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RESEARCH ARTICLE

Transcriptomic analysis of contrasting inbred lines and F_2 segregant of Chinese cabbage provides valuable information on leaf morphology

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Abstract

Background Leaf morphology influences plant growth and productivity and is controlled by genetic and environmental cues. The various morphotypes of *Brassica rapa* provide an excellent resource for genetic and molecular studies of morphological traits.

Objective This study aimed to identify genes regulating leaf morphology using segregating *B. rapa* p F_2 population.

Methods Phenotyping and transcriptomic analyses were performed on an F_2 population derived from a cross between Rapid cycling *B. rapa* (RCBr) and *B. rapa* ssp. *penkinensis*, inbred line Kenshin. Analyses focused on four target traits: lamina (leaf) length (LL), lamina width (LW), petiole length (PL), and leaf margin (LM).

Results All four traits were controlled by multiple QTLs, and expression of 466 and 602 genes showed positive and negative correlation with leaf phenotypes, respectively. From this microarray analysis, large numbers of genes were putatively identified as leaf morphology-related genes. The Gene Ontology (GO) category containing the highest number of differentially expressed genes (DEGs) was "phytohormones". The sets of genes enriched in the four leaf phenotypes did not overlap, indicating that each phenotype was regulated by a different set of genes. The expression of *BrAS2, BrAN3, BrCYCB1;2, BrCYCB2;1,4, BrCYCB3;1, CrCYCBD3;2, BrULT1*, and *BrANT* seemed to be related to leaf size traits (LL and LW), whereas *BrCUC1, BrCUC2*, and *BrCUC3* expression for LM trait.

Conclusion An analysis integrating the results of the current study with previously published data revealed that Kenshin alleles largely determined LL and LW but LM resulted from RCBr alleles. Genes identified in this study could be used to develop molecular markers for use in *Brassica* breeding projects and for the dissection of gene function.

Keywords Br135K microarray · DEGs · Leaf phenotype · Leaf size · *Brassica rapa* · Kenshin · Rapid cycling *Brassica rapa*.

Introduction

Leaves are the major biosynthetic organs of plants. As the function of a leaf is associated with its structure, leaf morphology influences plant growth and development (Tsukaya 2005; Fambrini and Pugliesi 2013). Leaf morphology varies between plant species and is under strong genetic and environmental control. The crop species *Brassica rapa* displays extreme morphological diversity, encompassing leafy vegetables, turnips, and oilseed rape. This diversity results from both genetic and epigenetic variation, and includes important traits for *Brassica* breeders (Zhao et al. 2005; Bonnema et al. 2011). Understanding morphological variation and development of leaves can help the production of novel leafy crops that attract consumers (Xiao et al. 2014)

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and provides insight into the adaptation of leaves to diverse environments (Kalve et al. 2014).

The determination of leaf shape and size is primarily controlled by meristem genes (including *CLAVATA1, CLAVATA3, WUSCHEL, KNOTTED1*, and *PHANTASTICA*), which are involved in leaf initiation; initiation is also regulated by hormone levels (Tsukaya 2005; Kessler and Sinha 2004). Final leaf size, however, is determined by the complex coordination of cell division and expansion (Kim and Cho 2006; Tsukaya 2006; Barkoulas et al. 2007; Micol 2009; Gonzalez et al. 2010, 2012).

A leaf is composed of the blade and the petiole, which differ from each other in terms of structure and physiology (Tsukaya 2006). The petiole supports the leaf blade and functions in a similar manner to the stem (Tsukaya et al. 2002; Kozuka et al. 2005, 2010). Petiole development is stimulated by shade-avoidance conditions (Tsukaya et al. 2002) and controlled by photoreceptors, such as phytochrome or cryptochrome (Kozuka et al. 2005). In addition, petiole development is regulated by hormones such as auxin, gibberellin, brassinosteroids (BRs), and ethylene (Kim et al. 2005). The proliferative zone, the region of the junction between the leaf blade and leaf petiole, produces both leaf blade and petiole cells and appears to be controlled by *ANGUSTIFOLIA3* (*AN3*) (Ichihashi et al. 2011). Leaf blade development, by contrast, is controlled by two major genes, the auxin transport-related *PINFORMED1* (*PIN1*) and a growth repressor *CUP-SHAPED COTYLEDON2* (*CUC2*) (Hay et al. 2006; Nikovics et al. 2006; Bilsborough et al. 2011), and a plant hormone, cytokinin (Shani et al. 2010). Several additional genes are known to be involved in blade growth: *BIG BROTHER* (*BB*), *ARP* [*ASYMMETRIC LEAVES1* (*AS1*), *ROUGH SHEATH2* (*RS2*), *PHANTASTICA*], *ASYMMETRIC LEAVES 2* (*AS2*), *CINCINNATA-TEOSINTE BRANCHED/CYCLOIDEA/PCF* (*CIN-TCP*), *WOX* (*WUSCHEL-RELATED HOMEOBOX*), and *JAGGED* (*JAG*) (Bar and Ori 2014). The genes *NO APICAL MERISTEM* (*NAM*)*/CUP-SHAPED COTYLEDON* (*CUC*), and *REDUCED COMPLEXITY* (*RCO*), together with auxin, are involved in the development of the leaf margin (Bar and Ori 2014; Vlad et al. 2014).

Leaf morphology, especially leaf size and shape, is a quantitative trait controlled by many genes. Quantitative trait loci (QTLs) are chromosomal regions that contain a gene or genes affecting a quantitative trait (Geldermann 1975). The most efficient method of analyzing QTLs is to examine transgression in an F_2 population derived from intra- or interspecific crosses (Tanksley 1993; Pérez-Pérez et al. 2010). Since the publication of the first linkage map constructed from a cross between Chinese cabbage and broccoli (Song et al. 1995), several groups have identified QTLs in Chinese cabbages and used them to unravel the genetic regulation of leaf architecture. Lou et al. (2007) identified ten leaf trait-related QTLs using an $F_{2/3}$ population derived from crosses between Chiifu, rapid cycling *Brassica rapa*-144 (RCBr-144), yellow sarson (oilseed rape), pak choi, and turnip. Li et al. (2009) identified candidate genes for control of the leaf lobe shape using an expressed sequence tagged (EST)-based single nucleotide polymorphism (SNP) marker linkage map and progenies derived from a cross between yellow sarson and *B. rapa* cv. Osome (heading type) (Li et al. 2009). Lim and colleagues generated large numbers of transgression lines from several crosses and identified several candidate genes that co-localized with QTLs in subsequent populations; these studies involved crosses between Chiifu and Kenshin (two inbred lines of *B. rapa* ssp. *pekinensis*) (Choi et al. 2017), Chiifu and Caixin (Li et al. 2015), and Chiifu and RCBr (Li et al. 2013). They found that several QTLs controlling leaf size co-localized with the genes *CYCLIN D3;1* (*CYCD3;1*), *CYCLIN B2;4* (*CYCB2;4*), *AN3, ULTRAPETALA1* (*ULT1*), and *AINTEGUMENTA* (*ANT*) (Choi et al. 2017). Xiao et al. (2014) identified a large number of genes regulating leaf development in *Brassica* species by integrating phenotypic QTLs and transcriptomic data of segregating populations derived from a cross between yellow sarson and pak choi. They established expression QTLs (eQTLs) by microarray analysis followed by qRT-PCR analysis (Xiao et al. 2014). More recently, restriction site-associated DNA sequencing (RAD-seq) of F_2 populations generated by a cross between *B. rapa* ssp. *chinensis* and *pekinensis* was used to generate genome-wide SNP-based genetic and QTL maps for Chinese cabbage (Huang et al. 2017).

We used an F_2 population derived from a cross between rapid cycling *Brassica rapa* (RCBr) and Kenshin, an inbred line of *B. rapa* ssp. *pekinensis*. RCBr is one of the Wisconsin fast plants (Goldman 1999; Slankster et al. 2012). A heading-type Kenshin was a parental line of a Chiifu and Kenshin double haploid (CKDH) mapping population used to construct a reference genetic map for sequencing the *Brassica rapa* genome (Vanjildorj et al. 2009). The parental lines and the F_2 population were subjected to phenotypic analysis to identify genes controlling leaf morphology. Next, the parental lines and four groups from the F_2 population, selected using the traits lamina length (LL), lamina width (LW), leaf margin (LM), and petiole length (PL), were subjected to microarray and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analyses. Candidate genes considered likely to control leaf morphology in *B. rapa* were further investigated by comparisons with previous studies.

Materials and methods

Plant materials

F_1 plants derived from a cross between a RCBr inbred line (#24001) and the *Brassica rapa* ssp. *pekinensis* inbred line

Kenshin were self-hybridized to generate an F_2 population. For phenotypic analysis, 15 seeds from each parental line and 200 F_2 seeds, which eventually gave more than 180 individuals, were sown on pots (15 cm diameter × 9 cm height) and grown in a greenhouse at Chungnam National University for 6 weeks. Leaf phenotype was analyzed using the fifth leaf. The seventh and ninth leaves were sampled from at least three individuals showing similar phenotypes, and the combined samples were used in microarray and qRT-PCR analyses. Experiments were conducted twice a year, in the spring (April to May) and fall (October to November). Samples were always collected 4 h after dawn to minimize the effect of the diurnal cycle on gene expression.

Leaf morphology

Four leaf morphology traits were analyzed: lamina (leaf) length (LL), lamina width (LW), petiole length (PL), and leaf margin (LM). The first three traits were graded into 1 cm intervals. LM was expressed as six grades (I: very little serration; II: slightly serrated; III: medium serrated; IV: highly serrated; V: slightly lobed; VI: lobed) according to the relative serration ratio and lobe phenotype.

Microarray experiment

Frozen samples (consisting of the seventh and ninth leaves from RCBr, Kenshin, and four F_2 groups with similar phenotypes: F_2-19, F_2-33, F_2-93, and F_2-100 types) were ground under liquid nitrogen. Total RNA was extracted from each sample with TRIzol reagent (Invitrogen, USA) and cleaned using a NucleoSpin® RNA Clean-up kit (Macherey–Nagel, Germany). The Br135K microarray (Brapa_V3_microarray, 3′-Tiling microarray) was used for transcriptome analysis. This is a high-density DNA array prepared by NimbleGen (http://www.nimblegen.com/) using Maskless Array Synthesizer (MAS) technology, as described in Jung et al. (2014). Probes were designed from 41,173 genes of *Brassica rapa* accession Chiifu-401-42 (http://brassicadb.org/brad/). Each length of three probes was 60 mers designed by 30 bp overlapped in 120 bp (60 bp coding sequence plus 60 bp 3′UTR of each gene), representing 123,647 features. Fifty features were also deposited from five markers (*GUS, GFP, Bar, Kan, Hyg*). Labeling, data processing, and background correction were performed as described previously (Jung et al. 2014). The raw data (.pair file) was subjected to RMA (Robust Multi-Array Analysis) (Irizarry et al. 2003), quantile normalization (Bolstad et al. 2003), and background correction as implemented in the NimbleScan software package, version 2.4.27 (Roche NimbleGen, Inc.). To assess the reproducibility of the microarray analysis, the experiment was repeated using independently prepared total RNA from two biological replicates. The microarray data, was deposited in The National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/) in USA, with the ID of (GSE114479, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114479). To understand the putative biological functions and biochemical pathways of differentially expressed genes (DEGs), enrichment analyses were carried out by searching Gene Ontology (GO) (Ashburner et al. 2000), agriGO (Du et al. 2010), and the Kyoto Encyclopedia of Genes and Genomes (Kanehisa et al. 2008).

Quantitative RT-PCR (qRT-PCR)

Cleaned RNA samples were subjected to first-strand cDNA synthesis using the Ace-α kit with Oligo-dT primers (TOYOBO, Japan). Target genes for qRT-PCR were selected following consultation of a previous report by Kalve et al. (2014) and the PhenoLeaf database (http://genetics.umh.es/phenoleaf/) (Wilson-Sánchez et al. 2014). Primers were designed against sequences in the Brassica database (BRAD, http://brassicadb.org/brad/). PCR, using SYBR® Green Real-time PCR Master Mix-Plus (TOYOBO, Japan) as a SYBR green dye, was performed as follows: 30 s at 95 °C, followed by 40 cycles of 95 °C for 5 s, 58 °C for 10 s, and 72 °C for 15 s.

Results and discussion

Analyses of leaf phenotypes

Trait analysis showed that Kenshin leaves were longer and wider, with shorter petioles and more rounded LMs, than leaves of RCBr (Fig. 1a). The F_2 population derived from an F_1 cross between the RCBr and Kenshin inbred lines displayed various morphological phenotypes (Table 1). The morphological characteristics of the fifth leaf of 6-week-old plants were analyzed (Fig. 1b). For phenotyping, 150 individuals were randomly selected from 180 F_2 plants (Fig. 2). The trait LL (length of leaf from apex to leaf base) was measured, and the values were grouped into 1 cm intervals (Fig. 2a). The mean values of LL for RCBr and Kenshin were less than 5 cm and 9–10 cm, respectively (Table 1). The most frequently observed values of LL were between 8 and 9 cm (34 plants; Fig. 2a). Generally, LL appeared to be a quantitative trait, but slightly shifted toward the Kenshin phenotype.

The values obtained for LW ranged from less than 3 cm to more than 8 cm (Fig. 2b). The mean values of LW for RCBr and Kenshin were less than 3 cm and 5–6 cm, respectively (Table 1). When grouped into 1 cm intervals, the most frequently observed values of LW in the F_2 population were between 5 and 6 cm (47 plants), indicating that LW was a

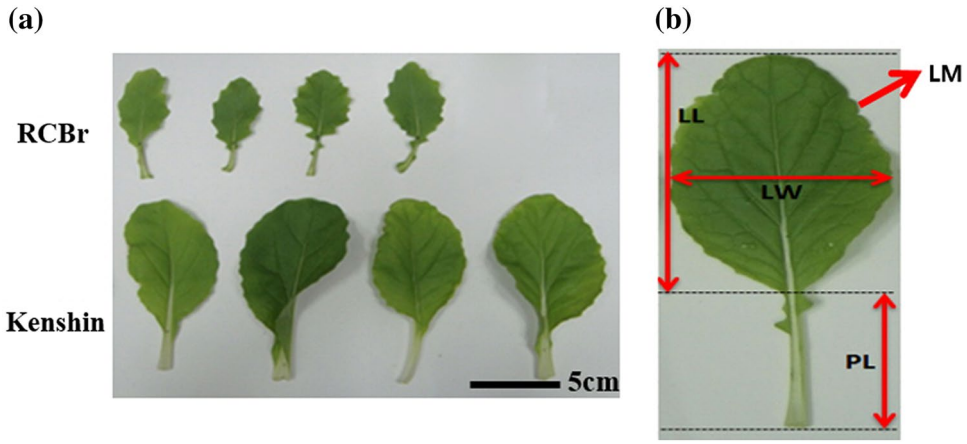

Fig. 1 Overall leaf morphology and specific leaf traits in RCBr and Kenshin lines. The fifth leaf from 6-week-old plants was measured. **a** Representative leaf morphologies of the fifth leaves from the parental lines RCBr and Kenshin. **b** The four leaf traits used in analyses of leaf phenotypes. *LL* lamina length (cm) from apex to base of blade, *LW* lamina width (cm) at the widest region of the lamina, *PL* petiole length (cm), *LM* leaf margin, scored from I to VI (I: very little serration; II: slightly serrated; III: medium serrated; IV: highly serrated; V: slightly lobed; VI: lobed)

Table 1 Description and values of four leaf traits measured from the fifth leaf of representative plants from each genotype

Sample	Description	Trait measurement			
		Lamina length (cm)	Lamina width (cm)	Petiole length (cm)	Leaf margin
Kenshin	Relatively big leaf size and entire leaf margin	9.3 ± 1.07	5 ± 0.80	2.8 ± 0.26	I
RCBr	Relatively small leaf size and lobed leaf margin	4.7 ± 0.89	2.8 ± 0.44	2.6 ± 0.35	VI
F_2-19	Lobed leaf margin	9.8	6.2	7.3	V
F_2-33	Short petiole length	11	6.3	2.9	II
F_2-93	Long lamina length and lamina width	13.7	8.5	2.7	II
F_2-100	Long petiole length	7.9	7.2	9.8	VI

The error bars indicate standard errors. Note that the microarray and qRT-PCR analyses used RNA extracted from the seventh and ninth leaves collected from three plants with similar phenotypes

quantitative trait and again shifted toward the Kenshin phenotype (Fig. 2b).

Both RCBr and Kenshin had mean values of PL of approximately 3 cm: 2.6 cm for RCBr and 2.8 cm for Kenshin (Table 1). The values obtained for PL in the F_2 population ranged from less than 2 cm to more than 9 cm. When these results were grouped into 1 cm intervals, 51 plants had PL values of between 4 and 5 cm (Fig. 2c). PL appeared to be a quantitative trait, but the PLs of most F_2 plants were longer than those of either parental line (Fig. 2c).

The LM trait was scored using a six-point scale ranging from I (very little leaf serration) to VI (leaf lobes present) (Fig. 2d). LMs of RCBr and Kenshin were scored as VI and I, respectively, and the F_2 population showed a left-shifted normal distribution (Fig. 2d). The most frequent LM score was II (45 plants), implying that a round margin was semi-dominant.

Most leaf phenotypes were normally distributed, implying regulation by QTLs. The values observed for LL, LW, and LM in the F_2 population were slightly shifted toward the Kenshin phenotype, but values of PL exceeded those of the parent phenotypes. This suggested that leaf morphology in F_2 individuals was controlled by alleles from Kenshin but PL was additive. The cauliflower *Orange* (*Or*) gene enhances petiole elongation by suppressing eukaryotic release factor 1 (Zhou et al. 2011). Expression of its orthologue *BrOr* (*Bra035916*) was elevated slightly in RCBr but did not correlate with PL in F_2 individuals. *ROTUNDIFOLIA3* (*ROT3*) encodes CYP90C1, which is involved in the late stages of BR biosynthesis and controls leaf petiole growth in the dark (Kim et al. 2005). A *BrROT3* (*Bra017757*) expression was 3.4-fold higher in Kenshin than in RCBr, and no correlation was found between *Bra017757* expression and phenotype in F_2 individuals. This suggests that regulation of PL is likely to be complex in *B. rapa* and differ from the mechanism known from *Arabidopsis thaliana*.

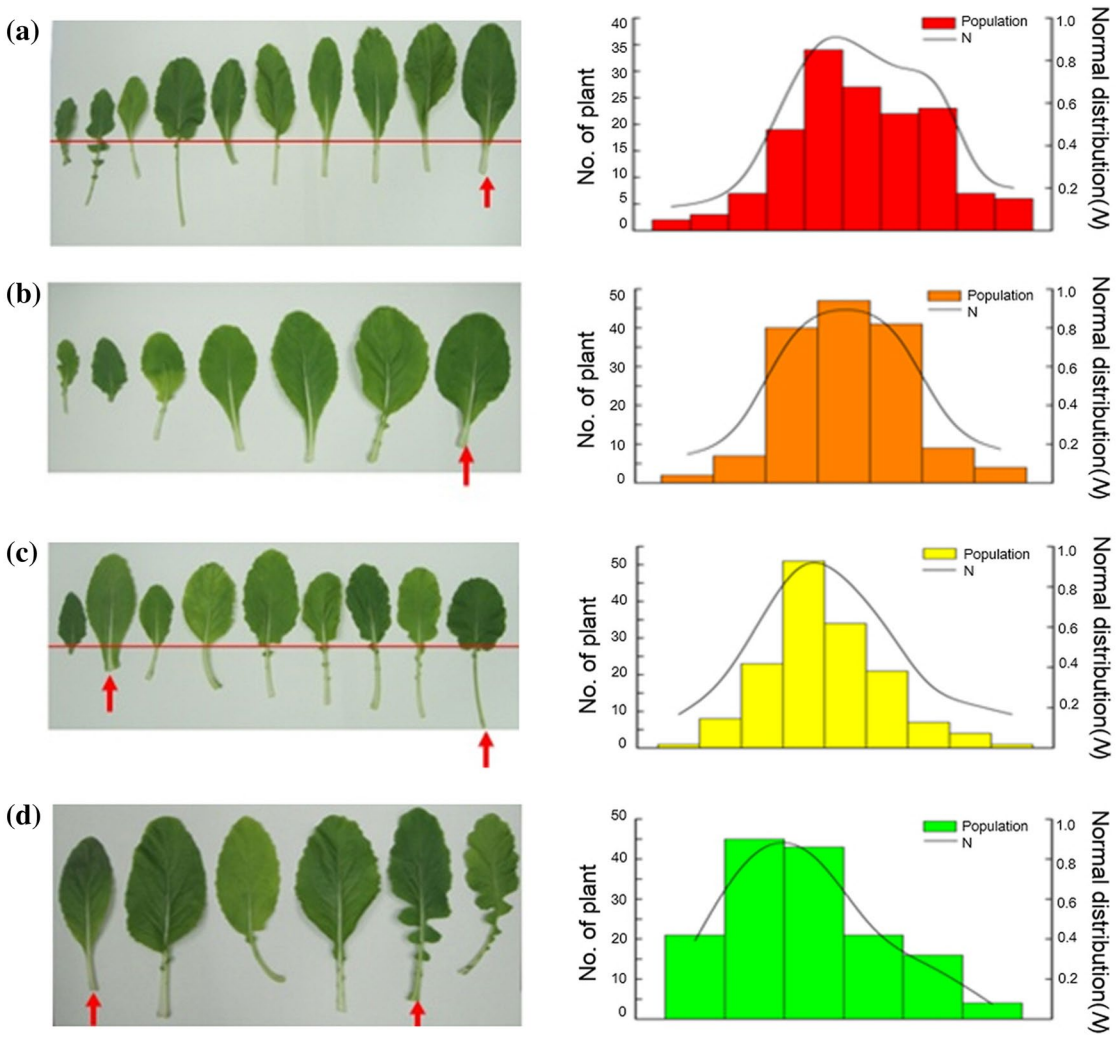

Fig. 2 Leaf phenotypes observed in the fifth leaf (left) and distribution of trait values for each character in the F_2 population (right). **a** Observed values of lamina length (LL) were classified into ten groups ranging from < 5 to > 13 cm. **b** Observed values of lamina width (LW) were classified into seven groups ranging from < 3 to > 8 cm. **c** Observed values of petiole length (PL) were classified into nine groups ranging from < 2 to > 9 cm. **d** Observed values for the leaf margin (LM) were classified into six groups, labeled I–VI. *N* normal distribution. Red arrows indicate the representative phenotype selected for microarray analysis. Horizontal red line indicated the base of leaf. (Color figure online)

Transcriptome analysis using the Br135K microarray

To identify genes whose expression were correlated with leaf phenotype values, six types of plants with different leaf characteristics were selected: the two parental lines (RCBr and Kenshin inbred lines) and four types of F_2 plants represented by F_2-93, F_2-33, F_2-100, and F_2-19 types. In the analyses, one representative plant plus two additional plants showing comparable phenotypes were pooled for each type (Fig. 3; Table 1). Although we have used a representative plant plus two additional individuals for experiments, only representative plant names were used to indicate each type. To identify genes involved in the determination of leaf morphology, the seventh and ninth leaves were harvested from representative plants and from two additional plants showing comparable phenotypes, while leaf phenotypes were determined with 5th leaves. The three samples were combined prior to extraction of RNA for use in the microarray experiment and real-time PCR analysis.

Data obtained from the Br135K microarray were expressed as probe intensity (PI) values for expression levels (Table S1) rather than log values to distinguish small differences in expression levels. Of the 41,173 genes present on the Br135K microarray, 5349 genes did not have a counterpart in *Arabidopsis* (i.e., could not be annotated using TAIR 10). A total of 28,679 genes showed PI values over 500 in at least one sample (Table S1); these levels of expression mean that transcript can be easily detected by

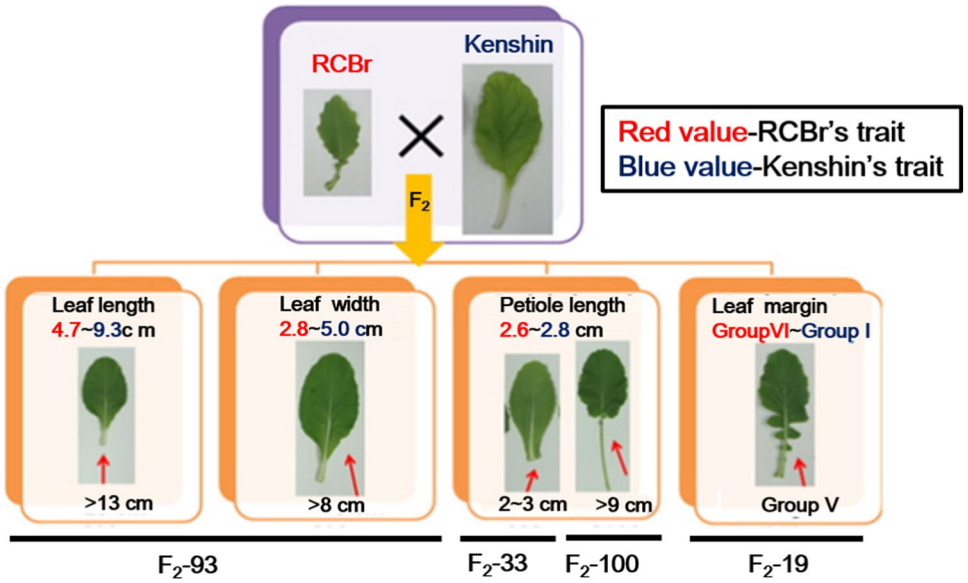

Fig. 3 Schematic explanation of RCBr, Kenshin, and F_2 leaves used in the microarray and qRT-PCR analyses. The trait values used for classification were obtained from measurements made on the fifth leaf of plants from the two parental lines and four F_2 plants. The seventh and ninth leaves were harvested from plants showing the phenotypes shown above, and RNA was extracted from these samples. Red and blue text indicates RCBr-like and Kenshin-like traits, respectively. (Color figure online)

25 cycles of RT-PCR. Genes whose expression correlated with phenotype were selected for further characterization (Figs. 4, 5, 6, 7).

The order of LL values, ranked from lowest to highest, was RCBr (4.7 cm), F_2-100 (7.9 cm), Kenshin (9.3 cm), and F_2-93 (13.7 cm) (Fig. 3; Table 1). Transcript levels of 96 genes were positively correlated with LL, and expression of 53 genes was negatively correlated with LL (Fig. 4; Table S2). Expression of most of these genes matched the LL phenotypes well. Several positively or negatively correlated genes were recognizable; for example, *BrGH3.5* (*Bra019060*), *BrAS2* (*Bra039733*), and *Bra002068*, encoding the auxin efflux carrier family protein, were positively correlated with LL, whereas *Bra031986*, encoding the tetratricopepetide repeat (TPR)-like superfamily protein, and *Bra019610*, encoding transducin/WD40 repeat-like superfamily protein, were negatively correlated. *ARABIDOPSIS AUXIN-RESPONSIVE GH3 FAMILY* 5 (*AtGH3.5*) increases leaf size and trichome number (Kryvych et al. 2008), *Arabidopsis* AS2-like/lateral organ boundary (LOB) domain family protein increases leaf size (Li et al. 2016), and *Arabidopsis* auxin efflux carrier family protein (*AT2G17500*) regulates auxin homeostasis. Orthologues of all three genes appeared to be associated with leaf size, especially LL, in *B. rapa*. On the other hand, expression of two negatively correlated genes might inhibit leaf growth. The *Arabidopsis* orthologue of *Bra031986* is important for RNA editing of a mitochondrial respiration gene (Arenas-M et al. 2014), and *Bra031986* encodes a protein possibly involved in a COMPASS-like complex, which was initially identified in *Caenorhabditis elegans* and is involved in the epigenetic regulation of development (Fisher et al. 2010). Moreover, *AT3G49660*, the *Arabidopsis* orthologue of *Bra031986*, interacts with transcription factors to regulate expression of specific genes (Song et al. 2015). Thus, these genes might be involved in determining leaf length in *B. rapa*.

To identify genes involved in determining LW, four plants were compared: RCBr (LW: 2.8 cm), Kenshin (LW: 5.0 cm), F_2-19 (LW: 6.2 cm), and F_2-93 (LW: 8.5 cm) (Fig. 3; Table 1). Transcript levels of 142 genes were proportional to LW values, and transcript levels of 82 genes were inversely proportional to LW values (Fig. 5; Table S3). Most genes whose expression positively associated with LW values were stress-responsive genes. Sixteen transcription factors, including *BrSPL11* (*Bra030040*), a SQUAMOSA PROMOTER-BINDING PROTEIN (SBP)-box family-like (*SPL*) gene, belonged to this category. In *Arabidopsis*, SPL10, SPL11, and SPL2 affect lamina shape and trichome formation by changing shoot maturation (Shikata et al. 2009). Expression of *GIBBERELLIN 20-OXIDASE* (*BrGA20OX3, Bra009285, Bra0033189*) and *GA-RESPONSIVE GATS1 HOMOLOGUE 1* (*BrGASA1, Bra029227*) was negatively correlated with LW. *Arabidopsis GA20OX1, -2*, and *-3* are involved in growth and fertility, and mutations of these genes lead to dwarfism (Plackett et al. 2012).

The same four plants used in the association analysis of PL were used to identify genes involved in determining PL: RCBr (PL: 2.6 cm), F_2-93 (PL: 2.7 cm), Kenshin (PL: 2.8 cm), and F_2-100 (PL: 9.8 cm) (Fig. 3; Table 1). Transcript levels of 174 genes were positively correlated with PL values, and expression of 381 genes was negatively correlated (Fig. 6; Table S4). Expression of only 25 and 36 genes, however, was exactly positively and negatively proportional, respectively, to the PL phenotypes; other genes showed a clear difference only between RCBr (short PL) and F_2-100 (long PL). *BrOPT6* (*Bra026311*), an orthologue

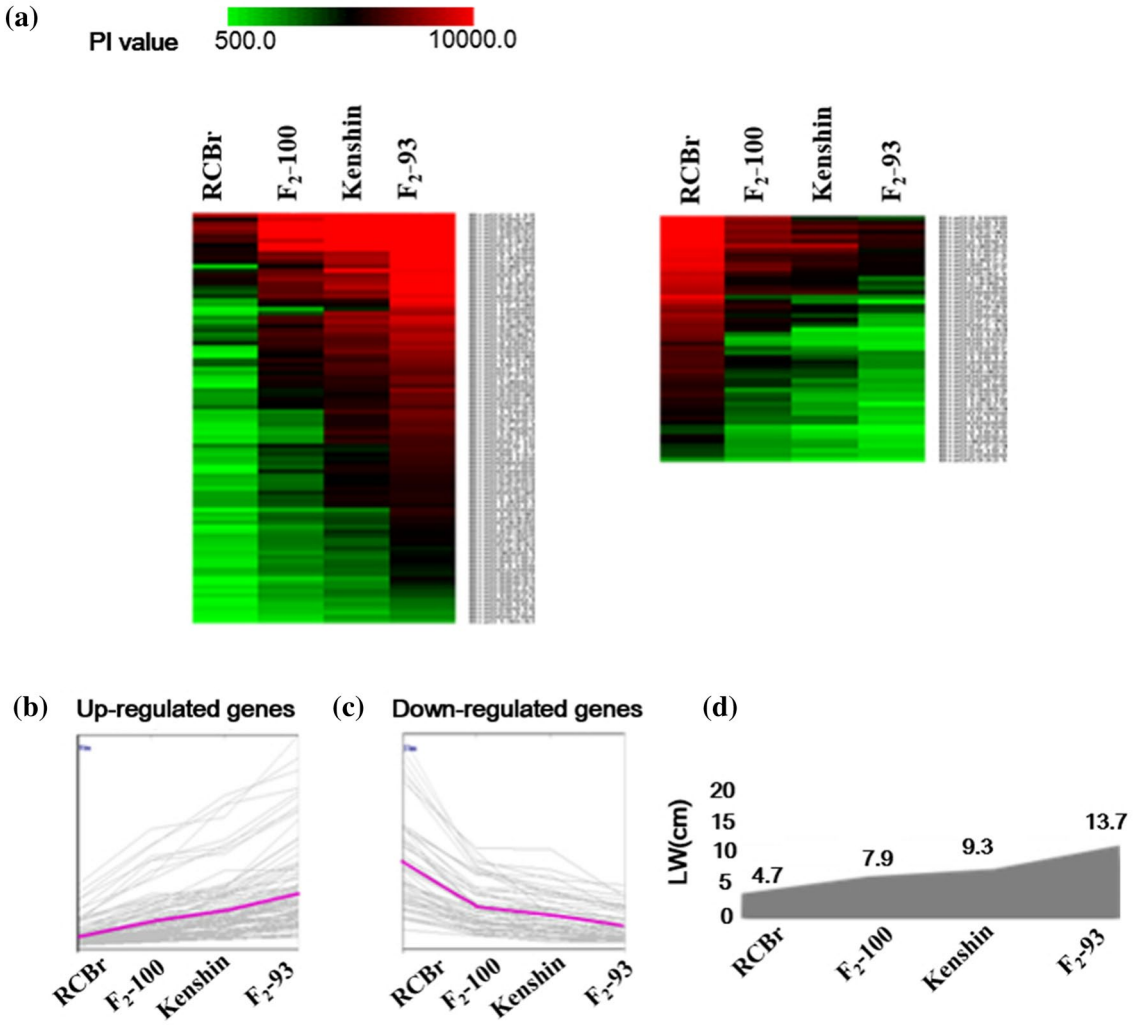

Fig. 4 Genes whose level of expression correlated with the extent of the phenotypic trait LL. Candidate genes were selected on the basis of (1) expression PI value > 500; and (2) similarity of patterns between gene expression and LL trait value. **a** Hierarchical clustering of 96 and 53 genes showing positive and negative correlations between expression levels and phenotype, respectively. **b, c** K-means clustering and expression patterns. Gray lines: Expression of individual genes. Pink lines: Mean expression of all genes. **d** Plot of lamina length against genotype. RCBr: 4.7 cm; F_2-100: 7.9 cm; Kenshin: 9.3 cm; F_2-93: 13.7 cm. (Color figure online)

of *ARABIDOPSIS OLIGOPEPTIDE TRANSPORTER 6* (*AtOPT6*), was a likely candidate gene for involvement in determining the PL phenotype; *AtOPT6* is expressed in vascular tissue and has broad substrate specificity (Pike et al. 2009). The functions of *Bra029651*2, encoding a Fe-2S ferredoxin-like superfamily protein, *Bra013540*, encoding an oxidoreductase, zinc-binding dehydrogenase family protein, and *Bra040419*, encoding a NPL4-like protein 1 (*BrNPL4L*), whose expression all showed a negative correlation with the PL phenotype, have not yet been identified.

To uncover genes involved in determining the LM phenotype, five plants were selected: Kenshin (class I), F_2-33 and F_2-93 (both class II), F_2-19 (class V), and RCBr (class VI) (Fig. 3; Table 1). The numbers of genes whose transcript levels were proportional and inversely proportional to the LM classification were 74 and 86, respectively (Fig. 7; Table S5). The most dramatic differences in expression levels were found between the parental lines, Kenshin, which showed no serration, and RCBr, whose leaves showed a high level of serration and lobing (Table S5). Expression of three genes showed a strong, positive correlation with the LM phenotype, *Bra016851*, encoding *LYSINE KETOGLUTARATE REDUCTASE TRANS-SPLICING-LIKE PROTEIN, Bra007991*, encoding *SUCROSE-PROTON SYMPORTER 1* (*BrSUC1*), and *Bra007197*, encoding *RECEPTOR-LIKE PROTEIN 47* (*BrRLP47*); the functions of these genes have not yet been studied. The expression of ten genes (*Bra012716, Bra012818, Bra020690, Bra008167, Bra020091, Bra033287, Bra029776, Bra001726, Bra037638*, and *Bra000189*) showed a strong negative

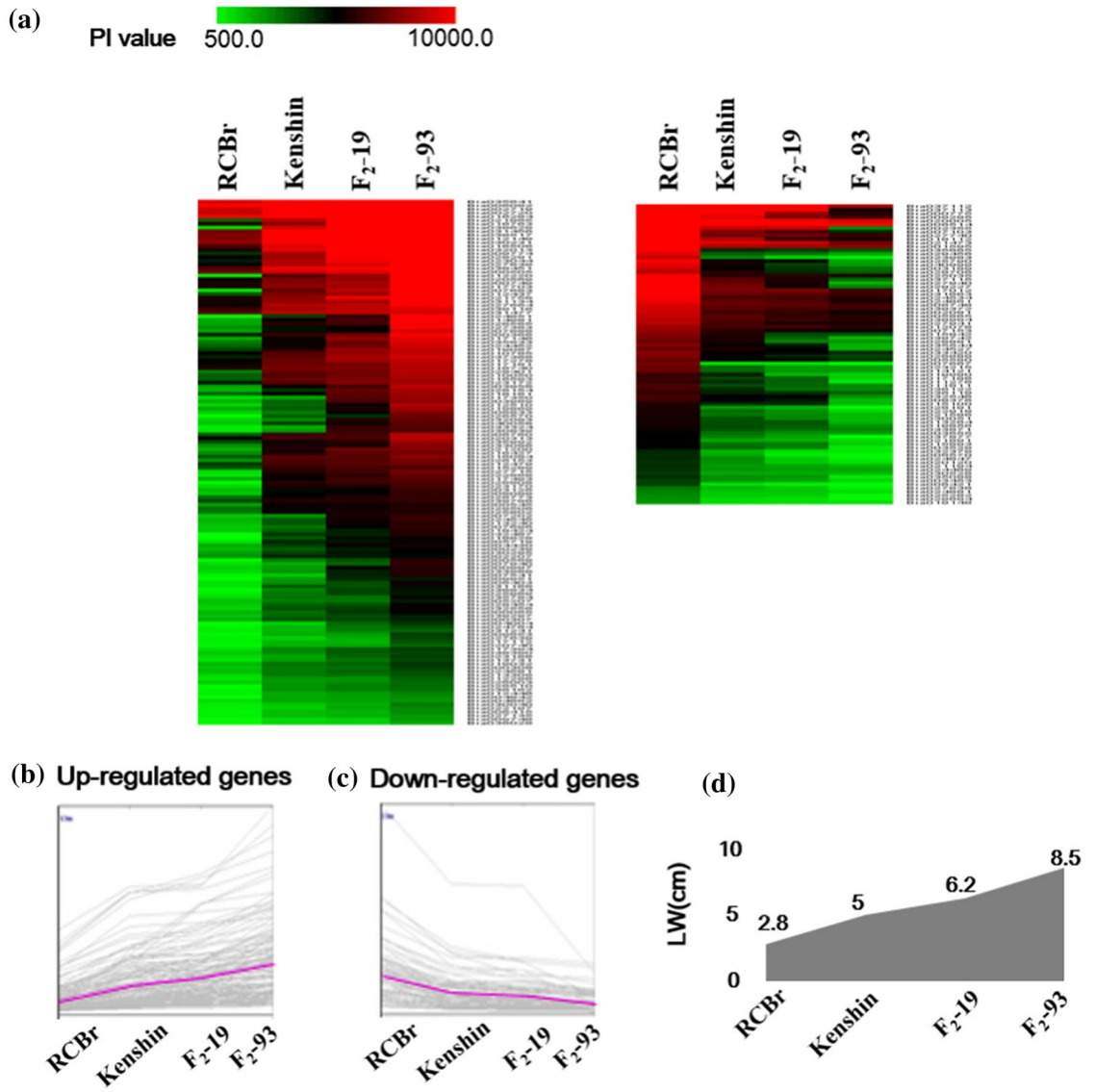

Fig. 5 Genes with expression levels correlated with the value of the phenotypic trait LW. Genes were selected on the basis of (1) expression PI value > 500; and (2) similarity of patterns between gene expression and LW trait value. **a** Hierarchical clustering of 142 and 82 genes showing positive and negative correlation between expression levels and phenotype, respectively. **b, c** K-means clustering and expression patterns. Gray lines: Expression of individual genes. Pink lines: Mean expression of all genes. **d** Plot of lamina width against genotype. RCBr: 2.8 cm; Kenshin: 5 cm; F_2-19: 6.2 cm; F_2-93: 8.5 cm. (Color figure online)

correlation with the LM phenotype. Moreover, expression of *LOB DOMAIN-CONTAINING PROTEIN 37/ASYMMETRIC LEAVES2-LIKE* (*ASL*) (*BrLBD37, Bra012164*), which is involved in leaf morphogenesis (Albinsky et al. 2010), was negatively correlated with the degree of the LM phenotype across all the genotypes, with the exception of F_2-33. *BrLBD37* is thus a good candidate for a gene determining the LM phenotype.

These microarray data are summarized in Table 2. Individuals resembling F_2-93 showed superior leaf phenotypes with respect to the traits LL and LW, indicating that they might be useful for generating molecular markers for breeding leafy vegetables. A previous study (Xiao et al. 2014) identified novel genes likely to be involved in leaf development, in addition to several known genes. That study used Agilent 105K *Brassica* species oligoarrays, which are not specific for *B. rapa* and do not contain a suitable number of *B. rapa* genes. Our microarray analysis used a chip specifically designed for *B. rapa* as well as 3′-specificity and revealed many genes whose expression correlated strongly with four selected leaf phenotypes. This has provided a large number of candidate genes likely to be important in determining leaf morphology.

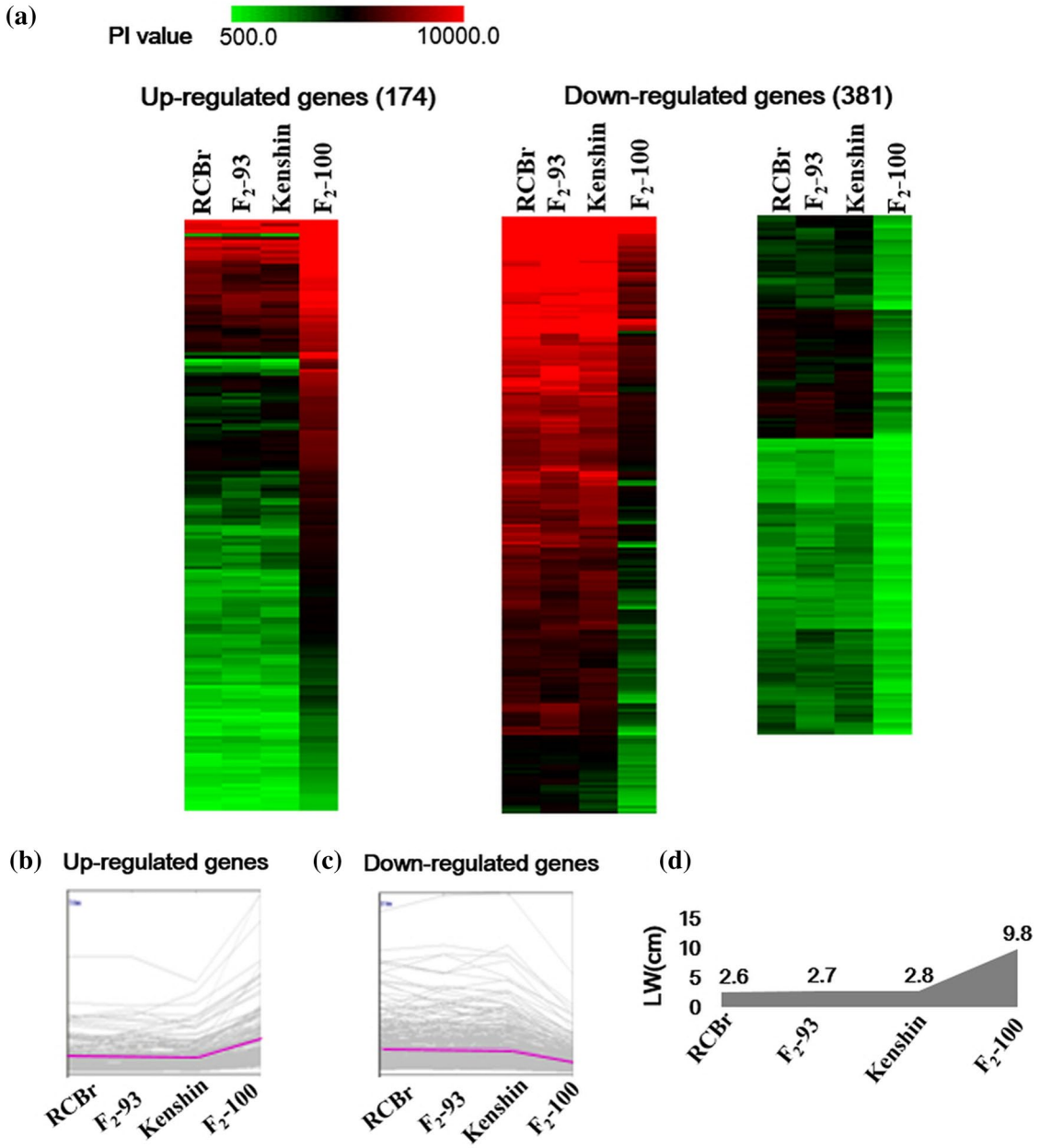

Fig. 6 Genes with expression levels correlated with the value of the phenotypic trait PL. Genes were selected on the basis of (1) expression PI value > 500; and (2) similarity of patterns between gene expression and PL trait value. **a** Hierarchical clustering of 174 and 381 genes showing positive and negative correlation between expression levels and phenotype, respectively. **b, c** K-means clustering and expression patterns. Gray lines: Expression of individual genes. Pink lines: Mean expression of all genes. **d** Plot of petiole length against genotype. RCBr: 2.6 cm; F_2-93: 2.7 cm; Kenshin: 2.8 cm; F_2-100: 9.8 cm. (Color figure online)

Analysis of selected genes by functional category

The DEGs associated with each leaf phenotype were annotated using GO in Biological Process in TAIR (The Arabidopsis Information Resource; http://arabidopsis.org/) (Table 3). DEGs were declared if expression levels in PI values between the lowest- and highest-ranked samples in Table 2 differed by more than 1.5-fold. A large number of genes were classified as unknown in all phenotypes. After this, "phytohormone" genes were found to be the most abundant (9.3%) GO category. The second to sixth categories by gene number were "developmental process", "enzyme activity", "transport", "cell division and elongation, differentiation", and "cell wall and cytoskeleton". The finding that phytohormone-associated genes formed the largest category of DEGs across all leaf

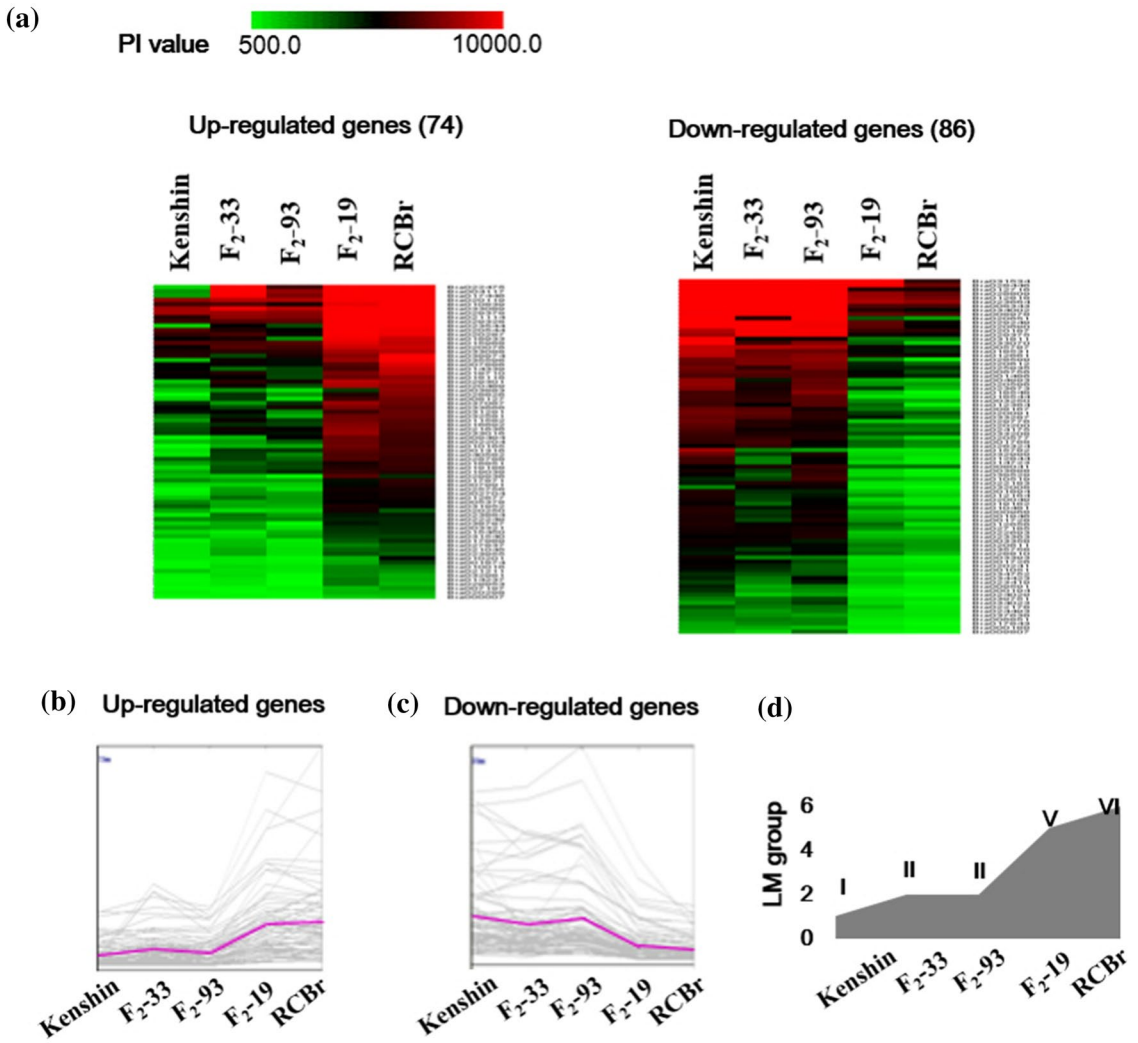

Fig. 7 Genes with expression levels correlated with the classification of the phenotypic trait LM. Genes were selected on the basis of (1) expression PI value > 500; and (2) similarity of patterns between gene expression and LM trait value. **a** Hierarchical clustering of 74 and 86 genes showing positive and negative correlation between expression levels and phenotype, respectively. **b, c** K-means clustering and expression patterns. Grey lines: Expression of individual genes. Pink lines: Mean expression of all genes. **d** Plot of leaf margin classification against genotype. Kenshin: I; F_2-33 and F_2-93: II; F_2-19: V; RCBr: VI. (Color figure online)

Table 2 Summary of number of differentially expressed genes associated with each leaf phenotype

Phenotype	Degree of phenotype (low → → → high)					Correlation (no. of genes)	
						Positive	Negative
LL	RCBr	F_2-100	Kenshin	F_2-93		96	53
LW	RCBr	Kenshin	F_2-19	F_2-93		142	82
PL	RCBr	F_2-93	Kenshin	100		174	381
LM	Kenshin	F_2-33	F_2-93	F_2-19	RCBr	74	86

This table is a summary of the results presented in Figs. 4, 5, 6 and 7

LL lamina length, *LW* lamina width, *PL* petiole length, *LM* leaf margin

phenotypes agreed with previous studies showing that phytohormones are involved in leaf development (Kim et al. 2006; Barkoulas et al. 2007; Cha et al. 2007; Micol 2009; Gonzalez et al. 2010; Kalve et al. 2014; Xiao et al. 2014). Other enriched genes also appear to play important roles in leaf growth and differentiation, as they are

Table 3 Gene Ontology (GO) categorization of selected genes based on the correlation between gene expression and leaf traits

GO categorization	Number of genes				SUM
	LL	LW	PL	LM	
Phytohormone	16	22	39	16	93
Developmental process	8	14	39	12	73
Enzyme activity	8	12	32	8	60
Transport	6	10	24	8	48
Cell division and elongation, differentiation	6	7	23	9	45
Cell wall and cytoskeleton	3	9	23	7	42
Kinase activity	5	8	17	8	38
Transcription factor	7	11	15	5	38
Metabolic process	7	3	9	5	24
Biosynthetic process	4	7	11	–	22
Zinc ion binding	–	3	13	4	20
Oxidation–reduction process	–	5	7	5	17
Catalytic activity	3	3	6	1	13
Response to stimulus	2	2	7	1	12
Translation	1	1	6	3	11
Defense response	2	3	5	1	11
Nucleic acid binding	–	–	11	–	11
DNA repair	2	1	4	1	8
Protein binding	1	3	2	1	7
Protein folding	1	2	3	–	6
ATP binding	1	2	3	–	6
RNA processing	1	3	1	–	5
Methylation	–	–	5	–	5
RNA methylation	2	2	–	–	4
DNA binding	1	2	–	1	4
Signal transduction	–	1	2	1	4
Phosphatase activity	–	–	4	–	4
Protein dephosphorylation	–	–	4	–	4
Transcription	–	–	4	–	4
Structural constituent of ribosome	1	1	1	–	3
N-terminal protein myristoylation	–	2	–	1	3
Cell redox homeostasis	–	1	–	2	3
Calcium-mediated signaling	–	–	3	–	3
DNA-directed RNA polymerase activity	–	–	3	–	3
Ubiquitination	–	–	2	1	3
Electron carrier activity	–	–	2	1	3
Nucleotide binding	–	–	–	3	3
Unclassified	8	12	35	11	66
Unknown	26	39	86	25	176
NA	23	12	41	17	93
Total	145	203	492	158	998

involved in developmental processes, cell division and elongation, differentiation, and cell wall and cytoskeleton (Cosgrove 2005; Wolf et al. 2012; Kalve et al. 2014).

Using our data and the findings of a previous report (Kalve et al. 2014), the genes involved in four biological process categories ("phytohormone", "cell division and elongation, differentiation", "developmental process", and "cell wall and cytoskeleton") were further characterized with respect to the four leaf traits (Tables S6–S9). These analyses were supplemented with information from the PhenoLeaf database (http://genetics.umh.es/phenoleaf/) and the phenotypes of Salk mutant lines (indicated by underlining and an asterisk in the Supplementary Tables).

First, DEGs associated with the phenotypic trait LL were considered (Table S6). Some important up-regulated genes shared a high level of sequence identity with *IAA-AMIDO SYNTHASE* (*WES1*), *PIN-LIKE 5* (*PILS5*), and *CYP74A*, while the down-regulated genes included homologues of *ABI FIVE-BINDING PROTEIN 2* (*AFP2*) and *STRICTOSIDINE SYNTHASE 2* (*SS2*), which are associated with phytohormone signaling and biosynthesis. Other up-regulated genes associated with LL were involved in cell division, elongation, and differentiation; these included *ARABIDOPSIS THALIANA HOMEOBOX 1* (*ATHB-1*), *KIP-RELATED PROTEIN 5* (*KRP5*), and *CHROMATIN REMODELING FACTOR 1* (*CHC1/SWP73B*). *HB-1* and *AS2*, two genes involved in developmental processes, were also up-regulated; however, another gene in this category, *TOPLESS/WUS-INTERACTING PROTEIN 1* (*TPL/WSIP1*), was down-regulated. Only one gene in the "cell wall and cytoskeleton" category, *XYLOSYL TRANSFERASE 1* (*XT1*), was associated with LL; this remains to be studied.

In the analysis of DEGs associated with the LW phenotype (Table S7), four genes related to well-known phytohormone-associated genes [*AFP1, GIBBERELLIN 2-XODASE 4* (*GA2OX4*), *ETHYLENE RESPONSE ELEMENT-BINDING FACTOR 5* (*ERF5*), and *RING DOMAIN LIGASE 1* (*RGLG1*)] were up-regulated. Three genes from the same category were down-regulated [two *GA2OX3* genes and *CYTOKININ OXIDASE 5* (*CKX6*)]. Additional well-characterized genes from other processes were identified as DEGs in the LW analysis. Genes associated with "cell division and elongation, differentiation" included *3x HIGH MOBILITY GROUP-BOX 2* (*3xHMG-BOX2*), which was up-regulated, and *GA-RESPONSIVE GAST1 HOMOLOGUE1* (*GASA1*), which was down-regulated. Other DEGs associated with the LW phenotypic trait were from the "developmental process" category. These included five up-regulated genes [*HB-1, UBIQUITIN-SPECIFIC PROTEASE 15* (*UBP15*), *CYTOCHROME C LYASE* (*CCMH/CycI*), and *ABL INTERACTOR-LIKE PROTEIN 3* (*ABIL3*)], and three down-regulated genes [TCP family transcription factor 4, *EMBRYONIC DEFECTIVE 1075* (*EMB1075*), and *EMBRYONIC DEFECTIVE 24* (*EMB24*)]. Genes from the "cell wall and cytoskeleton" category were also differentially expressed; *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 22*

(*XTH22*) and *XYG XYLOSYLTRANSFERASE 1* (*XT1*) were up-regulated, and *REVERSIBLY GLYCOSYLATED POLYPEPTIDE 1* (*RGP1*) and *IRREGULAR XYLEM 10* (*IRX10*) were down-regulated.

The PL phenotype was associated with the largest number of DEGs. The full list is given in Table S8, but a few genes are described here. In the "phytohormone" category, *SLOW MOTION* (*SLOMO*) and *RUB1 CONJUGATING ENZYME 1* (*RCE1*) were up-regulated, whereas *PIL6, NUCLEAR FACTOR Y SUBUNIT 4* (*NF-YA4*), *SMALL AUXIN UPREGULATED RNA32* (*SAUR32*), *ABERRANT LATERAL ROOT FORMATION 1* (*ALF1*), *BR-INSENSITIVE 1* (*BRI1*), *BRZ-INSENSITIVE-LONG HYPOCOTYLS 4* (*BIL4*), *HISTIDINE-CONTAINING PHOSPHOTRANSMITTER 1* (*HP1*), and *GALACTURONOSYLTRANSFERASE-LIKE 2* (*GATL2*) were all down-regulated. Several genes from the "developmental process" category were associated with PL, up-regulated genes included *PIN1, ACAULIS 5* (*ACL5*), *DIACYLGLYCEROL KINASE 7* (*DGK7*), *DsRNA-BINDING PROTEIN 1* (*DRB1*), *CONSTITUTIVE MORPHOGENESIS 2* (*COP2*), and *PIGMENT DEFECTIVE 191* (*PDF191*), whereas down-regulated genes included *BELL-LIKE HOMEODOMAIN 9* (*BLH9*), *ENDOPLASMIC RETICULUM-ADENINE NUCLEOTIDE TRANSPORTER 1* (*ER-ANT1*), *ARABIDOPSIS 6B-INTERACTING PROTEIN 1-LIKE 2* (*ASIL2*), and *LON PROTEASE 1* (*LON1*). All the genes associated with PL in the category of "cell division and elongation, differentiation" were down-regulated. These included *RETARDED ROOT GROWTH* (*RRG*), *MEMBRANE-RELATED BIGGER1* (*MRB1*), *AS1/2 ENHANCER 7* (*AE7*), *E2F TRANSCRIPTION FACTOR 1* (*E2F1/E2FB*), *COP9 SIGNALOSOME SUBUNIT 5 = 6B* (*CSN6B*), *RAN GTPASE ACTIVATING PROTEIN 2* (*RANGAP2*), *LONG AFTER FAR-RED 3* (*LAF3*), *LEUCINE-RICH REPEAT/EXTENSION 1* (*LRX1*), and *ARPC4*. In the "cell wall and cytoskeleton" category, *REF4-RELATED 1* (*RFR1*) was up-regulated while *CELLULOSE SYNTHASE-LIKE D3* (*CSLD3*), *GLUCURONIC ACID SUBSTITUTION OF XYLAN 3* (*GUX3*), *CINNAMYL ALCOHOL DEHYDROGENASE 5* (*CAD5*), and an unknown gene (possibly a Golgin family A protein) were down-regulated. Although many DEGs were associated with PL, no key transcription factors or core genes were identified.

DEGs associated with the LM trait also included large numbers of genes associated with the GO categories "phytohormone", "cell division and differentiation", "developmental process", and "cell wall and cytoskeleton" (Table S9). Some of these were examined with respect to their function. In the "phytohormone" category, *CHLOROPHYLLASE 1* (*CLH1*) was up-regulated and *G-PROTEIN GAMMA SUBUNITS 2* (*GG2*) was down-regulated. Up-regulated genes from the "developmental process" category included *CINNAMATE 4-HYDROXYLASE* (*C4H*) and *FLOWERING LOCUS T* (*FT*); down-regulated genes from the same category included *GATA TRANSCRIPTION FACTOR 21* (*GATA21*), *ASYMMETRIC LEAVES 2-LIKE 39* (*ASL39*), and RESPONSE REGULATOR 1 (*RR1*). Only one gene from the "cell wall and cytoskeleton" category, *MYOSIN 1*, was down-regulated.

The analysis of four leaf phenotypes focused on four biological process categories has identified 33 up-regulated and 42 down-regulated genes (Kalve et al. 2014 and websites). These genes were also identified in the present study, demonstrating that we had used an appropriate sampling regime (parents and F_2 population) and methodology (Br135K microarray). The genes enriched in each leaf morphology phenotype rarely overlapped, suggesting that each phenotype considered in the present study was regulated by a different set of genes.

Confirmation of microarray data with qRT-PCR analysis

To confirm the associations between identified genes and leaf morphology, transcript levels of 77 genes, selected from our microarray experiments, previous reports, and the PhenoLeaf database, were examined using qRT-PCR with gene-specific primers designed against sequences in BRAD (http://brassicadb.org/) (Table S10). A ribosomal protein gene from *B. rapa, BrRPL22*, was used as a control. Approximately half of the tested genes, including those presented in Fig. 8, showed the same or similar patterns of expression in both the microarray and qRT-PCR analyses.

Expression levels of *BrAS2, TERPENE SYNTHASE 10* (*BrTPS10*), and *XYLOTRANSFERASE1* (*BrXT1*) were positively correlated with LL, while *BrSS2* and *BrTPL* expression levels were negatively correlated with LL (Fig. 8, top panel). AS2 is known to be involved in leaf morphology and venation (Rédei and Hirono 1964; Semiarti et al. 2001; Iwakawa et al. 2007). The function of TPS10 is not yet known. XT1 is essential for cell wall biosynthesis (Park and Cosgrove 2012). An *xxt1*/*xxt2* double mutant shows a reduction in cell wall xyloglucan (Cavalier et al. 2008; Park and Cosgrove 2012) and a reduction in rosette leaves and petiole size (Park and Cosgrove 2012). The expression of *SS2* is controlled by the phytohormones auxin and jasmonic acid (Menke et al. 1999). TPL is involved in embryo formation, and in cotyledon and apex development (Szemenyei et al. 2008). Results obtained from *Arabidopsis* suggest that these genes are also likely to perform similar functions in leaf morphogenesis in *B. rapa*.

Expression of four genes (*BrAFP1, BrXTH22, BrERF5*, and *BrRGLG1*) was positively correlated with LW, whereas expression of two genes (*BrGA20OX3* and *BrIRX10*) was negatively correlated (Fig. 8, second panel from top). XTH22 modifies cell wall structure (Cosgrove 2005;

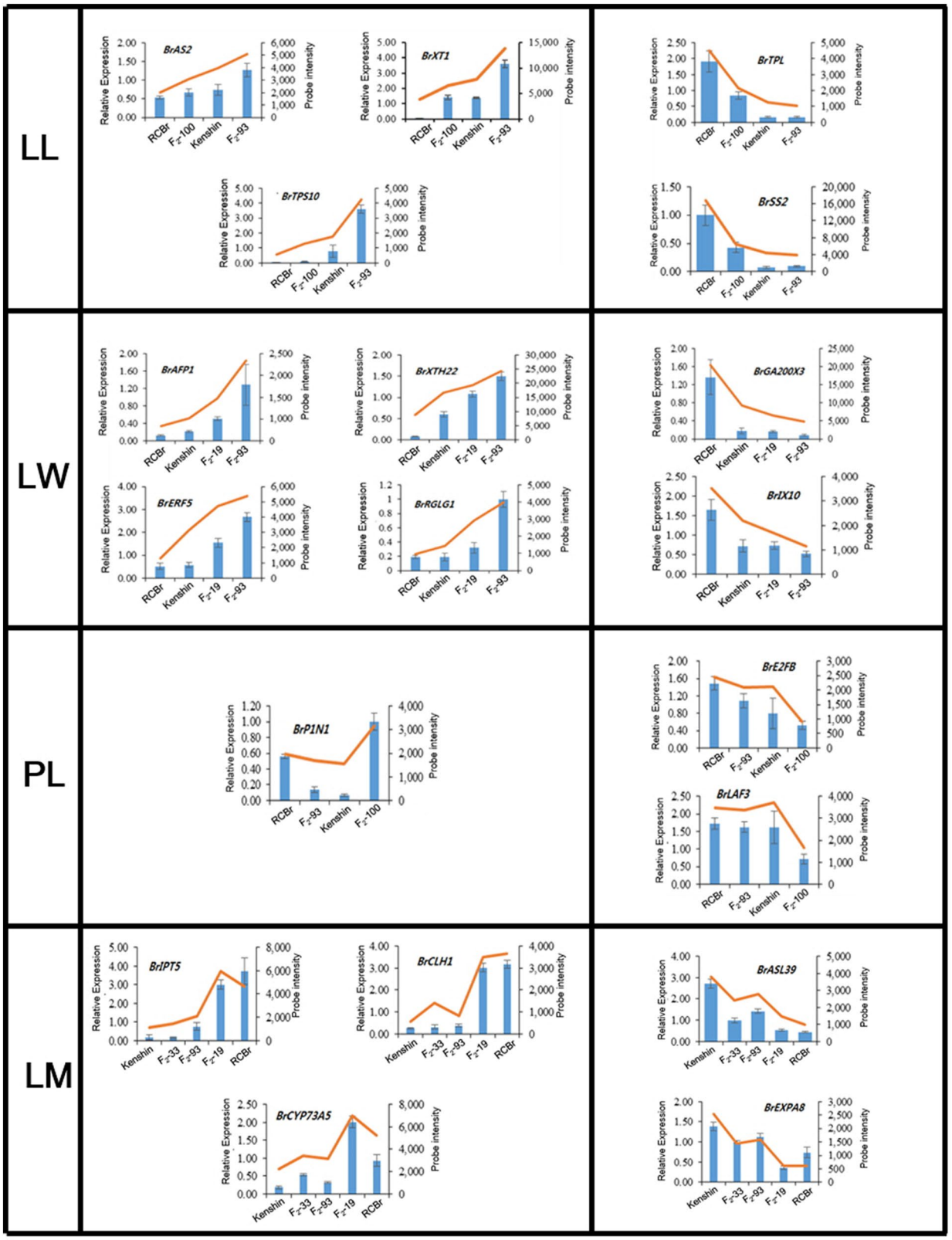

Fig. 8 qRT-PCR analysis to confirm the expression levels of selected genes. Genes positively correlated with a phenotypic trait are shown in the left-hand panel, and genes negatively correlated with a trait are shown in the right-hand panel. Expression levels of each gene were normalized against *BrRPL22* expression. Blue bars: Mean RNA expression levels measured using qRT-PCR. Orange line: Probe intensity (PI) values obtained from Br135K microarray analysis. Error bars: Standard error. K: Kenshin inbred line. (Color figure online)

Sasidharan et al. 2014). Although ERF5 inhibits leaf growth (Dubois et al. 2013), it showed elevated expression as LW values increased. Mutations in *RGLG1* change leaf morphology (Yin et al. 2007). GA20OX3 reduces gibberellin levels (Thomas et al. 1999), and overexpression reduces rice growth (Lo et al. 2008). IRX10, a member of the glycosyltransferase family, is involved in xylan biosynthesis (Brown et al. 2009). These observations all suggest that these genes are likely to affect LW.

The qRT-PCR analysis of genes associated with PL found similar patterns of up-regulation of *BrPIN1* and down-regulation of *BrE2FB* and *BrLAF3* to those seen in the microarray data (Fig. 8, second panel from bottom). The auxin transporter PIN1 is associated with leaf morphology and pattern formation of vascular tissues (Scarpella et al. 2006). E2FB (or E2F1) is the key target of auxin and regulates cell division for cell growth and differentiation (Magyar et al. 2005). LAF3 functions in phytochrome A signaling and affects photomorphogenesis (Hare et al. 2003). The expression patterns of these genes imply that determination of PL involves a complex mechanism. In *Arabidopsis, PHYTOCHROME B* (*Phy*B), *ROT3, ACAULIS 2* (*ACL2*), and *GA-INSENSITIVE* (*GAI*) are known to control PL (Tsukaya et al. 2002), but regulation of petiole development in *B. rapa* may differ from that in *Arabidopsis*.

Expression of *ISOPENTENYLTRANSFERASE 5* (*BrIPT5*), *BrCYP73A5*, and *CHLOROPHYLLASE 1* (*BrCLH1*), genes associated with the LM phenotype, increased in tandem with leaf serration, while *ASYMMETRIC LEAVES2-LIKE 39* (*BrASL39*) and *EXPANSIN 8A* (*BrEXPA8*) showed the opposite pattern (Fig. 8, bottom panel). IPT5 controls cytokinin biosynthesis (Miyawaki et al. 2006), and CYP73A5 is associated with lignin biosynthesis (Sundin et al. 2014). ASL39 affects leaf morphology, nitrogen transport, and anthocyanin biosynthesis (Rubin et al. 2009). EXPANSIN 8A (EXP8A) is associated with cell wall elongation (Esmon et al. 2005). These results suggest that these genes are involved in determining leaf shape.

Integrative analysis of leaf morphology-related genes

To narrow down the numbers of genes involved in leaf morphology in *B. rapa*, we compared our results with previously published data (Table 4). This table, which uses the same classification as Xiao et al. (2014), includes 24 genes up-regulated in Kenshin and 14 genes up-regulated in RCBr in our microarray data. The trends in gene expression were consistent with the observation that LL and LW in the F_2 population were slightly shifted toward the values of the Kenshin parental line (Fig. 2a, b). Genes associated with serration and lobe phenotypes were more likely to be up-regulated in RCBr. Expression of many of the listed genes did not differ between Kenshin and RCBr; however, ten genes mentioned by Choi et al. (2017) were up-regulated in Kenshin: *ARL, CLF, CycB2.3, CycB2.4, CycD3.1, ROT3, AN3, COW1, ULT1*, and *ANT*. Of the other genes examined, expression of *AS2* was strongly correlated with LL (Fig. 8) and up-regulated in Kenshin (Table 4). AS2 and AS1 regulate leaf development by repressing *BREVIPEDICELLUS* (*BP*/*KNAT1*) and *KNAT2*, members of the class I *KNOTTED1*-like homeobox (*KNOX*) family (Li et al. 2016). *KNOX* genes maintain shoot apical meristem activity but repress leaf development by inhibiting leaf initiation (Hay and Tsiantis 2010; Sluis 2015). The final leaf size depends on primodium size, the cell division or expansion rate, the duration of cell division or expansion, and meristemoid division (Bar and Ori wo14; Kalve et al. 2014). In particular, two overlapping phases of leaf development, cell proliferation and cell expansion, are closely related to leaf size (Golzalez et al. 2012). The increased expression of *BrAS2* (*Bra039733*) associated with Kenshin may reflect the fact that AS2 stimulates leaf initiation, which is thereby increased in LL. A high level expression of *BrAS2* gene may be responsible for the increased leaf size observed in the F_2 population, as they increase leaf size in *Arabidopsis* (Bar and Ori 2014).

BrCUC2 was highly expressed in RCBr (Table 4; Table S1), an inbred line that has a serrated and lobed LM. *BrCUC1* (*Bra021592, Bra001586*), *BrCUC2* (*Bra003023, Bra022685*), and *BrCUC3* (*Bra008259, Bra015750*) were much more highly expressed in RCBr than in Kenshin, with increases in expression of between 3- and eightfold. Increases in the expression levels of these genes may lead to more serration and lobed margins in F_2 populations, as described in *Arabidopsis* (Nikovics et al. 2006; Bar and Oir 2014; Vlad et al. 2014). Otherwise, it is possible that other regulatory factors are responsible for *CUC2* expression variation in *B. rapa*.

It is worth noting that the cyclin genes, *AN3, ULT1*, and *ANT*, were reported to overlap with leaf size QTLs by Choi et al. (2017) and that these genes were more highly expressed in Kenshin than in RCBr. In contrast to LM, values of LL and LW in the F2 population resembled those of Kenshin (Fig. 2; Table 4); this observation is supported by the expression levels of these genes (Table S1). Kenshin plants showed high expression levels of *BrAN3* (*Bra010002, Bra020616, Bra036131*), increased by 1.8- to 2.2-fold; of *BrCYCB1;2* (*Bra005880, Bra028741*), increased by 9- to 12-fold; of *BrCYCB2;1* (*Bra024504*), increased by eightfold; of *BrCYCB2;4* (*Bra003727, Bra015762*), increased by 9- to 28-fold; of *BrCYCD3;1* (*Bra034612, Bra017629, Bra011501*), increased by 10- to 15-fold; of *BrCYCD3;2* (*Bra012146*), increased by 15-fold; of *BrULT1* (*Bra0024219, Bra026276*), increased by Four- to fivefold; and of *BrANT 9* (*Bra011782, Bra017851*), increased by 2- to 2.5-fold. All these data suggest that these genes are essential

Table 4 Integrative analysis of genes identified in the current study as associated with leaf morphology with previously published data

Functional pathway	At_Locus	Gene	Full name	References	Remarks	In this study
Initiating primordium	AT4G08150	*KNAT1*	Knotted1-lik I-KNAT1 e homeobox (KNOT1)	1		ND
	AT1G70510	*KNAT2*	Knotted1-like homeobox I-KNAT2	1		ND
	AT1G23380	*KNAT6*	Knotted1-like homeobox I-KNAT6	1		2/4 R
	AT2G27100	*SE*	SERRATE	1		ND
	AT1G62360	*STM*	SHOOTMERISTEMLESS	1		–
	AT2G17950	*WUS*	WUSCHEL	1		–
Adaxial–abaxial polarity	AT5G60450	*ARF4*	AUXIN RESPONSE FACTOR 4	1		ND
	AT5G16560	*KAN 1*	KANADI 1	1		2/2 K
	AT1G32240	*KAN 2*	KANADI 2	1	Size (1)	ND
	AT4G17695	*KAN 3*	KANADI 3	1		1/2 K
	AT5G42630	*KAN 4*	KANADI 4	1		ND
	AT2G34710	*PHB*	PHABULOSA	1, 5	Vascular (5)	1/2 K
	AT1G30490	*PHV*	PHAVOLUTA	1, 5	Vascular (5)	ND
	AT5G60690	*REV*	REVOLUTA	1, 5	Vascular (5)	1/3 K
	AT2G45190	*YAB 1*	YABBY 1	1	Size (1)	2/3 K
	AT1G08465	*YAB 2*	YABBY 2	1		ND
	AT4G00180	*YAB 3*	YABBY 3	1		1/1 K
Lamina length	AT3G59900	*ARGOS*	AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE	1		1/1 R
	AT2G44080	*ARL*	ARGOS-LIKE	1, 2	LW (2)	1/2 K
	AT3G48750	*CDC2*	CELL DIVISION CONTROL 2	1		ND
	AT2G23380	*CLF*	CURLY LEAF	1, 2	LL (2), LW (1)	1/1 K
	AT4G38190	*CSLD4*	CELLULOSE SYNTHASE LIKE D4	1		ND
	AT4G37490	*CycB1*	CYCLIN B1;1	1		ND
	AT1G20610	*CycB2;3*	CYCLIN B2;3	1, 2		1/2 K
	AT1G76310	*CycB2;4*	CYCLIN B2;4	1, 2		2/2 K
	AT1G16330	*CycB3;1*	CYCLIN B3;1	1		2/2 K
	AT1G70210	*CYCD1;1*	CYCLIN D1;1	1, 2		ND
	AT4G34160	*CycD3;1*	CYCLIN D3;1	1, 2		3/3 K
	AT5G67260	*CycD3;2*	CYCLIN D3;2	1		1/1 K
	AT3G50070	*CycD3;3*	CYCLIN D3;3	1, 2		ND
	AT1G68480	*JAG*	JAGGED	1, 2	LW	1/3 R
	AT3G50630	*KRP2*	KIP-RELATED PROTEIN 2	1		2/2 R
	AT5G15580	*LNG1*	LONGIFOLIA1	1		1/3 K
	AT3G02170	*LNG2*	LONGIFOLIA2	1		1/3 K
	AT3G04740	*MED14*	MEDIATOR COMPONENTS 14	1		ND
	AT4G00100	*PFL2*	POINTED FIRST LEAF 2	1		1/3 K
	AT4G14713	*PPD1*	PEAPOD 1	1		–
	AT4G14720	*PPD2*	PEAPOD 2	1		ND
	AT5G63980	*RON1*	FRY1/SAL2	1		1/3 R
	AT2G39890	*ROT1*	ROTUNDIFOLIA 1	1		2/3 R
	AT2G36985	*ROT4*	ROTUNDIFOLIA4	1		1/2 R
	AT2G36590	*ATPROT3*	PROLINE TRANSFER 3	2		–
	AT4G36380	*ROT3*	CYP90C1/ ROTUNDIFOLIA 3	1, 2	Leaf shape (1) 2/2 K	
	AT1G03770	*RING1B*	RING 1B	2		1 K, 1R
	AT5G57380	*VIN3*	FIBRONECTIN TYPE III DOMAIN-CONTAINING PROTEIN	2		1/2 R

Table 4 (continued)

Functional pathway	At_Locus	Gene	Full name	References	Remarks	In this study
Leaf width	AT5G28640	*AN3*	ANGUSITFOLIA3	1, 2	LL (2)	1/3 K
	AT4G34580	*COW1*	CAN OF WORMS1	1, 2		2/3 K
	AT3G11220	*ELO1*	ELONGATA 1	1		ND
	AT5G66190	*FNR1*	FERREDOXIN-NADP(+)-OXIDORE-DUCTASE 1	1		1/2 K
	AT4G28190	*ULT1*	ULTRAPETALA	2		2/2 K
	AT5G55190	*RAN3*	RAN GTPase 3	2		ND
	AT5G13300	*VAN3*	ARF GPTase-ACTIVATION PROTEIN (SFC)	2		ND
Leaf shape	AT1G01510	*AN*	ANGUSTIFOLIA	1		ND
	AT4G37750	*ANT*	AINTEGUMENTA	1, 2	LW (2)	2/3 K
	AT5G53950	*CUC2*	CUP-SHAPED COTYLEDON2	1, 4	Leaf margin (4)	2/2 R
	AT5G07200	*GA20OX3*	GIBBERELLIN 20-OXIDASE 3	1, 3	Lobes (3)	3/3 R
	AT3G05120	*GID1A*	GA INSENSITIVE DWARF1A	1		1/1 R
	AT4G37740	*GRF2*	GROWTHREGULATING FACTOR 2	1		2/2 K
	AT3G13960	*GRF5*	GROWTH-REGULATING FACTOR 5	1	Size (1)	3/3 R
	AT1G23540	*IGI1*	INFLORESCENCE GROWTH INHIBI-TOR 1	1		1/2 R
	AT1G13710	*KLUH*	CYP78A5	1		1/2 K
	AT1G73590	*PIN1*	PINFORMED 1	1		ND
	AT3G44735	*PSK3*	PHYTOSULFOKINE 3 PRECURSOR	1		–
	AT3G47620	*TCP14*	TEOSINTE BRANCHED, CYCLOIDEA AND PCF (TCP) 14	1		ND
	AT1G69690	*TCP15*	TEOSINTE BRANCHED1/ CYCLOIDEA/PCF 15	1		ND
Proximal–distal polarity	AT3G57130	*BOP1*	BLADE ON PETIOLE 1	1		ND
	AT2G41370	*BOP2*	BLADE ON PETIOLE 2	1		ND
Symmetry	AT2G37630	*AS1*	ASYMMETRIC LEAVES 1	1, 3	Lobes (3)	ND
	AT1G65620	*AS2*	ASYMMETRIC LEAVES 2	1		1/1 K

ND no difference, –: no Br clone, *R* RCBr up, *K* Kenshin up

1: Xiao et al. (2014), 2: Choi et al. (2017), 3: Li et al. (2009), 4: Bar and Ori (2014), 5: Kalve et al. (2014)

for controlling leaf size in *B. rapa*, especially increases in leaf size.

Conclusion

To identify genes associated with leaf morphology in *B. rapa*, we generated an F_2 population from a cross between the inbred lines RCBr and Kenshin. We analyzed four leaf phenotypes, examined gene expression associated with phenotypic variation using the Br135K microarray, and finally confirmed these analyses by qRT-PCR. In addition, we determined the importance of the genes identified in this study by comparing them with genes known from QTL and eQTL mapping. All four phenotypes were quantitative traits in the F_2 population. Gene expression analysis usig the Br135K microarray identified 466 genes positively associated with the leaf phenotypes and 602 genes negatively correlated with the leaf phenotypes. Many of these DEGs had been previously identified as involved in leaf development. The GO categories containing the highest numbers of DEGs were, from highest to lowest, "phytohormone", "developmental process", "enzyme activity", "transport", "cell division and elongation, differentiation", and "cell wall and cytoskeleton". A qRT-PCR analysis further suggested that many selected DEGs had putative functions related to leaf morphology. An integrative analysis combining our results with previously published data revealed that the values of LL and LW in our RCBr/Kenshin-derived F_2 population were largely determined by alleles derived from Kenshin, but values for LM were determined by RCBr alleles. Genes previously identified as important determinants of leaf phenotypes were more highly expressed in Kenshin than in RCBr plants, suggesting that alleles from Kenshin determine large leaf size. Further research to dissect the roles of the candidate genes identified in this study in leaf

development is necessary for the development of molecular markers associated with leaf phenotype that can be used in molecular approaches to plant breeding.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest, financial or otherwise.

Human and animal rights No animals/humans were used for studies that are base of this research.

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