RapaNet: A Web Tool for the Co-Expression Analysis of *Brassica rapa* Genes

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**ABSTRACT:** Accumulated microarray data are used for assessing gene function by providing statistical values for co-expressed genes; however, only a limited number of Web tools are available for analyzing the co-expression of genes of *Brassica rapa*. We have developed a Web tool called RapaNet (http://bioinfo.mju.ac.kr/arraynet/brassica300k(query)), which is based on a data set of 143 *B. rapa* microarrays compiled from various organs and at different developmental stages during exposure to biotic or abiotic stress. RapaNet visualizes correlated gene expression information via hierarchical networks and phylogenetic trees using Pearson correlation coefficient (r). In addition, RapaNet provides hierarchical clustering diagrams, scatterplots of log ratio intensities, related pathway maps, and cis-element lists of promoter regions. To ascertain the functionality of RapaNet, the correlated genes encoding ribosomal protein (L7Ae), photosystem II protein D1 (psbA), and cytochrome P450 monoxygenase in glucosinolate biosynthesis (CYP79F1) were retrieved from RapaNet and compared with their *Arabidopsis* homologues. An analysis of the co-expressed genes revealed their shared and unique features.

**KEYWORDS:** *Brassica rapa*, microarray, network, L7Ae, psbA, CYP79F1

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**Introduction**

*Brassica rapa* comprises a variety of vegetables, including Chinese cabbage (*B. rapa* ssp. pekinensis), which is one of the most highly consumed vegetables throughout Eastern Asian countries, such as China, Japan, and Korea. The draft genome sequence of *B. rapa* accession Chifu-401-42, a Chinese cabbage, contains 41 174 protein-coding genes.¹ *Brassica rapa* is a member of the family Brassicaceae and considered a model plant for evolutionary research on genome polyplody. *Brassica* species contain 3 diploids in the classical triangle of U, namely, *B. rapa* (AA), *Brassica nigra* (BB), and *Brassica oleracea* (CC). The allotetraploids include *Brassica juncea* (AABB), *Brassica rapa* (AAC), and *Brassica carinata* (BBCC).²,³ The genome of *B. oleracea*, which belongs to the same Brassicaceae family, has revealed detailed genome triplification because of its split from *Arabidopsis* approximately 20 million years ago (Mya).⁴ The reshuffling of these triplicated genomic blocks was accompanied by massive gene loss during speciation, differential retention of the genes, and, presumably, changes in the regulation of gene expression.

Microarray analysis, one of the most commonly used methods for simultaneously measuring the expression levels of large numbers of genes, has helped biologists understand gene function on a genomic scale. Gene expression microarray data repositories, such as the Gene Expression Omnibus (GEO)⁵ and ArrayExpress,⁶ are publicly available. In *B. rapa* research, several microarrays have been used to study abiotic stress and Ogura cytoplasmic male sterility on a genome-wide scale.⁷,⁸ A microarray containing 90K expressed sequence tag consensus sequences from *B. rapa*, *B. rapa*, and *B. oleracea* was recently used to test genome-specific gene expression in *Brassica* species.⁹ The recent accumulation of data obtained using next-generation sequencing technology provides insightful information at the nucleotide sequence level and many research opportunities.¹⁰,¹¹

Many studies have attempted to obtain additional information from microarray data and to determine expression relationships between large numbers of genes. Several analysis tools and databases for analyzing co-expressed genes have been developed for individual model plants, such as *Arabidopsis* and rice, and for plants in general.¹²⁻²⁰ A co-expression analysis of the transcript abundance of developing seeds from 2 diverse *B. rapa* morphotypes, specifically pak choi (leafy-type) and yellow sarson (oil-type), with 2 of their doubled haploid progenies, has been conducted using an Agilent array representing 42 000 genes.²¹
However, a Web tool for analyzing gene co-expression based on microarray data was not available at the time of the study.

In this report, we present the **B rapa** Array Network (RapaNet; [http://bioinfo.mju.ac.kr/arraynet/brassica300k/query/](http://bioinfo.mju.ac.kr/arraynet/brassica300k/query/)), which was constructed using 143 *Brassica* microarrays based on the **Brassica rapa** 300k Microarray v2.0.7 In RapaNet, the correlation levels between gene pairs are evaluated using Pearson correlation coefficient (r)22 and are represented by a correlational network and phylogenetic tree. RapaNet provides hierarchical clustering diagrams, scatterplots of the log ratio intensities between 2 genes of interest, pathway maps, and cis-element lists of promoter regions. We examined the distributions of the r values between 47 548 unigenes on the microarray and found that the distributions followed a normal distribution and that 95% of the r values were within the range of -0.50 to 0.50. The co-expression of 3 representative genes was extracted from RapaNet and used to construct a gene regulatory profile: Brapa_ESTC000925 (L7Ac) encodes a chromosomally located ribosomal protein, Brapa_ESTC027946 (psbA) encodes photosystem II protein D1 and is located on the plastid chromosome, and Brapa_ESTC006895 (CYP79F1) encodes cytochrome P450 monooxygenase during the initial step of core glucosinolate (GS) biosynthesis. We also compared these co-expressed genes with their *Arabidopsis* homologues retrieved from CressExpress (cressexpress.org),23 and the results show the common and unique features of the co-expressed genes and their homologous genes in the Brassicaceae family.

**Materials and Methods**

**Microarray data normalization**

To build the database, we used 143 microarrays generated using the *Brassica rapa* 300k Microarray v2.0.7 Information on the microarray is available at NCBI GEO Platform GPL17248. Briefly, 10 probes (60 base pairs [bp] each) were extensively designed to cover the 3′ region of the coding sequence and the 3′ untranslated region. The probes were spaced 10 bp apart. In this manner, 507 495 oligonucleotides were designed from 47 548 consensus sequences. A BlastT analysis showed the 42 004 sequences matched to the 25 363 messenger RNAs (mRNAs) of *B rapa* genome v1.5 (brassicadb.org).

Microarray data sets typically have different levels of signal intensities and background noise depending on the conditions used in each experiment. To normalize the microarray data, the R statistical language and environment were used. First, XYS files were generated from Nimblegen pair files, and an annotation package for a Nimblegen microarray was built with the “pdlInfoBuilder” package prior to normalization (bioconductor.org). The XYS files were subsequently loaded using the “read.xysfiles” function in the “oligo” package. The loaded microarray data were then normalized with the Robust Multi-Chip Analysis (RMA) function24 in the Bioconductor package,25 and the normalized data were exported into a database for further analysis using the DBI and RSQLite packages.

**Evaluation of the expression relationships between genes**

To determine the degree of correlation between 2 genes, RapaNet calculates Pearson correlation coefficient (r), the value of which ranges from +1 to -1. Specifically, r values of +1 and -1 indicate perfect positive and negative linear relationships between 2 genes, respectively, whereas an r value of 0 indicates the absence of a linear relationship between 2 genes. To assess the significance of an r value calculated for a gene pair, a P value is calculated numerically using the Student t test. In addition to the P value of the r value, a z score based on the actual distribution of r values is calculated. To obtain the z score of the r value for each gene in a gene pair, the r values between the first gene and the other genes is first calculated, and the z score is then calculated based on the distribution of the r values. Because the sample size is suitably large, most of the distributions of the r values calculated between one gene and other genes showed a normal distribution. The z score, which is based on the actual r value distribution, is calculated using the following numerical formula:

\[
z = \frac{r - \mu}{\sigma}
\]

where μ is the mean of the population, and σ is the standard deviation of the population. The closer the absolute z score is to 0, the lower the significance.

**Development environment**

RapaNet was constructed using Django ([http://www.djangoproject.com/](http://www.djangoproject.com/)), a framework for Web application development that adopts an Model-View-Template (MVT) pattern in the Python programming language ([http://www.python.org/](http://www.python.org/)). In the pattern, the model contains the essential fields and behaviors of the database based on class definitions. Each class definition is translated by Django via a single database table into an equivalent SQL. The view consists of a Python function that responds to HTTP requests for specific URLs. The Django template manages the display of information and provides various built-in tags and filters. The MVT supports multiple templates for the same model.

The Database Management System (DBMS) PostgreSQL ([http://www.postgresql.org/](http://www.postgresql.org/)), a popular open-source DBMS available for various operating systems, such as Linux, Mac, and Windows, was employed. PostgreSQL uses a multiple-row data storage strategy, which makes it extremely responsive in high-volume environments. In addition, mod_python, which integrates the Python programming language into the
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relation between genes. A red edge indicates a positive correla-
(http://jtreeview.sourceforge.net/).
and the clustering diagrams are displayed by JAVA TreeView
malized intensity data using the PyCluster package in Python,
ule, and the color of the edges varies depending on the r value.
In addition, hierarchical clustering is performed on the normal-
ized intensity data using the PyCluster package in Python,
and the clustering diagrams are displayed by JAVA TreeView
(http://jtreeview.sourceforge.net/).
In the correlational network, the edges represent the corre-
lation between genes. A red edge indicates a positive correla-
whereas a green edge represents a negative correlation. As
the color of an edge deepens and the absolute r value, |r value|,
of the gene pair approaches 1, the closeness between the genes
increases. The phylogenetic tree also shows the relationships
among the correlated genes. The correlated genes are divided
into subgroups based on their distance in the tree. In addition
to correlational networks and phylogenetic trees, hierarchical
clustering diagrams are provided by RapaNet, enabling users to
examine gene expression patterns at a glance.

Comparison of commonly co-expressed genes
The genes co-expressed with L7Ae, Brapa_ESTC000925, and
AT5G20160 were retrieved from the RapaNet and CressExpress Web sites, respectively. Because CressExpress
gives r² values and RapaNet provides r values, the r values
obtained using RapaNet are squared prior to comparison with
the values found for Arabidopsis homologues with CressExpress.
Analysis of the top 5% r values yielded 2372 among 47 548
unigenes from RapaNet and 1141 among 21 810 unigenes
from CressExpress, and these unigenes were compared with
yield the list of 336 Arabidopsis homologous genes in
Supplementary 3.

Plant material
Brassica rapa L. (B rapa L. ssp pekinensis “Jangkang” AA, 2 m =
20) seeds were grown in 6.5 cm × 9 cm pots in a controlled
culture room at 22°C under long-day conditions (16 hours
light/8 hours dark) with white light (150 mol m⁻² s⁻¹). The
20-day-old seedlings were transferred into growth chambers at
4°C for vernalization. After 70 days, the plants were transferred
into larger pots (20 cm × 16 cm) and grown in a greenhouse
with 16 hours of light (sunlight + halogen lamps) at 27 ± 3°C
followed by 8 hours of darkness at 20 ± 4°C. The plants were
regularly watered and fertilized. Approximately 200 seeds from
each individual plant (or all the seeds if less than 200 were
available) were sown in separate trays in the greenhouse.

RNA isolation and reverse transcription polymerase
chain reaction
The total RNA from representative organs, such as the leaf, root,
and flower, was extracted using the TRI Reagent (Molecular
Research Center, www.mrcgene.com) and cleaned with the
Qiagen RNeasy mini kit (Qiagen, www.qiagen.com). The comp-
lementary DNA (cDNA) templates were synthesized using the
RevertAid H Minus M-MulV reverse transcriptase (Fermentas,
www.fermentas.com). The semiquantitative reverse transcription
polymerase chain reaction (RT-PCR) amplifications were
performed in 20-mL volumes with the following protocol: 1
cycle of 95°C for 2 minutes and 25 to 30 cycles of 94°C for 30
seconds, 60°C for 30 seconds, and 72°C for 30 seconds.
A semiquantitative RT-PCR analysis was conducted using real-time PCR Pre-mix with EvaGreen (SolGent, www.sol-
gen.com) in accordance with the manufacturer's recom-
ended protocol. The B rapa actin gene (Brapa_ESTC001256)
was used as an endogenous control. All the primer pairs are
listed in Supplementary 12.

Results
Visualization of gene relationships in RapaNet
To construct the RapaNet database, we used 143 microarray
data sets generated using the B rapa 300k microarray (NCBI
GEO Platform GPL17248) as described in the “Materials and
Methods” section. The sample information can be found in
Supplementary 1. To determine the degree of correlation
among the expression of 2 genes, RapaNet calculates Pearson
correlation coefficient (ρ), which is widely used for measuring
the correlation between 2 genes using normalized intensity val-
ues, as previously described. To assess the level of significance,
r, P, and z values were calculated numerically using Student t
test. RapaNet was constructed with Django (http://
www.djangoproject.com/), a framework for Web application development in the Python programming language (http://www.djangoproject.com/ Supplementary 2). PostgreSQL (http://www.postgresql.org/) was used as the DBMS. The statistical analysis modules were written in the C programming language to handle the enormous number of required calculations. The gene expression correlations are represented in 3 different ways: correlational trees, networks, and hierarchical clustering. As an example, Brapa_ESTC027946, the \textit{psbA} gene encoding photosystem II protein D1, was selected to analyze the gene regulatory network found by RapaNet (Figure 1A). In total, 9 genes were selected by setting $|r|$ value $\geq 0.8$ and depth = 1 in RapaNet. The depth represents the degree of the direct relationship of the related gene to a query or a “seed” gene;\textsuperscript{17} and a “0” indicates a direct relation to the seed gene. The query gene, \textit{psbA}, is marked with an asterisk. The (B) hierarchical clustering diagram and (C) correlational network were drawn using highly correlated genes (8 genes), which were identified by increasing the $r$ values to 0.8.

![Figure 1](image.png)

**Figure 1.** Visualization of the genes co-expressed with Brapa_ESTC027946, the \textit{psbA} gene encoding for photosystem II protein D1. (A) The phylogenetic tree was drawn based on 9 \textit{psbA}-related genes by setting $|r|$ value $\geq 0.8$ and depth = 1 in RapaNet. The depth represents the degree of the direct relationship of the related gene to a query or a “seed” gene,\textsuperscript{17} and a “0” indicates a direct relation to the seed gene. The query gene, \textit{psbA}, is marked with an asterisk. The (B) hierarchical clustering diagram and (C) correlational network were drawn using highly correlated genes (8 genes), which were identified by increasing the $r$ values to 0.8.

Distribution of $r$ values and correlation between \textit{P} values and \textit{z} scores

Because potential bias can be associated with specific resource tools,\textsuperscript{27} we tested the distributions of the $r$ values of all the possible gene pairs of the 47 548 \textit{B} genes in the RapaNet data set. The distribution of the $r$ values follows a normal distribution with a mean ($\mu$) $r$ value of 0.003 and a standard deviation ($\sigma$) of 0.26 (Figure 2A). Furthermore, 95% of the $r$ values were within the range of -0.50 to 0.50.

In RapaNet, 2 different indicators, \textit{z} score and \textit{P} value, are used to determine the statistical significance of the $r$ values. The \textit{P} value can be rapidly calculated because it is a type of...
statistical prediction, whereas the $z$ score is calculated with the mean and standard deviation of the actual $r$ value distributions and thus reflects the genuine character of the $r$ values. As the absolute $z$ score, $|z|$ score, increases and the $P$ value decreases, the significance of the $r$ value increases. To compare the $P$ value and absolute $z$ score indicators, 10 000 gene pairs were randomly selected and drawn as scatterplots (Figure 2B). The scatterplots show that the log of the absolute $z$ score, log $|z|$ score, is inversely related to the $P$ value. The linear model gives a slope of $-0.90$, indicating that the predicted $r$ value distribution is similar to the actual $r$ value distribution. Thus, the data show an unbiased distribution of the $r$ values.

Analysis of the co-expressed genes encoding ribosomal protein L7Ae and photosystem II protein psbA

After evolving from their ancestor, *Arabidopsis*, several events occurred in the genomes of the Brassicaceae family, such as the triplication of their genomes and the reshuffling of genomic blocks. These events resulted in differential retention of genes of the Brassicaceae family, and the regulation of gene expression might have undergone changes during speciation. To test how genes in the Brassicaceae family are co-expressed with other genes, we selected several genes and compared them with their homologues in *Arabidopsis*. First, the ribosomal protein L7Ae, Brapa_ESTC000925, was selected, and its co-expressed genes were identified using RapaNet. The $r^2$ value of the genes was found to equal 0.11 (SD: 0.10, Figure 3A). To confirm the reliability of the regulatory network of L7Ae, we searched for the co-expressed genes of its *Arabidopsis thaliana* gene homologue, AT5G20160, in the CressExpress database. The mean of the $r^2$ values was found to equal 0.074 (SD: 0.11, box plot in Figure 3C). The co-expressed genes of an *Arabidopsis thaliana* homologue, ATCG00020, were also retrieved from the CressExpress database. The number of co-expressed genes that ranked within 5% of L7Ae from *Arabidopsis* (1141) and *B rapa* (2372) were selected and compared with test the number of shared co-expressed genes in *Arabidopsis*. The results showed that 336 *Arabidopsis* genes were commonly co-expressed (Figure 3B and Supplementary 3). In contrast, Brapa_ESTC001256, an actin used as a control, has few genes in common (257) with any gene co-expressed with AT5G20160.

The *psbA* gene, which encodes photosystem II protein D1 and is located on the plastid chromosome, was selected for analysis of its gene regulatory network. The entire set of co-expressed genes was searched using Brapa_ESTC027946. The $r^2$ value of the genes was found to equal 0.048 (SD: 0.079, Figure 3C). The co-expressed genes of an *A thaliana* homologue, ATCG00020, were also retrieved from the CressExpress database. The number of co-expressed genes that ranked within 5% of *psbA* from *Arabidopsis* (1140) and *B rapa* (2371) revealed that 578 *Arabidopsis* genes were shared with those of *B rapa* (Figure 3D and Supplementary 4). More than half of the genes co-expressed with *Arabidopsis* ATCG00020 were found in those co-expressed with the *B rapa* homologue, which suggests that the critically conserved genes involved in photosynthesis exhibit very similar profiles of co-expressed genes over the speciation process. Interestingly, among these 578 genes, only 13 genes consist of plastid DNA, whereas the others are located on nuclear chromosomes, which implies that the regulation of the genes in chromosomes and chloroplasts is tightly regulated. In addition, the genes retrieved with Brapa_ESTC001256, an actin, did not include any of the 578 genes co-expressed with *psbA* in *Arabidopsis* and *B rapa* (Figure 3D).

We also tested the functional categories of the genes co-expressed with *psbA* by GO term enrichment. RapaNet retrieved 1504 genes using a maximum absolute $r$ value of 0.5,
and these were analyzed using the Singular Enrichment Analysis (SEA) tool in agriGO.\textsuperscript{28} The hierarchical tree graphs of the GO terms generated using the SEA tool showed that significant biological process GO terms were associated with \textit{psbA}-related genes, as shown in Supplementary 5. Most of the positively related genes are associated with light reactivity in photosynthesis, as expected. In addition, the negatively \textit{psbA}-related genes were not associated with any significant biological process GO term. Thus, the gene network obtained by RapaNet reflects the actual biological features of gene expression and can be used for constructing expression networks.

Unique profiling of a GS synthesis gene in \textit{B rapa} and \textit{Arabidopsis}

In addition, we profiled the co-expressed genes of the aliphatic GS biosynthesis pathway. Glucosinolates, a group of sulfur-rich secondary metabolites, play an important role in plant defense against herbivores and microbes. The 3 major GS groups are aliphatic, indolyl, and aromatic GS.\textsuperscript{29} In total, 91 GS biosynthesis genes (Supplementary 6) and 11 transcription factors have been identified in the \textit{B rapa} genome.\textsuperscript{30,31} Among the GS biosynthesis–related genes, 59 genes were unambiguously identified in the microarray used in this study, and 11
genes were involved in the synthesis of short aliphatic GS using methionine as the substrate (Supplementary 7). The genes involved in methionine chain elongation include BCAT4, MAM, and BCAT3. The initial step of core GS biosynthesis is catalyzed by cytochrome P450 monooxygenase (CYP79F1) and forms aldoxime. Subsequently, aldoxime is used for conjunction, C-S cleavage, glucosylation and sulfation by monooxygenase (CYP83A1), lyase (C-S lyase) and UGT74B1, respectively. The side chains are then modified by oxidation, elimination, alkylation, or esterification through the action of FMOGS-OX15, AOP, GSL-OH, and BZO1, respectively.

Several aliphatic GS biosynthesis pathway genes were selected (11 genes), and a network was drawn by setting the absolute $r$ value to greater than 0.71 and the depth of degree to 1 in RapaNet (Supplementary 8). The $r$ values between the genes in the pathway are presented in Table 1. In total, 77 genes clustered into 4 groups (Supplementary 9). Cluster 1 contains the genes (66) in the pathway, including Brapa_ESTC010094, Brapa_ESTC006895, Brapa_ESTC0206266, Brapa_ESTC006716, Brapa_ESTC002247, Brapa_ESTC011946, Brapa_ESTC022657, and Brapa_ESTC029776. The other 11 genes seeded with Brapa_ESTC034451, Brapa_ESTC007581, and Brapa_ESTC021288 were excluded from the main cluster (cluster 1) and 3 different clusters, suggesting that the regulation of gene expression diverged over time.

Brapa_ESTC006895 (CYP79F1) catalyzes the oxidation of amino acids to aldoximes, the initial step in GS biosynthesis. We compared the co-expressed genes of Brapa_ESTC006895 with those of an Arabidopsis homologue, AT1G16410. The genes were retrieved with CressExpress as described previously for the psbA gene. The mean $r$ values for Brapa_ESTC006895 and AT1G16410 were found to equal 0.037 (SD: 0.05) and 0.038 (SD: 0.053), respectively (Figure 4A). The top 5% of the ranked genes within each library share 215 genes (Figure 4B).

A GO analysis suggested that 389 terms related to biological process are enriched in this set (data not shown), including GO:0010439_regulation of GS biosynthetic process, GO:0019760_GS metabolic process, and GO:0019761_GS biosynthetic process. In addition, we tested all $r^2$ values of the GS pathway genes (59) with Brapa_ESTC006895 in B. rapa and directly compared them with those of the corresponding homologues of CYP79F1 in Arabidopsis (Supplementary 10 and Supplementary 11). The resulting linear model produces the line $y = 0.58x + 0.20$. The $P$ value for the slope is 2.25$^{10^{-7}}$, which indicates high significance. These data suggest that the regulation of the genes co-expressed with CYP79F1 is likely conserved during the speciation of the Brassicaceae family.

The $r$ values for the co-expression of the genes involved in the synthesis of the short aliphatic GS that were retrieved with Brapa_ESTC006895 (CYP79F1) ranged from −0.16 with Brapa_ESTC034451 to 0.83 with Brapa_ESTC026266 (Table 1). We validated the 11 genes involved in the pathway by semi-quantitative RT-PCR using the total RNA from representative organs, such as the leaf, root, and flower (Figure 5). Similar to the results obtained for Brapa_ESTC006895, their expression appears to be more consistent when the $r$ values are high and less consistent when the $r$ values are low. The $r$ values associated with Brapa_ESTC010094 and Brapa_ESTC026266 are 0.82 and 0.83, respectively, and their expression patterns are consistent across all of the organs. In contrast, the $r$ values associated with Brapa_ESTC034451 and Brapa_ESTC007581 were found to equal −0.16 and 0.32, respectively, and their expression patterns differed significantly from those of Brapa_ESTC006895.

**Discussion**

The accumulation of genome-wide gene expression data from microarrays and next-generation sequencing provides an opportunity to derive an understanding of the relationships between the genes involved in the various biological systems of an organism. Given the wide spectrum of databases available, specific types of gene regulation and function can potentially be ascertained through the correlation of gene expression profiles. Although RNA-Seq technology offers more advantages regarding this than microarrays; co-expression analysis is in its infancy and requires further refinement. In this article, we present RapaNet, a tool for analyzing the co-expression of B. rapa genes based on 143 data sets. RapaNet uses Pearson correlation coefficients for calculating edge weights. Multiple seeded genes can be selected, and the edges can be further extended by setting the number of degrees to range from 0 to 2, resulting in clusters. The clustered genes can then be visualized via network-like, tree-like, and cluster-shaped modules (Figure 1).

We used the RapaNet database to study the co-expression patterns of L7Ac, psbA, and CYP79F1. L7Ac is a ribosomal protein, and psbA and CYP79F1 are involved in primary and secondary metabolism, respectively. A comparison of L7Ac with an Arabidopsis homologue showed that approximately 27% (303/1141) of the Arabidopsis genes are included in the list of L7Ac co-expressed genes, and this number is comparable with that found for rice, a monocot plant. This number is greater than the 225 genes identified from a comparison with the genes co-expressed with an actin (Brapa_ESTC001256) and is markedly higher than those found from a randomized list (30) of these genes (data not shown), suggesting that the co-expressed genes of homologous genes are comparable throughout the plant kingdom (Figure 3). This finding was further confirmed using a gene, psbA, involved in primary metabolism. Of the genes co-expressed with psbA (top 5%) in Arabidopsis, more than half (578/1141) are shared with the genes co-expressed with psbA in B. rapa. Interestingly, although the psbA gene is located on the chloroplast genome, 13 of the 578 genes are located on the chloroplast genome, and the remaining genes are located on the chromosome (data not shown). It has been proposed that anterograde signals appear to be more consistent when the $r$ values are high and less consistent when the $r$ values are low. The $r$ values associated with Brapa_ESTC010094 and Brapa_ESTC026266 are 0.82 and 0.83, respectively, and their expression patterns are consistent across all of the organs. In contrast, the $r$ values associated with Brapa_ESTC034451 and Brapa_ESTC007581 were found to equal −0.16 and 0.32, respectively, and their expression patterns differed significantly from those of Brapa_ESTC006895.
Table 1. Correlation coefficients (r values) between the genes involved in the synthesis of short aliphatic glucosinolate.

|              | BRAPA_ESTC010094 | BRAPA_ESTC021288 | BRAPA_ESTC006895 | BRAPA_ESTC028266 | BRAPA_ESTC006716 | BRAPA_ESTC029776 | BRAPA_ESTC001256 (ACTIN) |
|--------------|------------------|------------------|------------------|------------------|------------------|------------------|--------------------------|
| Brapa_ESTC010094 | −0.19            | 0.82             | 0.71             | 0.65             | 0.42             | 0.39             | −0.01                    |
| Brapa_ESTC021288 | 0.06             | −0.05            | −0.13            | −0.07            | 0.23             | −0.05            | 0.004                    |
| Brapa_ESTC006895 | 0.83             | 0.77             | 0.49             | 0.60             | −0.16            | 0.50             | 0.32                     |
| Brapa_ESTC028266 | 0.82             | 0.65             | 0.56             | 0.001            | 0.60             | 0.20             | 0.81                     |
| Brapa_ESTC006716 | 0.73             | 0.51             | −0.35            | 0.44             | 0.24             | 0.78             | 0.19                     |
| Brapa_ESTC002247 | 0.60             | −0.06            | 0.62             | 0.09             | 0.53             | 0.20             |                          |
| Brapa_ESTC011946 | 0.06             | 0.74             | 0.16             | 0.43             | 0.01             |                  |                          |
| Brapa_ESTC034451 | 0.27             | −0.17            | −0.18            | −0.09            |                  |                  |                          |
| Brapa_ESTC022657 |                  | 0.11             | 0.47             | −0.08            |                  |                  |                          |
| Brapa_ESTC007581 | 0.34             | −0.01            |                  |                  |                  |                  |                          |
| Brapa_ESTC029776 |                  |                  |                  |                  |                  |                  | 0.00                     |

Among the GS biosynthesis–related genes, 59 genes were unambiguously identified in the microarray, and 11 genes identified in the synthesis of the short aliphatic glucosinolate and their gene correlations are shown. Brapa_ESTC001256 (an actin), a gene that is not involved in the pathway, is included for comparison purposes.
originating from the nucleus and retrograde signals from the chloroplast are used to achieve coordinated gene expression. As a part of photosystem II, the expression of \textit{psbA} might be regulated in both the nucleus and chloroplast to ensure the correct stoichiometric subunit composition of these complexes and to efficiently regulate the genes involved in the synthesis of other metabolites. The \textit{psbA} data described in this article suggest that the regulation of gene expression is tightly controlled through anterograde and retrograde signals. In contrast, none of the genes retrieved with Brapa\textunderscore ESTC001256, an actin, are shared with the genes co-expressed with \textit{psbA} in \textit{Arabidopsis} under the same conditions. These findings indicate that the co-expression analysis performed using RapaNet is consistent with the existing GO database. The genes co-expressed with \textit{psbA} were retrieved using RapaNet and subjected to SEA using agriGO, and most of the positively related genes were found to play relevant roles in the light reaction in photosynthesis, as expected (Supplementary 4).

We also applied RapaNet to analyze the genes co-expressed with \textit{CYP79F1}, which is involved in secondary metabolism. \textit{CYP79F1} catalyzes the oxidation of amino acids to aldoximes, the initial step in the short aliphatic glucosinolate pathway. The values were obtained as described above from RapaNet and CressExpress using Brapa\textunderscore ESTC006895 and AT1G16410, respectively. The mean $r^2$ values for Brapa\textunderscore ESTC006895 and AT1G16410 equal 0.037 (SD: 0.05) and 0.038 (SD: 0.05), respectively. (B) The top 5% of the ranked genes within each library were retrieved, and 215 genes were shared between Brapa\textunderscore ESTC006895 and AT1G16410.

Figure 4. Distribution of the squared Pearson correlation coefficients of all of the genes and the shared genes. (A) The distribution of the squared Pearson correlation coefficients of all of the genes co-expressed with Brapa\textunderscore ESTC006895 and AT1G16410 is box plotted. These genes are involved in catalyzing the oxidation of amino acids to aldoximes, the initial step in the short aliphatic glucosinolate pathway. The values were obtained as described above from RapaNet and CressExpress using Brapa\textunderscore ESTC006895 and AT1G16410, respectively. The mean $r^2$ values for Brapa\textunderscore ESTC006895 and AT1G16410 equal 0.037 (SD: 0.05) and 0.038 (SD: 0.05), respectively. (B) The top 5% of the ranked genes within each library were retrieved, and 215 genes were shared between Brapa\textunderscore ESTC006895 and AT1G16410.

Figure 5. Expression of the 11 genes involved in the synthesis of the short aliphatic glucosinolate in representative organs. The total RNA from the leaf, root, and flower was analyzed by semiquantitative reverse transcription polymerase chain reaction. The digits represent Pearson correlation coefficients for the correlations of Brapa\textunderscore ESTC006895, which catalyzes the oxidation of amino acids to aldoximes, with the genes involved in the synthesis of short aliphatic glucosinolate. L indicates leaf; F, flower after pollination; FB, flower before pollination; R, root.
Arabidopsis CYP79F1 homologues show a significant linear relationship (Supplementary 10). Tight coupling in the initial steps of the synthesis of methionine-derived GS34 might be important for achieving efficient control from the perspective of global regulation. Thus, the values found for the correlation of CYP79F1 with BA CT4 and BA T5 were quite high, in the range of 0.7 to 0.8, in both B rapa and Arabidopsis. The co-regulation of BA CT4 in the cytosol or of BA CT3 in the chloroplast might be essential because they immediately follow amino acid chain elongation. In addition, chain-elongated amino acids in the chloroplast must be exported to the cytosol for further modification, and BA T5 functions as a translocator of 2-keto acids between the chloroplasts and cytosol. The data also suggest that this pathway is highly comparable and conserved in B rapa and Arabidopsis.

The evolutionary split between the Brassicaceae family and A thaliana occurred ~20 Mya.1 Recent Brassicaceae spp genome sequencing has revealed that these genomes underwent segment inversions and translocations as well as fusions and fissions, resulting in Brassicaceae lineage-specific whole-genome triplication.4 Variation in the number of members of the gene families in the genome might have contributed to the remarkable morphological plasticity and presumably functional specification of these genes. A comparison of the co-expression profiles of B rapa and A thaliana genes suggests that the genes involved in primary pathways, such as photosynthesis, have been relatively strictly conserved throughout evolution. In contrast, the co-expressed genes of cell constituents or secondary metabolites, such as L7Ae and CYP79F1, have largely diverged throughout evolution. However, most of the key enzymes that play central roles in pathway regulation are strictly co-regulated, as demonstrated with CYP79F1.

Conclusions

RapaNet was designed to delineate the co-expressed genes in B rapa. The Web pages and programs associated with RapaNet can be accessed using any browser and operating system. The analysis of the genes co-expressed with a ribosomal protein, a photosystem II protein, and a protein in the GS synthesis pathway retrieved using RapaNet in this study highlight the functional characteristics of these genes. RapaNet can help researchers identify gene relationships and analyze gene functions. In addition, RapaNet was designed in a species-independent manner and can thus be expanded to construct similar Web-based tools for other organisms.

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Author Contributions

JK and KMJ designed the software architecture and wrote most of the manuscript. JK and T-HL built the database and implemented the software. JSK, SC, and Y-MP participated in the microarray analysis. S-IS, SIL, M-HL, C-KK, and YH provided the data. BHN assisted with project development. Y-KK inspired the overall work and revised the final manuscript. All the authors read and approved the final manuscript.

Supplementary Material

Supplementary 1. Sample information of microarray data in RapaNet.
Supplementary 2. The structure of RapaNet.
Supplementary 3. List of genes that are highly and commonly (336) co-expressed with L7Ae in B. rapa and Arabidopsis.
Supplementary 4. List of genes that are highly and commonly (1103) co-expressed with psbA in B. rapa and Arabidopsis.
Supplementary 5. Hierarchical tree graph of the significant GO terms of the genes positively related to psbA.
Supplementary 6. Metabolic pathway for hydroxyalkenyl glucosinolate biosynthesis from methionine.
Supplementary 7. Gene network of the metabolic pathway of hydroxyalkenyl glucosinolate synthesis from methionine.
Supplementary 8. The list of genes retrieved with the 11 genes involved in the synthesis of the short aliphatic glucosinolate from methionine.
Supplementary 9. r2-values of the genes involved in glucosinolate pathways with CYP79F1.
Supplementary 10. A linear model of the r2-values of the genes involved with CYP79F1 in glucosinolate pathways.
Supplementary 11. List of genes that are highly and commonly (215) co-expressed with CYP79F1 in B. rapa and Arabidopsis.
Supplementary 12. Primer pairs used for semi-quantitative RT-PCR analysis.

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