinese cabbage \( (B. \text{rapa} \text{ssp. pekinensis}) \) (Saeki et al. 2016). Saeki et al. (2016) reported that most genes show an additive expression pattern, and any expression level differences between parental lines were maintained in \( F_1 \) hybrids. This might be caused by somewhat similar phenotypes between parents used for \( F_1 \) hybrid production in the heading type Chinese cabbage. Two parents used in this study showed quite different phenotypes, such as growth (Fig. 1) and others (Hou et al. 2005), leading to non-additive expression in most genes. Conflicting results were also reported in previous maize \( F_1 \) studies. In one study, the majority (~75%) of genes exhibited additive expression in the hybrid and only small numbers of the non-additively expressed genes exhibited expression levels outside the parental range (Guo et al. 2006; Stupar and Springer 2006). In another study, much higher levels of non-additive expression and numerous examples of expression outside the parental range were observed (Auger et al. 2005; Meyer et al. 2007). It is not clear whether these differences are caused by biological differences between tissues, genotypes, or differences in the expression profiling platforms.

Identification of putative HRGs

It is not easy to determine which genes and how many genes are involved in heterosis, because large numbers of genes with complex roles are involved (Schnable and Springer 2013). We hypothesized that HRGs will be differentially expressed in \( F_1 \) hybrids compared to both parents: up-regulated or down-regulated. Consistent with our hypothesis up- and down-regulation of many genes were detected in \( F_1 \) hybrids (Fig. 2). However, only 14 genes and 1 gene were found to be up-regulated and down-regulated at all growth stages, respectively. These small numbers of putative HRGs might be explained, if developmental stage-dependent complex and different actions by large numbers of genes or alleles are required for heterosis (Schnable and Springer 2013). We further speculated that genes differently expressed at two different stages are HRGs with more important roles in heterosis. It turned out that there are 23 up-regulated DEGs overlapping between 15 and 30 DAG, another 23 DEGs between 30 and 60 DAG, and 124 DEGs between 15 and 60 DAG. In similar approach for down-regulated DEGs, 9 between 15 and 30 DAG, 18 between 30 and 60 DAG, and 51 between 15 and 60 DAG were identified. These DEGs, especially expressed in the early stages, were considered as HRG candidates for non-heading Chinese cabbage.

Among DEGs shown in Figure 2, we further analyzed (1) genes that were up-regulated in all stages, (2) up-regulated in both 15 and 30 DAGs, (3) up-regulated in both 30 and 60 DAGs and (4) down-regulated in all stages, as HRG candidates (Table 2). Interestingly, most up-regulated genes belonged to predicted mRNAs or uncharacterized \textit{Brassica} mRNAs, suggesting that heterosis of \textit{B. rapa} \text{ssp} \textit{chinensis} is affected by \textit{Brassica}-specific genes. Still, we found several genes, which might possess heterosis-related functions, in the list: \textit{Brapa}\_ESTC051755 for photosynthesis and \textit{Brapa}\_ESTC019040 for nucleic acid synthesis were up-regulated in all stages. In addition, 4 putative defense-related genes were found in up-regulated genes: \textit{Brapa}\_ESTC031864 encoding leucine-rich repeat protein kinase family protein, \textit{Brapa}\_ESTC002203 encoding 20S PROTEASOME BETA SUBUNIT PBF1, \textit{Brapa}\_ESTC014631 encoding NAD(P)-linked oxidoreductase superfamily protein and \textit{Brapa}\_ESTC024642 encoding disease resistance-responsive dirigent-like protein. Kumar et al. (2016) reported that \textit{Arabidopsis} orthologs for \textit{Brapa}\_ESTC014631 and \textit{Brapa}\_ESTC024642 exhibit defense response. For down-regulated genes in all stages, only one gene, \textit{Brapa}\_ESTC012945, which encodes bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein, was identified. The function of this protein has not been identified and requires functional studies.

Expression of genes showing homology to HRGs identified in other plants

Circadian rhythm-, chromatin remodeling- and stress responsive-related genes have been reported as HRGs in \textit{Arabidopsis thaliana}, and photosynthesis-related gene showed up-regulation in \textit{B. rapa} \( F_1 \) hybrid (references in Table 3). We summarized expression levels of \textit{B. rapa} homologos of
the critical roles of PIF4, CCA1 and LHY in heterosis were mechanisms are complex and species-specific. In others were not clear. This results implies that heterosis to be HRGs in non-heading cabbage, while conclusion for 3). According to probe intensity (PI) values, these known HRGs in non-heading Chinese cabbage (Table 2).

| Up/Down (Stages) | At_Locus | Br_SEQ_ID | BRAD_ID | Description, BlastN |
|------------------|----------|-----------|---------|-------------------|
| Up (All)         | No_hit_found | Brapa_ESTC048713 | Br006663 | PREDICTED: Brassica napus uncharacterized LOC10383461 |
|                  | Brapa_ESTC048714 | No_hit_found | PREDICTED: B. napus uncharacterized LOC10383461 |
|                  | ATIG09980 | Brapa_ESTC019540 | Br018822 | DNA/RNA polymerases superfamily protein |
|                  | No_hit_found | Brapa_ESTC048297 | - | PREDICTED: Brassica napus uncharacterized LOC10383461 |
|                  | Brapa_ESTC048298 | No_hit_found | PREDICTED: B. napus uncharacterized LOC10383461 |
|                  | No_hit_found | Brapa_ESTC049333 | - | Br sequence |
|                  | ATIG47110 | Brapa_ESTC051755 | Br022915 | AtSpf7110 gene (light-harvesting-like protein 3 (LH3)) |
|                  | No_hit_found | Brapa_ESTC0342529 | Br038841 | PREDICTED: B. napus uncharacterized LOC10433243 |
|                  | No_hit_found | Brapa_ESTC051614 | Br010245 | PREDICTED: B. napus uncharacterized LOC106418084, mRNA |
|                  | No_hit_found | Brapa_ESTC051622 | - | Br sequence |
|                  | ATIG09370 | Brapa_ESTC044073 | Br038841 | PREDICTED: B. napus uncharacterized LOC10436163 |
|                  | ATIG6620 | Brapa_ESTC020203 | Br034468 | 25S PROTEASOME BETA SUBUNIT PBF1 |
|                  | No_hit_found | Brapa_ESTC049934 | - | Br sequence |
|                  | ATIG31120 | Brapa_ESTC051120 | Br027213 | PREDICTED: B. napus putative F-box protein At3g7620 |

| Up (15/30) |
|------------|
| No_hit_found | Brapa_ESTC051613 | Br010245 | PREDICTED: B. napus uncharacterized LOC103849825 |
| No_hit_found | Brapa_ESTC037377 | Br006426 | PREDICTED: B. napus uncharacterized LOC103836270 |
| No_hit_found | Brapa_ESTC030869 | Br013078 | PREDICTED: B. napus uncharacterized LOC103836270 |
| No_hit_found | Brapa_ESTC030723 | Br040000 | PREDICTED: B. napus probable protein S-xylosyltransferase 22 |
| No_hit_found | Brapa_ESTC048680 | Br019236 | PREDICTED: B. napus probable protein S-xylosyltransferase 22 |
| No_hit_found | ATIG09760 | Brapa_ESTC011203 | Br006516 | Mitochondrial substrate carrier family protein |
| ATIG60701* | Brapa_ESTC014631 | Br027140 | NAD(P)-linked oxidoreductase superfamily protein (ATR3/Atx1-inducible) |
| No_hit_found | Brapa_ESTC033736 | Br013078 | PREDICTED: B. rapa uncharacterized LOC103856270 |
| No_hit_found | Brapa_ESTC043214 | Br031342 | PREDICTED: B. napus splicing factor 12a large subunit B-like (LOC100364061) |

| Up (30/60) |
| ATIG65280 | Brapa_ESTC019092 | Br016859 | Arabidopsis thaliana other RNA IncRNA |
| ATIG47370 | Brapa_ESTC029421 | Br000277 | Pectin lyase-like superfamily protein |
| ATIG46060* | Brapa_ESTC024642 | Br027681 | Disease resistance-responsive (dirigent-like) protein family protein (DIPS) |
| ATIG64700 | Brapa_ESTC018664 | Br035694 | Leucine-rich repeat protein kinase family protein (LRR-RLK) |
| No_hit_found | Brapa_ESTC080899 | - | PREDICTED: B. napus uncharacterized LOC10436163 |
| No_hit_found | Brapa_ESTC046000 | Br035221 | PREDICTED: B. napus uncharacterized LOC10436163 |
| ATIG6300 | Brapa_ESTC041995 | Br017014 | Pectin lyase-like superfamily protein |
| No_hit_found | Brapa_ESTC051749 | - | PREDICTED: B. napus uncharacterized LOC10436163 |

| Down (All) |
| ATIG62510 | Brapa_ESTC012895 | Br027039 | Bilobaline inhibitory lip-transfer protein/seed storage 25 albumin superfamily protein |

List of differentially expressed genes in F1 hybrid compared to its parent line over two fold. Genes showing over two fold up-regulation at least two stages of Chinese cabbage growth (15 DAG, 30 DAG, and 60 DAG) and down-regulated in all stages were selected for analysis (see Fig. 2)(No_hit_found: no Arabidopsis homolog present, - : no cDNA found in BRAD, *: gene identified by Kumar et al. (2016) as defense role against cotton bollworp.)

these known HRGs in non-heading Chinese cabbage (Table 3). According to probe intensity (PI) values, LHCA2 and stress-responsive genes, such as COR78 and COR47, appeared to be HRGs in non-heading cabbage, while conclusion for others were not clear. This results implies that heterosis mechanisms are complex and species-specific. In Arabidopsis, critical roles of PIF4, CCA1 and LHY in heterosis were reported (Shen et al. 2012; Miller et al. 2015; Ko et al. 2016), but their roles in non-heading Chinese cabbage are not supported by mRNA expression analysis. In maize, the early activation of morning-phased genes by CCA1 in the maize hybrids promotes photosynthesis and growth vigor (Ko et al. 2016). This temporal shift of ZmCCA1-binding targets correlated with non-additive and additive gene ex-
Table 3  Expression of *B. rapa* genes homologous to genes showing heterosis-related expression in other plants

| At_Locus | Gene description | Br_SEQ_Id       | Probe intensity  |
|----------|------------------|-----------------|------------------|
|          |                  |                 | 15 DAG | 30 DAG | 60 DAG | Reference       |
| AT1G01060 | LHY (LATE ELONGATED HYPOCOTYL) | Brapa_ESTC008780 | 480 270 396 | 307 266 295 | 1583 3235 1958 | Ni et al. 2009 |
|          |                  | Brapa_ESTC027456 | 95 39 43 | 35 77 76 | 1457 1354 1457 |               |
|          |                  | Brapa_ESTC028354 | 1531 1484 960 | 1227 870 | 1185 3884 4653 |               |
|          |                  | Brapa_ESTC28906  | 509 378 247 | 321 361 443 | 2945 3187 3291 |               |
|          |                  | Brapa_ESTC029140 | 220 90 183 | 100 84 103 | 1295 2145 1297 |               |
| AT1G01510 | AN (ANGUSTIFOLIA) | Brapa_ESTC014581 | 8823 10283 9862 | 9672 12697 12208 | 11634 13445 16742 | Kim et al. 2002 |
|          |                  | Brapa_ESTC027456 | 4304 4017 | 3004 4437 | 3812 4102 5597 |               |
|          |                  | Brapa_ESTC028354 | 1531 1484 960 | 1227 870 | 1185 3884 4653 |               |
| AT1G22770 | GI (GIGANTEA) | Brapa_ESTC017304 | 15282 17247 11276 | 17041 16639 | 11515 13092 15503 | Ni et al. 2009 |
|          |                  | Brapa_ESTC029140 | 220 90 183 | 100 84 103 | 1295 2145 1297 |               |
| AT2G43010 | PIF4 (PHYTOCHROME INTERACTING FACTOR 4) | Brapa_ESTC012017 | 1438 2463 4227 | 2040 3237 3449 | 1863 2494 6262 | Wang et al. 2017 |
|          |                  | Brapa_ESTC018786 | 7303 8497 | 11187 | 8071 10934 | 6198 8463 16801 |               |
| AT2G46830 | CCA1 (CIRCADIAN CLOCK ASSOCIATED 1) | Brapa_ESTC006966 | 200 179 288 84 | 99 126 | 1913 1194 891 |               |
|          |                  | Brapa_ESTC018786 | 7303 8497 | 11187 | 8071 10934 | 6198 8463 16801 |                |
| AT3G18000 | ATG2 (G2p-related protein)(EBP1) | Brapa_ESTC009728 | 8864 8878 10174 | 10754 12800 | 11249 9869 | Wang et al. 2016 |
|          |                  | Brapa_ESTC009728 | 16033 14047 15178 | 18601 20514 | 21149 19476 1920 |               |
|          |                  | Brapa_ESTC009892 | 14493 14210 | 17826 15503 | 18251 16788 |               |
| AT4G19020 | CMT2 (Chromomethylase 2) | Brapa_ESTC009064 | 2915 3337 3167 | 2479 2006 | 2499 3223 | Zemach et al. 2013 |
| AT5G05450 | RHI8 (DEAD/DEAH box helicase, putative) | Brapa_ESTC030024 | 2565 2964 3105 | 2847 2102 | 2437 2622 | Plotter et al. 2017 |
| AT5G49160 | MET1 (DECREASED METHYLATION 2DNA) | Brapa_ESTC014151 | 6107 5762 4380 | 8626 3442 | 5289 6703 | Kawanabe et al. 2016 |
| AT5G61380 | TOC1 (TIMING OF CAB1 1) | Brapa_ESTC012017 | 1438 2463 4227 | 2040 3237 3449 | 1863 2494 6262 | Wang et al. 2017 |
| AT5G66750 | DDM1 (DECREASED DNA METHYLATION 1) | Brapa_ESTC029914 | 1798 1305 1356 | 2802 953 | 1624 2544 | Zemach et al. 2013 |
|          |                  | Brapa_ESTC030555 | 4050 2966 2595 | 5163 2537 | 2736 4415 | Sasaki et al. 2016 |
| AT4G25080 | CHLM (MAGNESIUM-PROTOPORPHYRIN IX METHYLTRANSFERASE) | Brapa_ESTC02086 | 13691 18396 14496 | 14269 12665 | 13189 14831 16039 |               |
| AT3G61470 | LHCA2 (Photosystem I light harvesting complex gene 2) | Brapa_ESTC007961 | 40044 45566 36797 | 42413 37810 | 41103 40901 42387 | Sasaki et al. 2016 |


pression in early and late stages of seedling development. Because we did not examine expression levels throughout a day, we do not exclude a possibility that expression level of circadian rhythm-related genes correlate to heterosis phenotype in non-heading Chinese cabbage.

Expression of NBS-LRR class genes

According to original breeders, F₁ hybrid Shulu shows resistance to various diseases, such as TuMV, downy mildew and alternaria leaf spot (Hou et al. 2005). Therefore, we analyzed expression of disease resistance genes like NBS-LRR class. Among 99 NBS-LRR class protein genes included in the Br300K microarray, 17 genes corresponding 13 Arabidopsis genes were highly expressed in F₁ hybrids (Table 4). Only three Arabidopsis genes have been functionally characterized so far. ATSG11250, homolog of Brapa_ESTC009435 and Brapa_ESTC032144, is known as BURNOUT1 (BNT1) and encodes a TIR-NBS-LRR protein responsible for disease resistance (Sarazin et al. 2015). AT5G11250 affects the levels of stress hormones, such as jasmonic acid, salicylic acid, abscisic acid and ethylene. AT1G15890, homolog of Brapa_ESTC034248 encodes putative CC-NBS-LRR class protein that causes bacterial cell death (Yang et al. 2016). AT3G50950, homolog of Brapa_ESTC036172, encodes Arabidopsis R protein HOPZ-activated resistance 1 (ZAR1) required for recognition of HopZ1a, Pseudomonas syringae type III secreted effector (Lewis et al. 2010). Examination of expression pattern and sequence variations in 17 disease resistance genes, especially homologs of above mentioned three Arabidopsis genes, could be useful to develop molecular markers for disease resistance in Brassica species.
Table 4 NBS class defense-related genes showing high expression in F1 hybrids at least two growing stages

| At_Locus     | Description, blastN (Disease Resistant Protein) | Br_SEQ_ID     | BRAD_ID     | Probe intensity |
|--------------|-------------------------------------------------|---------------|-------------|-----------------|
| AT1G3350     | CC-NBS-LRR class, putative                      | Brapa_ESTC015346 | Br037448 | 277 4520 2420 406 1119 2610 625 6549 1265 |
| AT1G72890    | TIR-NBS class, putative                         | Brapa_ESTC036158 | Br016029 | 246 280 10 175 373 54 328 236 52  |
| AT5G18350    | TIR-NBS-LRR class, putative                     | Brapa_ESTC037594 | Br001161 | 886 1749 990 768 838 526 628 764 302  |
| AT5G29890    | TIR-NBS-LRR class, putative                     | Brapa_ESTC042689 | Br001160 | 5848 5689 4363 4273 4893 3867 3076 4010 2814 |
| AT5G11250*   | TIR-NBS-LRR class, putative                     | Brapa_ESTC031244 | Br006146 | 2851 3805 2565 1739 2010 1919 1608 2204 1326 |
| AT5G66900    | CC-NBS-LRR class, putative                      | Brapa_ESTC008403 | Br025290 | 867 2003 1682 1430 1837 1437 1491 1783 1162 |
| AT5G46450    | TIR-NBS-LRR class, putative                     | Brapa_ESTC0346013 | Br022036 | 194 108 28 107 118 83 87 164 133 |
| AT5G41540    | TIR-NBS-LRR class, putative                     | Brapa_ESTC011594 | AT5G1110 | 473.5 1011.8 1547.8 1010.7 m |
| AT1G5890*    | CC-NBS-LRR class, putative                      | Brapa_ESTC034248 | Br018835 | 5532 4963 4243 5604 6479 5486 3854 4015 3145 |
| AT1G69550    | TIR-NBS-LRR class, putative                     | Brapa_ESTC034328 | Br029936 | 65 119 132 95 169 102 54 164 134  |
| AT4G27190    | NBS-LRR class, putative                         | Brapa_ESTC008807 | Br026368 | 3914 4404 4023 6671 8044 5932 6100 5244 4694 |
| AT5G26990    | TIR-NBS-LRR class, putative                     | Brapa_ESTC021828 | Br034079 | 1499 1075 1727 1629 3193 2402 923 1369 1278 |
| AT5G50950*   | CC-NBS-LRR class, putative                      | Brapa_ESTC036172 | Br036845 | 17874 13266 11186 11327 11777 10183 7026 8846 8979 |
| AT5G41750    | TIR-NBS-LRR class, putative                     | Brapa_ESTC042275 | Br013144 | 9878 10607 8205 1520 1995 1716 331 384 1336 |

Table 5 Select genes for the RT-PCR experiment with additive or non-additive expression; f, high PI value in female parent; m, high PI value in male parent; +, higher PI value in F1 hybrid outside the parental values; -, lower PI value in F1 compared to parental values

| Gene expression pattern | Stage | Br_SEQ_ID | At_Locus | Description, blastN | PI value | F1 hybrid | Male | Mid-parent (80+83)/2 | Remarks |
|-------------------------|-------|-----------|----------|---------------------|----------|-----------|------|---------------------|---------|
| Additive                | 15 DAG| Brapa_ESTC034378 | AT3G10690 | DNA gyrase subunit A family protein | 7362.4 5731.5 4099.3 5730.8 f |
|                         |       | Brapa_ESTC005544 | AT5G66190 | RCA (RUBISCO ACTIVASE) | 11021.2 9163.8 7307.6 9164.4 f |
| Additive                | 30 DAG| Brapa_ESTC011189 | AT5G11110 | ATP5P2F (sucrose phosphate synthase 2F) | 473.5 1011.8 1547.8 1010.7 m |
|                         |       | Brapa_ESTC012536 | AT4G01230 | Ribosomal protein L5 family protein | 18408.4 15713.8 13017.2 15712.8 f |
|                         |       | Brapa_ESTC022226 | AT1G27190 | LRR transmembrane protein kinase, putative | 9591.3 8719.6 7847.5 8719.4 f |
|                         |       | Brapa_ESTC005539 | AT5G66190 | RCA (RUBISCO ACTIVASE) | 11021.2 9163.8 7307.6 9164.4 f |
| Non-additive            | 60 DAG| Brapa_ESTC046155 | AT5G6630 | Clathrin adaptor complexes medium subunit family protein | 17451.9 15204.9 12958.5 15205.2 m |
|                         |       | Brapa_ESTC059491 | AT2G09730 | ACTR (ATP BINDING COTYLEDON 2) | 39951.7 45713.9 39491.5 39721.6 + |

Expression profiling of selected genes

To confirm microarray results, several classes of genes were selected and subjected to RT-PCR (Fig. 3). Gene description and PI values were presented in Table 2 for up-regulated genes at all three stages and Table 5 for additively and non-additively expressed genes in F1 plants. Although there were some variaitions were detected, RT-PCR signals were similar to that observed with PI values, indicating that microarray experiments reliably reflected transcription levels in general. Particularly, all up-regulated genes were confirmed to be predominantly expressed in F1 hybrids, while genes
Fig. 3 RT-PCR confirmation of expression patterns for selected genes. A, Up-regulated genes in F1 hybrid at all stages; B, Genes with additive expression at the indicated stage (right side); C, Genes with non-additive expression at the indicated stage (right side). Showing either additive or non-additive expression displayed stage-specific expression patterns. Since all up-regulated genes in Fig. 3A have not been functionally characterized, the identification of these genes by heterosis-related expression pattern provides a good starting point to characterize their functions, such as growth and photosynthesis.

In conclusion, F1 hybrids generated by crosses between genetically distinct individuals show heterosis phenotypes through complex mechanisms, in terms of numbers of genes and timing of their actions. Multiple loci seem to be involved in heterosis for different traits and in different hybrids (Schnable and Springer 2013). Identification of genes governing heterosis mechanism is therefore very challenging. We have obtained valuable information from Br300K microarray by comparing gene expression patterns in F1 hybrid and its parents. We identified (1) several putative HRGs that are highly up-regulated in F1 hybrids and possibly responsible for heterosis phenotype and (2) some B. rapa homologs of Arabidopsis HRGs promising to play similar roles in non-heading Chinese cabbage, and (3) several NBS-LRR class genes showing heterosis-related expression and possibly involved in defense signaling. Our finding will facilitate to improve hybrid breeding and to develop molecular markers for the disease resistance.

Acknowledgement

This work was supported by Research Fund of Chungnam National University (CNU), Daejeon, Korea, to Yoonkang Hur (2015-1115-01).

Supplementary materials

Supplementary Table 1 List of primer sequences used in RT-PCR

Supplementary Table 2 Br300K microarray results annotated with Arabidopsis TAIR7.

References

Auger DL, Gray AD, Ream TS, Kato A, Coe EH Jr, Birchler JA (2005) Nonadditive gene expression in diploid and triploid hybrids of maize. Genetics 169:389-397

Baranwal VK, Mikkilineni V, Zehr UB, Tyagi AK, Kapoor S (2012) Heterosis: emerging ideas about hybrid vigour. J Exp Bot 63:6309-6314

Basunanda P, Radoev M, Ecke W, Friedt W, Becker HC, Snowdon RJ (2010) Comparative mapping of quantitative trait loci involved in heterosis for seedling and yield traits in oilseed rape (Brassica napus L.). Theor Appl Genet 120: 271-281

Charlesworth D, Willis JH (2009) The genetics of inbreeding depression. Nat Rev Genet 10:783-96

Chen ZF (2013) Genomic and epigenetic insights into the molecular bases of heterosis. Nat Rev Genet 14:471-482

Dong X, Feng H, Xu M, Lee J, Kim YK, Lim YP, Piao Z, Park YD, Ma H, Hur Y (2013) Comprehensive analysis of genic male fertility-related genes in Brassica rapa using a newly developed Br300K oligomeric chip. PLoS One 8: e72178

Eitas TK, Nimchuk ZL, Dangl JL (2008) Arabidopsis TAO1 is a TIR-NB-LRR protein that contributes to disease resistance induced by the Pseudomonas syringae effector AvrB. Proc Natl Acad Sci USA. 105:6475-6480

Fu D, Xiao M, Hayward A, Jiang G, Zhu L, Zhou Q, Li J, Zhang M (2015) What is crop heterosis: new insights into an old topic. J Appl Genetics 56:1-13
Groszmann M, Greaves IK, Albertyn ZI, Scofield GN, Peacock WJ, Dennis ES (2011) Hormone-regulated defense and stress response networks contribute to heterosis in Arabidopsis F1 hybrids. Proc Natl Acad Sci USA 112:E6397-E6406

Groszmann M, Greaves IK, Fujimoto R, Peacock WJ, Dennis ES (2013) The role of epigenetics in hybrid vigour. Trends Genet 29:684-690

Guo M, Rupe MA, Yang X, Crasta O, Zinselmieier C, Smith OS, Bowen B (2006) Genome-wide transcript analysis of maize hybrids: allelic additive gene expression and yield heterosis. Theor Appl Genet 113:831-845

Hatsugai N, Hillmer R, Yamaoka S, Hara-Nishimura I, Katagiri F (2016) The μ subunit of Arabidopsis adaptor protein-2 is involved in effector-triggered immunity mediated by membrane-localized resistance proteins. Mol Plant Microbe Interact 29:345-351

Hochholdinger F, Hoecker N (2007) Towards the molecular basis of heterosis. Trends Plant Sci 12:427-432

Hou XL, Cao SC, Zhang SN, Zhang ZC, Wang JJ, Sun HX (2005) Selection of non-heading Chinese cabbage cultivar Shulu with high-quality. J Nagling Agr Univ 28:30-33

Irizarry RA, Hobbs B, Collin F, Beazer-barclay YD, Antonellis KJ, Scherf U, Speed TP (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4:249264

Jeong SY, Ahmed NU, Jung HJ, Kim HT, Park JI, Nou IS (2017) Discovery of candidate genes for heterosis breeding in Brassica oleracea L. Acta Physiol Plant 39:180

Kawanabe T, Ishikura S, Miyaji N, Sasaki T, Wu LM, Itabashi E, Takada S, Shimizu M, Takasuki-Yasuda T, Osabe K, Peacock WJ, Dennis ES, Fujimoto R. (2016) Role of DNA methylation in hybrid vigor in Arabidopsis thaliana. Proc Natl Acad Sci USA. 113:E6704-E6711

Kim GT, Shoda K, Tsuge T, Cho KH, Uchimiya H, Yokoyama R, Nishitani K, Tsukaya H (2002) The ANGUSTIFOLIA gene of Arabidopsis, a plant CtbP gene, regulates leaf-cell expansion, the arrangement of cortical microtubules in leaf cells and expression of a gene involved in cell-wall formation. EMBO J 21:1267-1279

Ko DK, Rohozinski D, Song Q, Taylor SH, Juenger TE, Harmon FG, Chen ZJ (2016) Temporal shift of circadian-mediated gene expression and carbon fixation contributes to biomass heterosis in maize hybrids. PLoS Genet 12:e1006197

Kumar S, Kanakachari M, Gurusamy D, Kumar K, Narayanasamy P, Kethireddy Venkata P, Solanke A, Gamanagatti S, Hiremath V, Katageri IS, Leelavathi S, Kumar PA, Reddy VS (2016) Genome-wide transcriptomic and proteomic analyses of bollworm-infested developing cotton bolls revealed the genes and pathways involved in the insect pest defense mechanism. Plant Biotechnol J 14:1438-1455

Lewis JD, Wu R, Gutman DS, Desveaux D (2010) Allele-specific virulence attenuation of the Pseudomonas syringae HopZ1a type III effector via the Arabidopsis ZAR1 resistance protein. PLoS Genet 6:e1000894

Li A, Fang MD, SongWQ, Chen CB, Qi LW, Wang CG (2012) Gene expression profiles of two intraspecific Larix lines and their reciprocal hybrids. Mol Biol Rep 39:3773-3784

Li D, Huang Z, Song S, Xin Y, Mao D, Lv Q, Zhou M, Tian D, Tang M, Wu Q, Liu X, Chen T, Song X, Fu X, Zhao B, Liang C, Li A, Liu G, Li S, Hu S, Cao X, Yu J, Yuan L, Chen C, Zhu L (2016) Integrated analysis of phenotype, genome, and transcriptome of hybrid rice uncovered multiple heterosis-related loci for yield increase. Proc Natl Acad Sci USA 113: E6026-E6035

Lippman ZB, Zamir D (2007) Heterosis: revisiting the magic. Trends Genet 23:60-66

Miller M, Song Q, Shi X, Juenger TE, Chen ZJ (2015) Natural variation in timing of stress-responsive gene expression predicts heterosis in intraspecific hybrids of Arabidopsis. Nat Commun 6:7453

Ni Z, Kim ED, Ha M, Lackey E, Liu J, Zhang Y, Sun Q, Chen ZJ (2009) Altered circadian rhythms regulate growth vigour in hybrids and allopolyploids. Nature 457:327-331

Plötner B, Nurmi M, Fischer A, Watanabe M, Schneebeger K, Holm S, Vaid N, Schöttler MA, Walther D, Hoeftiger R, Weigel D, Laitinen RAE (2017) Chlorosis caused by two recessively interacting genes reveals a role of RNA helicase in hybrid breakdown in Arabidopsis thaliana. Plant J 91:251-262

Romagnoli S, Maddalon M, Livini C, Motto M (1990) Relationship between gene expression and hybrid vigor in primary root tips of young maize (Zea mays L) plantlets. Theor Appl Genet 80:767-775

Saeki N, Kawanabe T, Ying H, Shimizu M, Kojima M, Abe H, Okazaki K, Kaji M, Taylor JM, Sakakibara H, Peacock WJ, Dennis ES, Fujimoto R (2016) Molecular and cellular characteristics of hybrid vigour in a commercial hybrid of Chinese cabbage. BMC Plant Biol 16:45

Sarazin V, Duclecreq J, Mendou B, Aubanelle L, Nicolas V, Aono M, Pillard S, Guerineau F, Sangwan-Norrel B, Sangwan RS (2015) Arabidopsis BNT1, an atypical TIR-NBS-LRR gene, acting as a regulator of the hormonal response to stress. Plant Sci 239:216-229

Schnable PS, Springer NM (2013) Progress toward understanding heterosis in crop plants. Annu Rev Plant Biol 64:71-88

Shen H, He H, Li J, Chen W, Wang X, Guo L, Peng Z, He G, Zhong S, Qi Y, Terzaghi W, Deng XW (2012) Genome-wide analysis of DNA methylation and gene expression changes in two Arabidopsis ecotypes and their reciprocal hybrids. Plant Cell 24:875-892

Song H, Yi H, Do c, Han CT, Nou IS, Hur Y (2017) Genome-wide analysis of gene expression to distinguish photoperiod-dependent and -independent flowering in Brassicaceae. Genes Genom 39:207-223
Stupar RM, Springer NM (2006) *Cis*-transcriptional variation in maize inbred lines B73 and Mo17 leads to additive expression patterns in the F1 hybrid. Genetics 173:2199-2210
Stupar RM, Gardiner JM, Oldre AG, Haun WJ, Chandler VL, Springer NM (2008) Gene expression analyses in maize inbreds and hybrids with varying levels of heterosis. BMC Plant Biol 8:33
Tsaftaris AS (1995) Molecular aspects of heterosis in plants. Physiol Plant 94:362-370
Wang L, Wu LM, Greaves IK, Zhu A, Dennis ES, Peacock WJ (2017) PIF4-controlled auxin pathway contributes to hybrid vigor in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 114:E3555-E3562
Wang T, Sui Z, Liu X, Li Y, Li H, Xing J, Song F, Zhang Y, Sun Q, Ni Z (2016) Ectopic expression of a maize hybrid up-regulated gene, ErbB-3 binding Protein 1 (ZmEBP1), increases organ size by promoting cell proliferation in *Arabidopsis*. Plant Sci 243:23-34
Workman C, Jensen LJ, Jarmer H, Berka R, Gautier L, Nielsen HB, Saxild HH, Nielsen C, Brunak S, Knudsen S (2002) A new non-linear normalization method for reducing variability in DNA microarray experiments. Genome Biol 3:research0048
Yang Y, Wu X, Xuan H, Gao Z (2016) Functional analysis of plant NB-LRR gene *L3* by using *E. coli*. Biochem Biophys Res Commun 478:1569-1574
Zemach A, Kim MY, Hsieh PH, Coleman-Derr D, Eshed-Williams L, Thao K, Harmer SL, Zilberman D (2013) The *Arabidopsis* nucleosome remodeler DDM1 allows DNA methyltransferases to access H1-containing heterochromatin. Cell 153:193-205