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A Co-expression Network of Drought Stress-related Genes in Chinese Cabbage

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Abstract

Plants have evolved to adapt to abiotic stresses, such as salt, cold, and drought stress. In this study, we conducted an in-depth analysis of drought resistance mechanisms by constructing a gene co-expression network in Chinese cabbage (*Brassica rapa* ssp. *pekinensis* L.). This drought stress co-expression network has 1,560 nodes, 4,731 edges, and 79 connected components. Based on genes that showed significant co-expression in the network, drought tolerance was associated with the induction of reactive oxygen species removal by raffinose family oligosaccharides and inositol metabolism. This network could be a useful tool for predicting the functions of genes involved in drought stress resistance in Chinese cabbage.

Additional key words: abiotic stress, microarray, raffinose family oligosaccharides, reactive oxygen species

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Introduction

In terms of food safety issues, drought is one of the critical menaces to the biosphere, owing to its impact on the growth and survival of crop plants. As there is a limited supply of water worldwide, many countries are concerned by the growing demand for water for agricultural and human use (Somerville and Briscoe, 2001). Many previous studies have provided considerable information regarding the mechanisms of drought resistance in plants (Hasegawa et al., 2000). The major effects of drought stress that reduce the yield of crops are the reduction of canopy absorption of incidental photosynthetically active radiation, reduction in radiation use efficiency, and decrease in the harvest index (Earl and Davis, 2003; Moon et al., 2017; Park et al., 2017). While plant responses to singular abiotic stresses such as drought, cold, and salt, have been extensively studied, the complex response mechanisms to abiotic stress have received limited attention. Thus, we have an incomplete picture of plant responses to abiotic stress because plants need to respond simultaneously to multiple stresses in the environment (Lee et al., 2016; Yu et al., 2016; Yu and Park, 2016; Zhou et al., 2007).

Drought stress is one of the major factors affecting the growth, survival, and ultimately the yield of harvested crops (Agarwal et al., 2006; Chen et al., 2002; Rabbani et al., 2003). Recently, to reduce the damage caused by drought stress, studies on the regulation of transcription factors, such as dehydration-

responsive element binding 1A (DREB1A) and C-box binding factor 1 (CBF1), have been conducted; the aim was to enhance drought resistance by understanding and modulating the complex changes in the expression of downstream genes in *Arabidopsis* (Kasuga et al., 1999; Kreps et al., 2002). A second approach was to over-express drought resistance genes, including *calcium-dependent protein kinase* (*CDPK*), *stress-responsive NAC 1* (*SNAC1*), *Arabidopsis Na+/H+antiporter* (*AtNHX5*), and rat *neuronal NO synthase* (*nNOS*), to enhance drought resistance in rice (Kreps et al., 2002; Saijo et al., 2000), cotton (Liu et al., 2014), paper mulberry (*Broussonetia papyrifera* L. Vent; Li et al., 2011), and *Arabidopsis* (Shi et al., 2014), respectively.

In this study, we used the *Brassica* expressed sequence tag (EST) and microarray database (RDA, 2008) to obtain microarray data related to drought stress, and constructed gene co-expression networks for drought stress responses in *Brassica* plants through statistical analysis and visualization of the molecular interactions.

Materials and Methods

Microarray data collection and construction of a gene co-expression network

For this study, the microarray data for drought stress treatments of *Brassica* plants were obtained from *BrEMD* (*Brassica rapa* EST and microarray database, http://www.brassica-rapa.org/BrEMD; currently maintained by the national agricultural biotechnology information center, http://nabic.rda.go.kr). These data were originally obtained from an experiment on inbred Chinese cabbage (*Brassica rapa* ssp. *pekinensis* L.) grown in growth chambers (16 h day/8 h night photoperiod, 40–70% relative humanity, 290 μmol $m^{-2} \cdot sec^{-1}$ light intensity) for three weeks, after which drought stress was applied and total RNA was isolated 0.5, 3, 12, 24, and 48 h after the application of the stress. This experiment was conducted with two biological replicates. Gene expression in *B. rapa* was then analyzed using a KBGP-24K microarray chip version 1.0 with the NimbleGen System (Lee et al., 2008).

PlantArrayNet (GreenGene BioTech Inc.; http://bioinfo.mju.ac.kr/arraynet) is a tool that analyzes correlation coefficients (*r* values) between the expression of different genes based on *Brassica* 300K microarray data. This tool was used to analyze the interactions between genes expressed in *B. rapa* in response to drought stress, using a statistical RiceArrayNet protocol (Lee et al., 2009). Genes showing at least a 2-fold change in expression compared to the control treatment (0 h) were identified for all time-points. Genes with correlation coefficient values higher than 0.85 in the PlantArrayNet analysis were selected for the network. The co-expression network for genes related to drought stress was constructed with the Cytoscape program (version 3.4.0, Cytoscape Consortium; Smoot et al., 2011), and the structure of the network was visualized using a spring embedded layout (Barnes and Hut, 1986).

Analysis of changes in gene expression in *B. rapa* during drought stress

The gene co-expression network was used to analyze the major changes in gene expression caused by drought stress and their associated function. Changes in gene expression in the co-expression network were ascertained using KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg) and TAIR (The Arabidopsis Information Resource, http://www.arabidopsis.org). DAVID (The Database for Annotation, Visualization and Integrated Discovery, http://david.abcc.ncifcrf.gov) was used for functional annotation of *B. rapa* genes (Huang et al., 2009; Kanehisa et al., 2012). Based on these data, gene ontology was analyzed and visualized using the Cytoscape plug-in program ClueGO (Bindea et al., 2009).

Results and Discussion

Construction of the drought stress-related gene co-expression network

A gene co-expression network for drought stress response genes in *B. rapa* was constructed using the *Brassica* 300K microarray data from PlantArrayNet, and microarray data from a collaborative study between our research team and the *Brassica* genomics team at the National Institute of Agricultural Biotechnology. Genes with expression correlation values higher than 0.85 in response to drought stress were selected. From a total of 23,937 probes, 1,560 probes that showed significant correlation under the drought stress conditions were selected. To analyze the functions of these genes, the bioinformatics resource DAVID was used for functional annotation clustering. Of the 176 functional clusters analyzed, the four clusters with the highest enrichment scores were selected (Tables 1). Cluster one had the highest enrichment score and contained genes associated with abiotic stress, osmotic stress, and salt stress responses. The other clusters comprised genes associated with the response to inorganic compounds, carboxylic acid catabolism, microbodies, and peroxisomes. These results indicate a close relationship between drought stress and salt stress resistance mechanisms. Drought and salt stress have been reported to be associated with the ionic and osmotic homeostasis signaling pathway, phospholipid signaling, photosynthesis rate, and cell growth (Chaves et al., 2009; Zhu, 2002). These observations are consistent with the functional annotation of the genes and gene expression clusters discovered in our study. This indicates that the group of genes selected to construct the network were significantly associated with drought resistance.

The spring embedded layout in Cytoscape was used to visualize the results of the analysis (Fig. 1). Each node in the co-expression network corresponds to a gene, and a significant correlation between the expression of two genes is represented by an edge. After constructing the co-expression network, multi-edge node pairs and self-loop nodes were identified and eliminated, resulting in a network with 1,560 nodes, 4,969 edges, and 79 connected components. The largest independent network among the suborder-connected components contained 82.82% of all nodes (1,292 nodes) and 95.21% of all edges (4,731 edges).

Table 1. Functional annotation clustering of genes related to drought stress in Chinese cabbage.

Cluster number	Cluster enrichment score	GO code[z]	Functional category	Count	*p*-value	Benjamini value
Cluster 1	15.32	GO:0009628	Response to abiotic stimulus	140	1.84E-23	2.42E-20
		GO:0006970	Response to osmotic stress	57	5.41E-13	2.37E-10
		GO:0009651	Response to salt stress	52	1.12E-11	2.45E-9
Cluster 2	6.88	GO:0010038	Response to metal ion	46	3.59E-8	5.90E-6
		GO:0046686	Response to cadmium ion	40	1.88E-7	1.90E-5
		GO:0010035	Response to inorganic substance	55	3.30E-7	2.45E-9
Cluster 3	4.48	GO:0009514	Glyoxysome	6	2.09E-5	3.00E-1
Cluster 4	3.75	GO:0016054	Organic acid catabolic process	19	6.82E-8	8.97E-6
		GO:0046395	Carboxylic acid catabolic process	19	6.82E-8	8.97E-6
		GO:0005777	Peroxisome	23	1.82E-5	1.46E-3
		GO:0042579	Microbody	23	1.82E-5	1.46E-3
		GO:0009062	Fatty acid catabolic process	9	1.39E-4	9.08E-3
		GO:0006635	Fatty acid beta-oxidation	8	2.97E-4	1.68E-2
		GO:0044242	Cellular lipid catabolic process	11	3.68E-4	2.00E-2
		GO:0034440	Lipid oxidation	8	5.80E-4	2.90E-2
		GO:0019395	Fatty acid oxidation	8	5.80E-4	2.90E-2
		GO:0030258	Lipid modification	9	9.43E-4	3.81E-2
		GO:0016042	Lipid catabolic process	22	9.94E-4	3.89E-2

[z] Gene ontology code number

Analyzing temporal response mechanisms using the drought stress gene expression network

The gene co-expression network was used to analyze the expression patterns of co- expressed genes during drought stress (dehydration). From the total of 1,560 genes showing significant correlations, 131 genes (8.40%), 28 genes (1.80%), 3 genes (0.19%), 293 genes (18.78%), and 1,105 genes (70.83%) showed significant correlations after 0.5, 3, 12, 24, and 48 h of drought stress, respectively (Fig. 1).

The genes expressed after 0.5 h of drought stress were associated with water-soluble vitamin metabolism, photosynthetic light harvesting, and glycerol metabolism. This suggests that water-soluble vitamins are associated with the drought stress response (Fig. 2A). A KEGG pathway analysis of the selected genes showed an association with the carotenoid pathway of water-soluble vitamin biosynthesis. Continuous drought stress leads to deficiencies in protein, phosphorus, and provitamin A. The expression of genes associated with ascorbate biosynthesis such as *Phosphomannomutase* (*PMM*), *ThiaminC* (*THIC*), and *Thiamine thiazole synthase* (*THI1*) were 4.98-, 3.09-, and 2.19-fold higher than the control, respectively. Previous studies have indicated that continuous abiotic stress triggers oxidative stress and the formation of reactive oxygen species. This leads to the production of non-enzymatic antioxidants such as vitamins, glutathione, and carotenoids that act by scavenging reactive oxygen species (ROS) by donation of an electron or hydrogen (Asada, 1999; Jaleel et al., 2009). Moreover, in mammalian research, vitamin A biosynthesis likely involves aldehyde dehydrogenase (ALDH), which plays a role in the detoxification of stress-generated aldehydes, and protection against osmotic imbalance (Perozich et al., 1999). Thus, carotenoid biosynthesis may affect drought tolerance in *B. rapa*. Notably, Zhang *et al.* (2010) reported that drought stress caused a reduction in relative water content

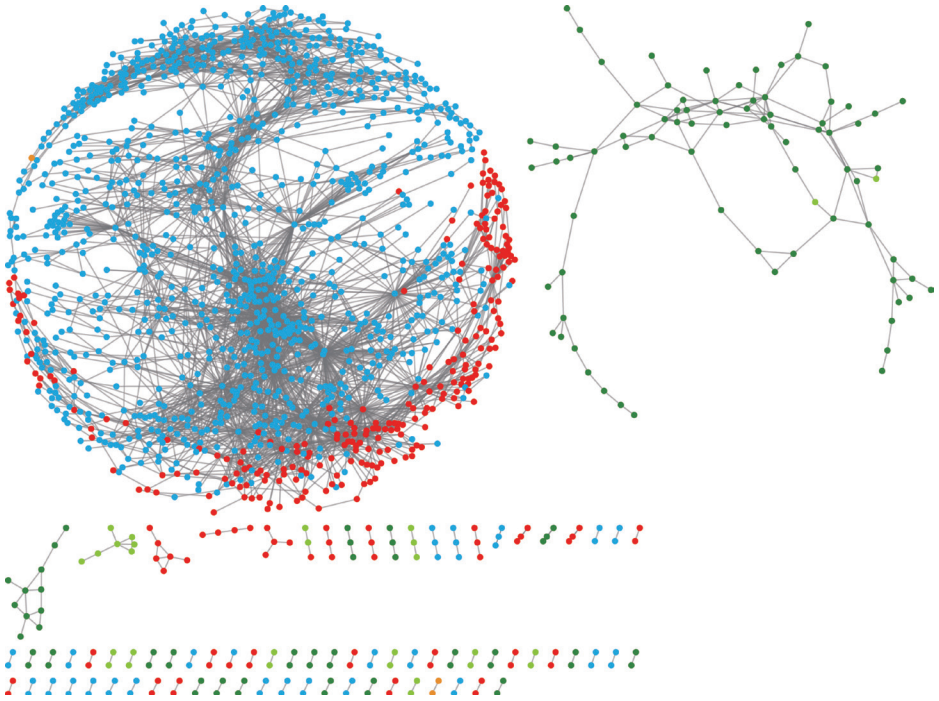

Fig. 1. Properties and topologies of the co-expression networks of genes related to drought stress in Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*). The data in the co-expression network indicates that the average correlation coefficient (*r* value) is higher than 0.85. For statistical analysis of the data, RiceArrayNet protocol (Lee et al., 2009) was used. The network is displayed in a Cytoscape spring embedded layout. Nodes represent genes, while edges represent significant co-expression relationships between the genes. Colored dots indicate significantly expressed genes at 0.5 h (131 nodes; green dot ●), 3 h (28 nodes; yellow-green dot ●), 12 h (3 nodes; orange-yellow dot ●), 24 h (293 nodes; red dot ●), and 48 h (1,105 nodes; turquoise blue dot ●) after dehydration treatment.

(RWC) in plants by preventing absorption of phosphorus (P) and nitrogen (N), inhibiting photosynthesis and transpiration. Hence, gene changes related to photosynthesis inactivation and an increase in provitamin A biosynthesis that occurred after 0.5 h of drought stress may promote drought tolerance.

Many genes expressed after 3 h of drought stress were related to glycoside biosynthesis (Fig. 2B). A correlation between glycoside biosynthesis and drought stress signaling has not been reported, but it has been observed in some studies that drought stress leads to an increase in total phenolic glycoside concentrations (Hale et al., 2005; Roth et al., 1997). The results from our study on *B. rapa* also showed that the expression of *Branched-chain aminotransferase4* (*BCTA4*), *AOP3*, *Reduced epidermal fluorescence 2* (*REF2*), and *Arabidopsis sulfotransferase 5b* (*AtSOT18*) were 2.42-fold, 1.99-fold, 1.82-fold, and 1.98-fold higher, respectively, after 3 h of drought treatment than that at 0 h.

The genes expressed after 12 h of drought stress were associated with glycoside biosynthesis, thus exhibiting a pattern similar to that observed at 3 h. Therefore, the genes responding after 12 h of drought stress did not have a significant effect on the correlation analysis of the co-expression network. Functional annotation clustering using the bioinformatics resource DAVID indicated that genes expressed after 12 h of drought stress were clustered in the RNA binding and mRNA processing group. More specifically, these genes were related to alternative and pre-mRNA splicing. RNAs are highly sensitive to abiotic stress, and drought stress affects RNA tertiary structure by affecting the both concentration and types of ions and osmolytes present

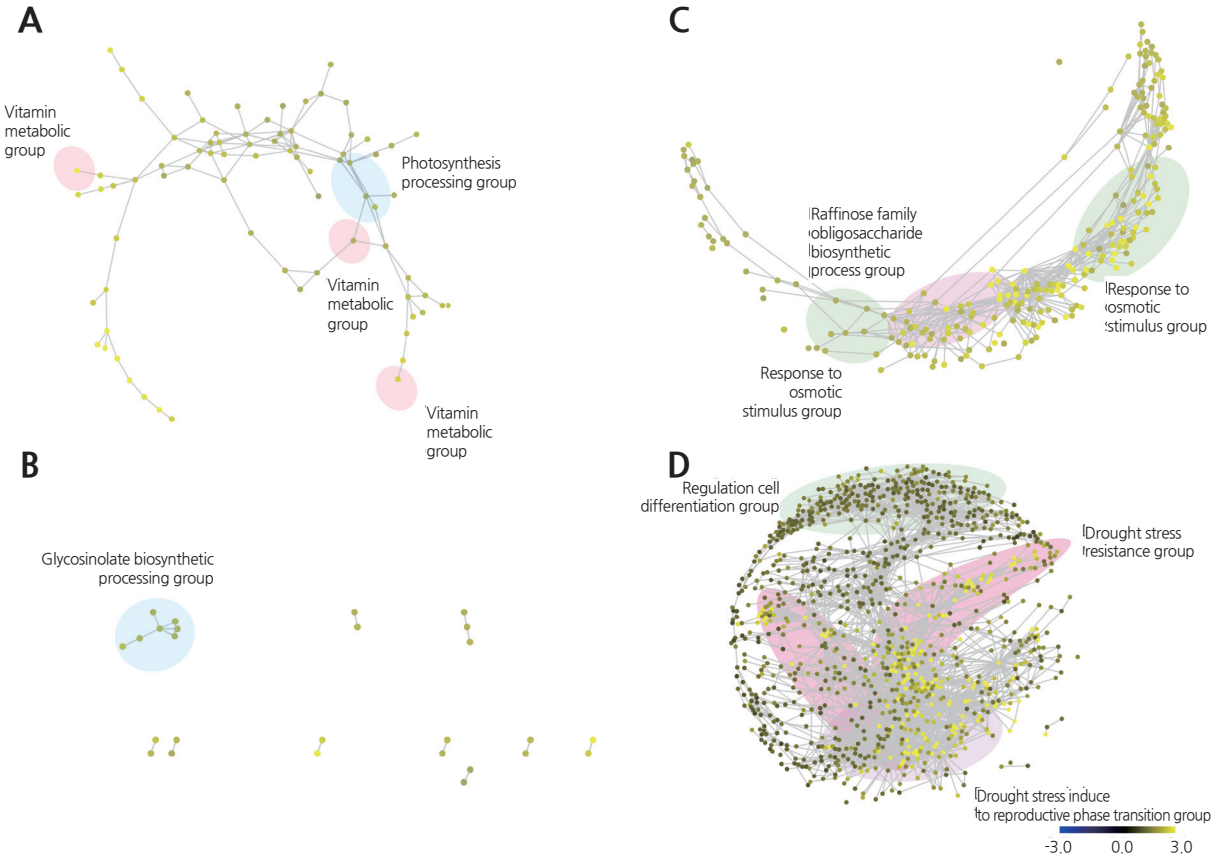

Fig. 2. Classification and topologies of co-expression networks of drought stress-related genes in Chinese cabbage. Functional clustering is associated with 0.5 h (A), 3 h (B), 24 h (C), and 48 h (D) after dehydration treatment. Co-expression networks show gene interactions with correlation coefficient values higher than 0.85. The color scale indicates the relative expression on a $\log_2$ scale. Yellow indicates higher and blue indicates lower transcript levels than those of the control. Networks are displayed in Cytoscape spring embedded layouts.

(Chan et al., 2013; Reddy et al., 2013). These changes in RNA stability and structure may activate glycoside biosynthesis and promote other mechanisms to counteract abiotic stress.

The genes that were expressed after 24 h of drought stress were associated with osmotic and salt stress responses, and belonged to three major groups (Fig. 2C). The first group contained genes involved in raffinose family oligosaccharide (RFOs) biosynthesis. This group of genes is linked to the positive regulation of defense responses, and their expression indicates that genes in *B. rapa* involved with the biosynthesis of RFOs are associated with drought response. RFOs are important polysaccharides for sugar vitrification in plants. Vitrification has been reported to be a mechanism of cell membrane protection as it increases tolerance to elevated temperatures and impedes liposome fusion under drought stress (Hincha et al., 2003; Valluru and Ende, 2008). The expression of several genes associated with the biosynthesis of RFOs including *Raffinose synthase 5* (*RS5*), *GA insensitive dwarf1b* (*AtGid1b*), *Trehalose-6-phosphate phosphatase J* (*TPPJ*), and *Trehalose phosphate synthase* (*TPS10*) were 4.42-, 2.37-, 2.75-, and 2.42-fold higher, respectively, than the control. The second group contained genes associated with proline metabolism and response to osmotic stimulus. This group of genes is associated with the metabolism of oxygen and reactive oxygen species, indicating that the genes involved in RFO-mediated membrane protection in *B. rapa* are ultimately linked to the ROS-scavenging mechanism. The last group of genes that responded to drought stress at 24 h were associated with organic acid catabolism, porphyrin catabolism, and leaf senescence, and showed an expression pattern like the RFOs-mediated ROS detoxification mechanism.

Finally, genes expressed after 48 h of drought stress were associated with transport and protein targeting and belonged to three major groups including the drought stress-mediated reproductive phase transition group, regulation of cell differentiation group, and drought stress resistance group (Fig. 2D). At this time point, 70.84% of all genes in the network were expressed (1,105 genes); this was the largest number of significantly expressed genes at any time-point in the experiment. Genes belonging to the first group were associated with the response to water deprivation, two-component signal transduction system (phosphorelay), and vegetative to reproductive phase transition. Results from this study also confirmed that abscisic acid (ABA) and ethylene signaling were activated in *B. rapa* after 48 h of drought stress, and this signaling led to the activation of carboxylic acid metabolism, phospholipid dephosphorylation, and lipid modification, as well as activation of a signal transduction cascade to develop systemic acquired resistance via salicylic acid. In response to drought stress, an increase in the intracellular concentration of ABA was observed. This increase negatively affected yield and caused a delay in female organ development without a major effect on the male inflorescence. An increase in ABA levels in reproductive organs as a result of drought stress may inhibit cell division, thereby impairing the development of flowers and seeds (Barnabás et al., 2008; Yang et al., 2001). The accumulation of ABA has been shown to regulate *FCS-Like Zinc finger* (*FLZ*) genes during the transition from the vegetative to reproductive phase, and Jamsheer and Laxmi (2015) reported that ABA mediates growth under drought stress conditions. The results from our study on *B. rapa* also showed that the expression of *Protein arginine methyltransferase 4B* (*PRMT4B*), *Vernalization5/Vin3-like 1* (*VEL1*), *Histone acetyltransferase of the CBP family 1* (*HAC1*), *BCL-2-associated athanogene 6* (*BAG6*), and *Histone mono-ubiquitination 1* (*HUB1*) were 2.61-, 2.35-, 2.48-, 2.85-, and 2.13-fold higher, respectively, after 48 h of drought treatment than that at 0 h. Moreover, the ABA signaling was further transduced to provide resistance to drought stress; this group of drought stress-related genes is closely associated with the biosynthesis of RFOs. This indicates that the same drought stress response mechanisms were active at 24 h and 48 h. In this study, the expression of RFO biosynthesis-associated genes, such as *Raffinose synthase 5* (*RS5*), *Trehalose-6-phosphatase synthase S6* (*TPS6*), *Sucrose synthase 3* (*SUS3*), and *Sucrose-6F-phosphate phosphohydrolase family* (Best *Arabidopsis thaliana* gene match: AT2G35840) were 7.50-, 1.98-, 25.77-, and 11.94-

fold higher, respectively, at 48 h than that at 0 h. In summary, accumulation of ABA and ROS occurred after 48 h of drought treatment, followed by an increase in RFOs that led to ROS scavenging and the protection of the cell membrane. Thus, an increase in ABA promoted drought resistance in the plant.

The major response mechanisms in response to drought stress in *B. rapa* can be summarized as follows: after 0.5 h of drought stress, vitamin A is produced as a result of the inactivation of photosynthesis. This is followed by the activation of glycoside biosynthesis, and a negative effect on growth and photosynthesis rate at 3 h. After 12 h of drought stress, there are changes in the global RNA tertiary structure caused by changes in the concentration of ions and osmolytes. After 24 h of drought stress, there is an activation of ROS scavenging by RFOs to provide drought resistance. If drought stress continues for 48 h, the concentration of ABA and ROS increases, plant growth is inhibited, female organ development is suppressed, transition to reproductive phase is affected, and yield is negatively affected. However, ABA can provide long-term drought resistance through the synthesis of RFOs (Fig. 3).

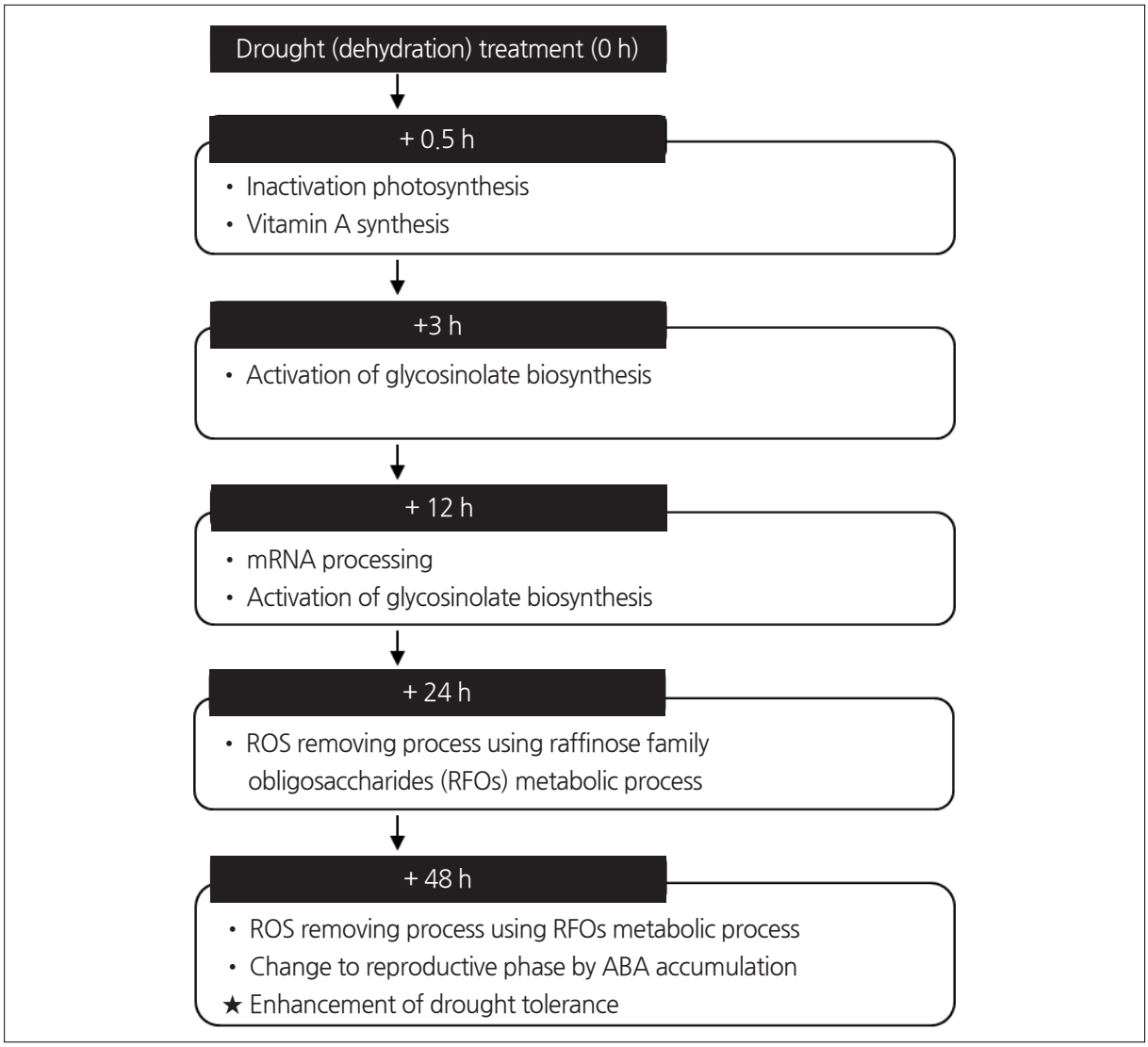

Fig. 3. A hypothetical model depicting drought stress-related gene expression at 0.5, 3, 12, 24, and 48 h after dehydration treatment in Chinese cabbage.

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