J Plant Biotechnol (2017) 44:208–219
DOI:https://doi.org/10.5010/JPB.2017.44.3.208

ISSN 1229-2818 (Print)
ISSN 2384-1397 (Online)

Research Article

Genome-wide analysis of heterosis-related genes in non-heading Chinese cabbage

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Received: 18 September 2017 / Revised: 19 September 2017 / Accepted: 21 September 2017

Abstract Heterosis or hybrid vigor describes a phenomenon that superior phenotypes compared to the two parents are observed in the heterozygous F_1-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 F_1 hybrids and non-additive expression was prominent. Genes that are expressed specifically for F_1 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 F_1 hybrid-specific. Thirteen NBS-LRR class genes showed high and specific expression in F_1 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, F_1 hybrid, Non-heading Chinese cabbage, NBS-LRR, microarray

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Introduction

Hybrid vigor or heterosis is a phenomenon in which the heterozygous F_1-hybrid plants exhibit superior phenotypes in biomass, growth, size and stress resistance, over their homozygous parent inbred lines. Due to superior phenotypes of F_1 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 F_1 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; Tsaftaris 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

level is equivalent to the mid-parent values while non-additive expression occurs whenever the hybrid expression level deviates from the mid-parent level (Stupar et al. 2006). The expression levels outside the parental range in non-additive expression will greatly contribute heterosis phenotypes. However, it is challenging to determine how many genes or what kinds of genes are involved in heterosis, because large numbers of genes differentially contribute to the superior performance of hybrid, depending on growth stages or organs (Schnable and Springer 2013).

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 *Arbiosopsis* 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. *chinenssis* F_1 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. *chinenssis* F_1 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), themicroarray 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′- GAACCGGGTG CTCCTCAGGA-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 F_1 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 F_1 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 F_1 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).

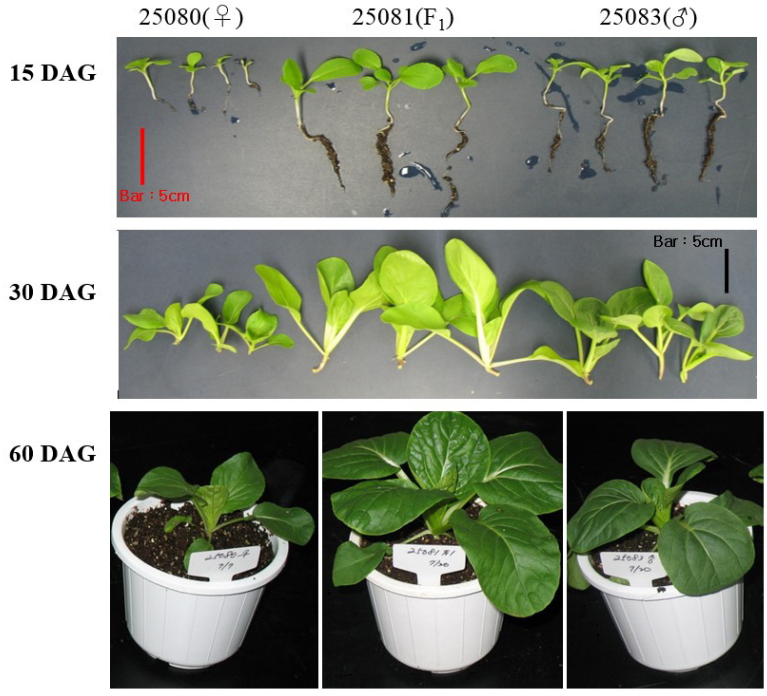

Fig. 1 Morphology of plants used in this experiment. Self-incompatible Aijiaohung line (25080) and Suzhouqing inbred line (25083) were used as female parent male parent, respectively. '25081' indicates F_1 hybrid

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 F_1 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 (41)			952 (33)			808 (50)		
Non-additive expression		13,695 (6,900)			14,358 (7,918)			12,692 (5,561)		

( ): 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. rapa* ssp. *pekinensis*) (Saeki et al. 2016). Saeki et al. (20106) 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 F1 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 hybirds (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.

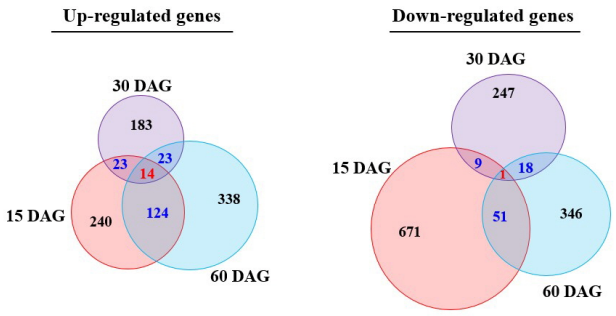

Fig. 2 Distribution of DEGs in F_1 hybrids showing more than two fold expression change, when compared with both parents. DAG, days after germination

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 *Brassica* mRNAs, suggesting that heterosis of *B. rapa* ssp *chinenesis* is affected by *Brassica*-specific genes. Still, we found several genes, which might possess heterosis-related functions, in the list: *Brapa_ESTC051755* for photosynthesis and *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: *Brapa_ESTC031864* encoding leucine-rich repeat protein kinase family protein, *Brapa_ESTC002203* encoding 20S PROTEASOME BETA SUBUNIT PBF1, *Brapa_ESTC014631* encoding NAD(P)-linked oxidoreductase superfamily protein and *Brapa_ESTC024642* encoding disease resistance-responsive dirigent-like protein. Kumar et al. (2016) reported that *Arabidopsis* orthologs for *Brapa_ESTC014631* and *Brapa_ESTC024642* exhibit defense response. For down-regulated genes in all stages, only one gene, *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 *Arabidopdopsis thaliana,* and photosynthesis-related gene showed up-regulation in *B. rapa* F_1 hybrid (references in Table 3). We summarized expression levels of *B. rapa* homolgos of

Table 2 List of differentially expressed genes in F_1 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)

Up/Down (Stages)	At_Locus	Br_SEQ_ID	BRAD_ID	Description, BlastN	Fold change					
					15 DAG		30 DAG		60 DAG	
					81/80	81/83	81/80	81/83	81/80	81/83
UP (All)	No_hits_found	Brapa_ESTC048713	Bra006663	PREDICTED: B. rapa QWRF motif-containing protein 7-like	**18.4**	**138.0**	**15.1**	**205.1**	**25.5**	**227.8**
		Brapa_ESTC048714	-	..	**5.6**	**8.8**	**6.7**	**9.4**	**9.5**	**9.1**
	AT1G49980	Brapa_ESTC019040	Bra018822	DNA/RNA polymerases superfamily protein	**57.5**	**51.2**	**40.3**	**40.3**	**47.8**	**41.6**
	No_hits_found	Brapa_ESTC048297	-	PREDICTED: Brassica rapa uncharacterized LOC103834461	**37.1**	**34.6**	**15.3**	**17.0**	**103.1**	**141.1**
		Brapa_ESTC048298	-	..	**3.3**	**2.8**	**3.5**	**4.9**	**28.8**	**24.2**
	No_hits_found	Brapa_ESTC049333	-	Br sequence	**30.4**	**25.7**	**29.3**	**28.7**	**35.5**	**29.9**
	AT5G47110	Brapa_ESTC051755	Bra023915	At5g47110 gene (light-harvesting-like protein 3 (LIL3))	**28.5**	**32.6**	**15.5**	**15.2**	**58.1**	**51.3**
	No_hits_found	Brapa_ESTC042529	Bra038041	PREDICTED: B. napus uncharacterized LOC106433243	**24.4**	**4.5**	**8.1**	**2.8**	**11.8**	**11.2**
	No_hits_found	Brapa_ESTC051614	Bra010245	PREDICTED: B. napus uncharacterized LOC106418084, ncRNA	**21.9**	**26.2**	**9.7**	**9.4**	**46.2**	**2.7**
	No_hits_found	Brapa_ESTC001522	-	Br sequence	**19.4**	**21.6**	**23.1**	**9.8**	**42.2**	**2.1**
	AT2G13970	Brapa_ESTC044073	Bra038041	PREDICTED: B. napus uncharacterized LOC106431633	**11.6**	**3.8**	**6.4**	**3.8**	**11.4**	**10.0**
	AT3G60820	Brapa_ESTC002203	Bra014468	20S PROTEASOME BETA SUBUNIT PBF1	**6.9**	**7.3**	**7.8**	**7.9**	**7.2**	**33.0**
	No_hits_found	Brapa_ESTC049334	-	Br sequence	**3.5**	**6.1**	**4.5**	**4.8**	**7.4**	**7.3**
	No_hits_found	Brapa_ESTC031120	Bra027213	PREDICTED: B. napus putative F-box protein At3g17620	**2.8**	**3.7**	**3.4**	**2.9**	**5.0**	**4.2**
Up (15/30)	No_hits_found	Brapa_ESTC051613	Bra010245	PREDICTED: B.a rapa uncharacterized LOC103849825	**42.9**	**48.9**	**14.6**	**18.0**	34.1	1.5
	No_hits_found	Brapa_ESTC033737	Bra006426	PREDICTED: B. rapa uncharacterized LOC103856270	**17.6**	**29.0**	**32.1**	**25.8**	29.7	0.9
	No_hits_found	Brapa_ESTC003869	Bra013078	PREDICTED: B. rapa uncharacterized LOC103856270	**15.0**	**7.3**	**18.0**	**10.1**	25.4	0.5
	No_hits_found	Brapa_ESTC003723	Bra004000	PREDICTED: B. rapa probable protein S-acyltransferase 22	**12.7**	**2.6**	**6.3**	**3.6**	1.6	2.0
	No_hits_found	Brapa_ESTC004680	Bra019236	PREDICTED: B. rapa probable protein S-acyltransferase 22	**8.3**	**7.7**	**6.3**	**7.8**	1.8	2.1
	AT5G19760	Brapa_ESTC011203	Bra006516	Mitochondrial substrate carrier family protein	**2.0**	**2.2**	**2.1**	**2.2**	1.9	2.0
	AT1G60710*	Brapa_ESTC014631	Bra027140	NAD(P)-linked oxidoreductase superfamily protein (ATB2)(Auxin-inducible)	**21.1**	**35.7**	**8.0**	**9.7**	14.7	1.0
	No_hits_found	Brapa_ESTC033736	Bra013078	PREDICTED: B. rapa uncharacterized LOC103856270	**11.0**	**3.0**	**22.6**	**6.9**	16.4	0.5
	No_hits_found	Brapa_ESTC043214	Bra031342	PREDICTED: B. napus splicing factor U2af large subunit B-like (LOC106346061)	**6.9**	**2.2**	**9.8**	**3.5**	14.6	0.7
Up (30/60)	AT3G52860	Brapa_ESTC001902	Bra016859	Arabidopsis thaliana other RNA lncRNA	1.0	1.4	**2.1**	**8.6**	**2.7**	**4.2**
	AT4G13760	Brapa_ESTC020421	Bra000327	Pectin lyase-like superfamily protein	1.3	2.5	**5.3**	**5.0**	**11.4**	**9.7**
	AT1G64160*	Brapa_ESTC024642	Bra027681	Disease resistance-responsive (dirigent-like protein) family protein (DIR5)	2.2	0.5	**8.0**	**2.5**	**3.1**	**2.4**
	AT3G47090	Brapa_ESTC031864	Bra035694	Leucine-rich repeat protein kinase family protein (LRR-RLK)	1.4	0.7	**12.3**	**18.1**	**11.6**	**11.0**
	No_hits_found	Brapa_ESTC038099	-	PREDICTED: B. napus uncharacterized LOC106386659, ncRNA	0.3	2.6	**3.4**	**6.3**	**2.3**	**2.9**
	No_hits_found	Brapa_ESTC040600	Bra035021	PREDICTED: B. napus uncharacterized LOC106403229	2.1	1.4	**2.1**	**2.6**	**4.5**	**4.2**
	AT1G43090	Brapa_ESTC041995	Bra017014	Pectin lyase-like superfamily protein	0.7	0.6	**22.1**	**10.4**	**31.3**	**38.2**
	No_hits_found	Brapa_ESTC051749	-	PREDICTED: B. napus uncharacterized LOC106433400	1.8	1.3	**7.2**	**3.7**	**7.2**	**4.1**
	No_hits_found	Brapa_ESTC051750	-	PREDICTED: B. napus uncharacterized LOC106433400	1.7	1.2	**14.3**	**8.9**	**16.0**	**13.6**
Down (All)	AT1G62510	Brapa_ESTC012945	Bra027039	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	**-5.0**	**-4.3**	**-2.4**	**-7.7**	**-3.8**	**-7.8**

List of differentially expressed genes in F_1 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 bollworm.)

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									Reference
			15 DAG			30 DAG			60 DAG			
			25080	25081	25083	25080	25081	25083	25080	25081	25083	
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	4341	
		Brapa_ESTC028906	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_ESTC015231	4304	4017	3004	4437	3812	4102	5597	6491	6791	
		Brapa_ESTC047153	3610	3984	4698	3525	4169	5220	3738	4304	7589	
AT1G22770	*GI* (GIGANTEA)	Brapa_ESTC017304	10582	13343	17247	11276	17041	16639	11515	13092	15503	Ni et al. 2009
		Brapa_ESTC039985	14641	14131	17624	13652	18088	17106	10937	12892	13800	
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	11878	8071	10934	12229	6198	8463	16081	
AT2G46830	*CCA1* (CIRCADIAN CLOCK ASSOCIATED 1)	Brapa_ESTC006966	617	197	288	84	99	126	1913	1194	891	Ni et al. 2009
		Brapa_ESTC008890	945	280	309	164	249	166	3656	2975	1858	
AT3G51800	*ATG2* (G2p-related protein)(EBP1)	Brapa_ESTC007218	8864	8878	10174	10754	10830	12012	12180	11249	9869	Wang et al. 2016
		Brapa_ESTC007283	16033	14047	15178	18170	18601	20514	21149	19476	19020	
		Brapa_ESTC008892	14493	14210	14921	17826	15503	18299	17708	18251	16788	
		Brapa_ESTC017696	6663	5780	6951	9232	10731	10114	12313	11761	12483	
		Brapa_ESTC020359	9573	7849	11016	11578	10661	11844	14132	13022	12074	
		Brapa_ESTC021489	10939	9850	13452	12292	11871	13230	16308	14754	13506	
		Brapa_ESTC025539	4032	3531	6893	3853	5519	5702	5197	3238	2533	
		Brapa_ESTC043540	10325	12035	11256	12677	13211	14065	12451	15279	13073	
AT4G19020	*CMT2* (Chromomethylase 2)	Brapa_ESTC009064	2915	3337	3167	2479	2006	2499	3125	3424	3223	Zemach et al. 2013
AT5G05450	*RH18* (DEAD/DEAH box helicase, putative)	Brapa_ESTC030024	2565	2964	3105	2847	2102	2437	2622	2540	2169	Plötner et al. 2017
		Brapa_ESTC045372	6793	6539	7459	7947	5535	7071	7744	7255	7546	
AT5G49160	*MET1* (DECREASED METHYLATION 2DNA)	Brapa_ESTC014151	6107	5762	4380	8626	3442	5289	6703	5063	3701	Kawanabe et al. 2016
AT5G61380	*TOC1* (TIMING OF CAB1 1)	Brapa_ESTC002126	2773	3582	4766	3066	2649	3641	944	766	1112	Ni et al. 2009
		Brapa_ESTC006958	7045	8226	9979	12056	10903	10955	2257	2138	3015	
		Brapa_ESTC033209	16000	19937	20292	18860	18627	19981	7451	7250	9041	
AT5G66750	*DDM1* (DECREASED DNA METHYLATION 1)	Brapa_ESTC020388	1798	1305	1356	2802	953	1624	2544	2018	1738	Zemach et al. 2013
	SWI2/SNF2-like protein; Chromatin remodeling 1 (CHR1)	Brapa_ESTC030555	4050	2966	2595	5163	2537	2736	4415	3853	3338	
AT4G25080	*CHLM* (MAGNESIUM-PROTOPORPHYRIN IX METHYLTRANSFERASE)	Brapa_ESTC002086	13691	18396	14496	14269	12665	13189	14831	16039	14486	Saeki et al. 2016
		Brapa_ESTC015556	14397	13943	11646	12510	9260	8697	15347	12276	9566	
		Brapa_ESTC047649	6196	5880	6028	6742	5821	3960	7479	6888	7527	
AT3G61470	*LHCA2* (Photosystem I light harvesting complex gene 2)	Brapa_ESTC007961	40044	45566	36797	42413	37810	41103	40901	42837	37873	Saeki et al. 2016
		Brapa_ESTC010415	38879	41868	37548	36718	33981	38168	37282	35083	36177	
		Brapa_ESTC012915	22986	28174	24231	22768	19967	19510	24822	22579	22955	
		Brapa_ESTC023178	3647	3505	2885	2886	2833	2375	2543	2964	2952	
		Brapa_ESTC027380	4004	5096	5055	2954	2592	2174	5670	4264	4704	

Table 3 Continued

At_Locus	Gene description	Br_SEQ_Id	Probe intensity									Reference
			15 DAG			30 DAG			60 DAG			
			25080	25081	25083	25080	25081	25083	25080	25081	25083	
		Brapa_ESTC029407	23250	23845	24536	19038	24518	18430	23398	31942	28986	
		Brapa_ESTC031617	102	72	12	195	133	23	168	72	15	
		Brapa_ESTC051898	90	148	369	71	69	53	409	379	437	
AT3G22231	PCC1 (PATHOGEN AND CIRCADIAN CONTROLLED 1)	Brapa_ESTC028145	121	10803	12587	129	5754	13964	84	12832	15138	Miller et al. 2015
		Brapa_ESTC047280	149	12667	15914	156	12142	16511	34	17737	24380	
AT2G40000	HSPRO2 (HOMOLOG OF SUGAR BEET HS1 PRO-2)	Brapa_ESTC011718	20546	11481	10834	8698	8817	6869	2015	2417	1754	Miller et al. 2015
		Brapa_ESTC012195	19188	19022	22703	10708	11129	13634	5408	7166	9784	
		Brapa_ESTC014954	6867	3389	4766	2478	4776	3257	1020	802	1033	
		Brapa_ESTC021307	22562	18144	22090	9919	10494	11943	3862	4634	8553	
		Brapa_ESTC021617	25259	18414	13942	14969	16658	11849	7868	7790	6467	
		Brapa_ESTC024564	8822	4388	5638	2509	5218	4074	818	627	1049	
		Brapa_ESTC025072	16419	15051	17053	8610	9287	11446	4059	5230	7798	
		Brapa_ESTC038750	23748	20199	22408	12753	12716	15184	5145	6741	11178	
AT2G14610	PR1 (PATHOGENESIS-RELATED GENE 1)	Brapa_ESTC002102	24976	7546	16429	19659	14660	8789	14617	17595	18051	Miller et al. 2015
		Brapa_ESTC013481	7451	6349	6681	7800	7648	8177	2569	1400	1203	
		Brapa_ESTC042390	31300	11521	21992	25887	18717	12265	18855	21498	21687	
AT5G52310	COR78 (COLD REGULATED 78)	Brapa_ESTC003116	29897	33849	29168	35598	24688	30845	22402	19684	19596	Miller et al. 2015
		Brapa_ESTC007756	22484	26325	23312	20265	22952	19805	18859	22770	22197	
AT1G20440	COR47 (COLD REGULATED 47)	Brapa_ESTC047060	29034	29501	32189	25268	21550	21905	26515	26981	26378	Miller et al. 2015
		Brapa_ESTC018725	19163	18060	20708	15488	14960	11953	17599	18980	17988	
AT5G25610	RD22 (RESPONSIVE TO DESSICATION 22)	Brapa_ESTC001082	21734	21850	20960	27979	37840	37698	24547	27274	28453	Miller et al. 2015
		Brapa_ESTC010889	15824	12213	12688	21638	17455	21897	19309	16196	13363	
		Brapa_ESTC025981	22113	20015	17356	30697	39296	36578	26766	25407	23135	
		Brapa_ESTC032166	255	135	52	232	170	121	295	307	121	
		Brapa_ESTC049783	6212	5183	5883	7006	8241	7307	7224	6590	6255	

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, F_1 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 F_1 hybrids (Table 4). Only three *Arabidopsis* genes have been functionally characterized so far. *AT5G11250*, 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 F_1 hybrids at least two growing stages

*At*_Locus	Description, blastN (Disease Resistant Protein)	Br_SEQ_ID	BRAD_ID	Probe intensity								
				15 DAG			30 DAG			60 DAG		
				25080	25081	25083	25080	25081	25083	25080	25081	25083
AT1G53350	CC-NBS-LRR class, putative	Brapa_ESTC015346	Bra037448	277	4520	2420	406	1119	2610	625	6549	1265
AT1G72890	TIR-NBS class, putative	Brapa_ESTC036158	Bra016029	246	280	10	175	373	54	328	236	52
AT5G18350	TIR-NBS-LRR class, putative	Brapa_ESTC037594	Bra001161	886	1749	990	766	838	526	628	764	302
		Brapa_ESTC042689	Bra001160	5848	5689	4363	4273	4893	3867	3076	4010	2814
		Brapa_ESTC029890	Bra006487	279	417	243	261	304	324	176	468	378
AT5G11250*	TIR-NBS-LRR class, putative	Brapa_ESTC032144	Bra006146	2851	3805	2565	1739	2010	1919	1608	2204	1326
		Brapa_ESTC009435	Bra006556	3459	3886	3750	2819	3352	2620	3486	3439	3281
AT5G66900	CC-NBS-LRR class, putative	Brapa_ESTC008403	Bra025290	867	2003	1682	1430	1837	1437	1491	1783	1162
		Brapa_ESTC025668	Bra018057	9875	12594	10126	8353	9561	11576	4918	6728	4967
AT5G46450	TIR-NBS-LRR class, putative	Brapa_ESTC046013	Bra022036?	194	108	28	107	118	83	87	164	113
AT5G41540	TIR-NBS-LRR class, putative	Brapa_ESTC018846	Bra001162	3595	3731	1929	2624	2481	1933	2191	2890	2021
AT1G15890*	CC-NBS-LRR class, putative	Brapa_ESTC034248	Bra018835	5532	4963	4243	5609	6472	5486	3854	4051	3145
AT1G69550	TIR-NBS class, putative	Brapa_ESTC034328	Bra020936	65	119	132	95	169	102	54	164	134
AT4G27190	NBS-LRR class, putative	Brapa_ESTC008807	Bra026368	3914	4404	4023	6671	8044	5932	6100	5244	4694
AT5G22690	TIR-NBS-LRR class, putative	Brapa_ESTC021828	Bra034079	1499	1075	1727	1629	3193	2402	923	1369	1278
AT3G50950*	CC-NBS-LRR class, putative	Brapa_ESTC036172	Bra036845	17874	13266	11186	11327	11777	10183	7026	8846	8697
AT5G41750	TIR-NBS-LRR class, putative	Brapa_ESTC042275	Bra013144	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				Remarks
					25080	25081	25083	Mid-parent value	
					Female	F1 hybrid	Male	(80+83)/2	
Additive expression	15 DAG	Brapa_ESTC034378	AT3G10690	DNA gyrase subunit A family protein	7362.4	5731.5	4099.3	5730.8	f
		Brapa_ESTC011594	AT5G11110	ATSPS2F (sucrose phosphate synthase 2F)	473.5	1011.8	1547.8	1010.7	m
	30 DAG	Brapa_ESTC012536	AT4G01310	Ribosomal protein L5 family protein	18408.4	15713.8	13017.2	15712.8	f
		Brapa_ESTC022326	AT1G27190	LRR transmembrane protein kinase, putative	9591.3	8719.6	7847.5	8719.4	f
		Brapa_ESTC005539	AT4G29010	AIM1 (ABNORMAL INFLORESCENCE MERISTEM)	1554.5	2467.4	3379.4	2466.9	m
	60 DAG	Brapa_ESTC046155	AT5G46630	Clathrin adaptor complexes medium subunit family protein	17451.9	15204.9	12958.5	15205.2	f
		Brapa_ESTC050491	AT2G39730	RCA (RUBISCO ACTIVASE)	11021.2	9163.8	7307.6	9164.4	f
Non-additive expression	15 DAG	Brapa_ESTC011189	AT2G05100	LHCB2.1 (Photosystem II light harvesting complex gene 2.1)	39951.7	45713.9	39491.5	39721.6	+
		Brapa_ESTC016074	AT1G29930	CAB1 (CHLOROPHYLL A/B BINDING PROTEIN 1)	51284.7	44655.3	55324.5	53304.6	–
		Brapa_ESTC025522	AT5G66190	ATLFNR1 (LEAF FNR 1); NADPH dehydrogenase/ oxidoreductase	36986.1	38352.5	27943.2	32464.6	+
	30 DAG	Brapa_ESTC047891	AT1G20620	CAT3 (CATALASE 3)	38890.8	51640.6	45377.4	217134.1	+
		Brapa_ESTC032082	AT5G09440	Phosphate-responsive protein, putative	12234.3	28554.8	16204.8	14219.6	+
		Brapa_ESTC016880	AT2G26230	Uricase / urate oxidase / nodulin 35, putative	6084.3	17723.4	14794.1	10439.2	+
		Brapa_ESTC021056	AT5G25540	CID6 (CTC-Interacting Domain 6); protein binding	16785.4	17366.5	8675.0	12730.2	+
	60 DAG	Brapa_ESTC022798	AT1G06680	PSBP-1 (OXYGEN-EVOLVING ENHANCER PROTEIN 2)	39278.9	50232.5	39308.9	39293.9	+
		Brapa_ESTC013408	AT5G04590	SIR (sulfite reductase)	6239.6	14146.4	4784.8	5512.2	+

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 variatinos were detected, RT-PCR signals were similar to that observed with PI values, indicating that micro-array experiments reliably reflected transcription levels in general. Particularly, all up-regulated genes were confirmed to be predominantly expressed in F_1 hybrids, while genes

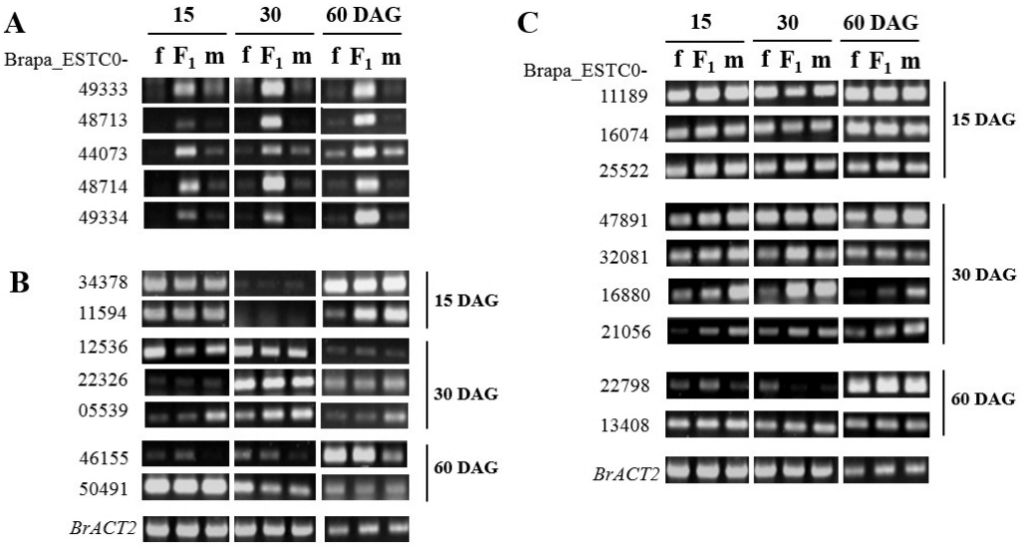

Fig. 3 RT-PCR confirmation of expression patters for selected genes. A, Up-regulated genes in F1 hybrid at all stages; B, Genes with additive expression at the indicated stage (right side); C, Genes with non-additive expression at the indicated stage (right side)

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, F_1 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 F_1 hybrid and its parents. We identified (1) several putative HRGs that are highly up-regulated in F_1 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.

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

Expression pattern	Stages	*Br*_SEQ_ID	Primer Sequence	
			Forward (5'-)	Reverse (5'-)
Up-regulated	All stages	Brapa_ESTC049333	CTAGCCGCCTCCGTCGATCT	GTCGGACTGGACCTCCATGAA
		Brapa_ESTC048713	GGCATCCCAGAAGGTGCTCTAA	ATTGCCACATGAGAAGGAAGCC
		Brapa_ESTC044073	CAACAAGACCATCAGGGAGGCT	CTTTTCGCACCAAGGATGACCT
		Brapa_ESTC048714	CCAATGACCCAAAGGAGGTCTG	CCGTGGACTTTGTTGCTTGTTG
		Brapa_ESTC049334	GTCGGACTGGACCTCCATGAAC	TCCGTCGATCTGTGGTAGATCC
Additive	15 DAG	Brapa_ESTC034378	TGGAAGACTCCGACAGTGGTGA	CTGGTCTTCGGAAAACTCGCTC
		Brapa_ESTC011594	CGGAAGAAACGGGTCTTCGATA	AAGCTCTCTCCTCCCCACCTGT
	30 DAG	Brapa_ESTC012536	CCGCGTACCTGGAGAGAATCAT	GACACCAATGCTGTAGTTGCCG
		Brapa_ESTC022326	AGCTGAACCTGATCCCGACTTG	GACCGATCATCAGCATGCAAAG
		Brapa_ESTC005539	CAATATAATGCCCGGTGGGAAG	GCATTCCATTCATTGCCCTTTC
	60 DAG	Brapa_ESTC046155	CAGAGGGAGTGAATCTGCCGTT	CACCTTGAGGAACCGAACTCGT
		Brapa_ESTC050491	AGGGGATCTTACAATATTAATGGTTTGC	GACAACTTTGATCCAACAGCTAGAAGTG
Non-additive	15 DAG	Brapa_ESTC011189	CAGCAATCCAGCAATCCTCCTT	TCTTAGCGAATGTCTCCGGGTC
		Brapa_ESTC016074	CTGCTCCAAACAACAACCAATAAAC	CTTTCGGACCCGTACCATGG
		Brapa_ESTC025522	CACATCGTCTTCACCACCGAAG	TGGTCCAGTGATCTTTGCCTCA
	30 DAG	Brapa_ESTC047891	CTTTGATCTTGTTGGTAACAACACTCC	CGACGTCATCAAACATCCAGC
		Brapa_ESTC032082	GAGCTTACGTCTGGGTGGGAAA	CTTCTTGATTCCCGCCGTTAAA
		Brapa_ESTC016880	TCGTCGCTACCGATACCATGAA	CCACGGTATGGTTCTCTGACCC
		Brapa_ESTC021056	CACGTGCAGTACCAACCCTACG	GAGGATGTTGATGATGCGCTTG
	60 DAG	Brapa_ESTC022798	ACAGCGCGTGTTTCCTACATCA	TGTGTTTGTCTTTGGCTTCCCA
		Brapa_ESTC013408	GTCGACCCATTAAACCAAACGG	TCGGTGTTCCTCCTAGCCAAAC