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Chlorosis of Ogura-CMS *Brassica rapa* is due to down-regulation of genes for chloroplast proteins

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**Abstract**
Cytoplasmic male sterility (CMS) is a maternally inherited trait leading to loss of the ability to produce fertile pollen and is extensively used in hybrid crop breeding. Ogura-CMS was originally generated by insertion of *orf138* upstream of *atp8* in the radish mitochondrial genome and transferred to *Brassica* crops for hybrid breeding. Gene expression changes by dysfunctional mitochondria in Ogura-CMS result in pollen developmental defects, but little is known about gene expression patterns in vegetative tissue. To examine the interaction between nuclear and organelar regulation of gene expression, microarray and subsequent gene expression experiments were conducted with leaves of *F*$_1$ hybrid Chinese cabbage derived from self-incompatible (SI) or Ogura-CMS parents (*Brassica rapa* ssp. *pekinensis*). Out of 24,000 genes deposited on a KBGP24K microarray, 66 genes were up-regulated and 26 genes were down-regulated by over 2.5 fold in the CMS leaves. Up-regulated genes included stress-response genes and mitochondrial protein genes, while genes for ascorbic acid biosynthesis and thylakoid proteins were down-regulated. Most of the major component genes for light reactions of photosynthesis were highly expressed in leaves of both SI and CMS plants, but most of the corresponding proteins were found to be greatly reduced in leaves of CMS plants, indicating posttranscriptional regulation. Reduction in thylakoid proteins and chlorophylls led to reduction in photosynthetic efficiency and chlorosis of Ogura-CMS at low temperatures. This research provides a foundation for studying chloroplast function regulated by mitochondrial signal and for using organelle genome introgression in molecular breeding.

**Keywords**
Ogura-CMS, microarray, chlorosis, Chinese cabbage, photosynthesis

**Introduction**
Cytoplasmic male sterility (CMS) is a maternally-inherited trait that produces either aborted or infertile pollen grains. CMS is a consequence of miscoordination between nuclear and cytoplasmic gene products from different origins (Aviv and Galun 1980). These changes are usually caused by mutations, rearrangements, and/or recombinations in the mitochondrial genome, but not by nuclear gene mutations (Carlsson and Glimelius 2011). At least 14 mitochondrial genes that induce CMS have been characterized in plants (Chase 2007; Kojima et al. 2010). CMS is a valuable tool for commercial production of hybrid seeds in crops (Pelletier and Budar 2007), and is an excellent subject for the study of anterograde and retrograde signaling (Fujii and Toriyama 2008).

Ogura-CMS, originally identified in wild radish (*Raphanus sativus*) (Ogura, 1968), is controlled by a mitochondrial *orf138* locus that consists of two co-transcribed open reading frames: *orf138* and *orfB* (also called *atp8*, encoding ATP synthase subunit 8) (Bonhomme et al. 1991; Bonhomme et al. 1992; Krishnasamy and Makaroff 1993; Grelon et al. 1994). *Brassica napus* that contains Ogura-type CMS was originally produced by protoplast fusion (Pelletier et al. 1983) and transferred to Chinese cabbage in the 1980s (Yamagishi and Bhat 2014). Its first *F*$_1$ hybrid seeds were produced from the CMS lines (Ke et al. 1992); however, these seeds have not been widely used because *F*$_1$ plants showed a negative effect, chlorosis at low temperature (LT), instead of heterosis. To eliminate these undesirable effects, *B. rapa* breeders produced new hybrids by protoplast fusion and repeated backcrossing successful in *B. napus* and *B. juncea* (Yamagishi and Bhat 2014).
To understand mechanisms of Ogura-CMS in *B. rapa*, omics approaches have been recently conducted. Using *B. rapa* 300K microarray, Dong et al. (2013) analyzed genes specific for pollen development stage and concluded that the retrograde signal from Ogura-CMS mitochondria delays expression of large number of nuclear genes involved in pollen development. Wei et al. (2015) identified important miRNAs and their target genes in Ogura-CMS Chinese cabbage using several omics data. However, these two researches have focused on pollen development in floral buds and no omics approaches have been applied to dissect gene expression profiles in vegetative tissues, such as leaf of Ogura-CMS Chinese cabbage.

Mitochondrial influence on the nuclear gene expression is referred to as mitochondrial retrograde regulation (MRR) and it occurs in CMS lines via CMS-inducing genes (Carlsson and Glimelius 2011), making CMS a useful system to study MRR (Chase 2007). Chloroplastic retrograde signaling changes both nuclear (Fernández and Strand 2008; Liao et al. 2016; Woodson 2016) and mitochondrial gene expression (Liao et al. 2016). However, little is known about the regulation of chloroplast genes and nuclear genes for chloroplast proteins by mitochondrial retrograde signaling. Especially, the role of retrograde pathway specific for CMS has never been described for plant vegetative tissues.

Chinese cabbage (*B. rapa* ssp. *pekinesis*) is one of the most important leafy vegetables in Asia and exhibits strong heterosis. Application of Ogura-CMS to produce *F*$_1$ seeds in Chinese cabbage has a high economic potential in seed industry, once accompanying problems like chlorosis have been resolved. To understand chlorosis development in Ogura-CMS Chinese cabbage under LT, we have examined gene expression profiles using KBGP24K microarray and compared chloroplast gene expression and photosynthetic activity. We concluded that chloroplast function was greatly inhibited in Ogura-CMS leaves due to the reduction of chloroplast gene expression by dysfunctional mitochondria.

**Materials and Methods**

**Plant materials**

Chinese cabbages (*B. rapa* ssp. *pekinesis*) were *F*$_1$ hybrids obtained using either SI or Ogura-CMS (CMS) in BioBreeding Institute, Korea. Seeds were sown in pots on Aug. 10 and 3 week seedlings were transplanted to bigger pots and field. At 10-leaf stage (Fig. 1A) before the exposure to low temperature (LT) (mid-September), 7$^{th}$ to 9$^{th}$ leaves were sampled from three individual plants and frozen in liquid nitrogen until use. Leaves from same developmental and environmental conditions were used for measurement of photosynthesis and western blot analysis.

**KBGP-24K microarray (Version 1)**

Using approximately 24,000 unigenes derived from EST analysis, oligomeric microarray was designed with 12 probes (six sense and six antisense) per gene (Lee et al. 2008). A set of 180,156 probes were designed, and duplicated in two separated block on a single chip. The 60-nucleotide probes with Tm values of 75 to 85°C were synthesized on the slide using NimbleGen System (http://www.nimblegen.com/). Random GC probes to monitor the hybridization efficiency and four corners to overlay the grid on the image were included.

Two biological replicates of total RNA were prepared from each plant sample and 10 μg of total RNA were used for cDNA synthesis with Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen, USA). Subsequent procedures for chip assay were followed as described (Lee et al. 2008). After normalization of probe intensity (Cy3 intensity), perfect match (PM) values of the six probes were used for selection of responsive genes. After removing genes with less than 1,000 PM value at all time points, genes specifically expressed or up-regulated in either tissue were selected and analyzed.
Table 1 List of polyclonal antibodies used in this study. All antibodies were purchased from Agrisera Co. Ltd (Vännäs, Sweden)

| Gene location | Antibody name | Protein name | Classification |
|---------------|---------------|--------------|----------------|
| Plastid       | PsbA          | Photosystem II protein D1 | PSI           |
|               | Cytf          | Cytochrome f protein (PetA) of thylakoid Cytb6/f-complex | Electron transport |
|               | PsaA          | Photosystem I P700 chlorophyll a apoprotein A1 | PSI           |
|               | PsaC          | Photosystem I iron-sulfur center | PSI           |
| Nucleus       | LhcB1         | LHCII chlorophyll a/b binding protein 1-(1-5) | LHCII         |
|               | LhcB2         | LHCII type II chlorophyll a/b-binding protein | LHCII         |
|               | LhcA1         | PSI type I chlorophyll a/b-binding protein | LHCI          |
|               | LhcA2         | PSI type II chlorophyll a/b-binding protein | LHCI          |

Determination of chlorophyll fluorescence parameters

Changes in in vivo chlorophyll fluorescence were monitored through Xe-pulse amplitude modulated fluorometry (Walz, Germany) using cabbage leaf disc that were dark-adapted for 20 min before measurement. The $F_v/F_m$ value, which is an indicator for maximum PS II efficiency, was calculated as $(F_m - F_o)/F_m$, where $F_v$ is the dark-adapted variable fluorescence, $F_m$ is the maximum fluorescence and $F_o$ is the dark-adapted fluorescence. The actual quantum yield of PSII photochemistry in light-adapted cabbage leaf was calculated as $1 - F/F_m'$, where $F$ is steady-state fluorescence and $F_m'$ is maximal fluorescence under illumination. Fluorescence quenching parameters were determined by qP, the coefficient of photochemical quenching, as defined by Schreiber et al. (1994), and NPQ (non-photochemical quenching: $(F_m/F_m' - 1)$ during illumination at 800 $\mu$mol photons m$^{-2}$s$^{-1}$).

Determination of photosynthetic O$_2$ evolution

Light-response curves of photosynthetic O$_2$ evolution during illumination were determined with a leaf-disc O$_2$ electrode (Oxygraph system, Hansatech, UK) in air with 5% CO$_2$ at 25°C. Various irradiances (50 and 800 $\mu$mol photons m$^{-2}$s$^{-1}$) were provided using neutral density filters. The temperature was kept constant at 25°C. The Chlorophyll content in leaf segments was determined from aqueous buffered 80% acetone extracts (25 mM Heps, pH 7.5), as in Porra et al. (1989).

Analysis of proteins related to photosynthesis

Thylakoid protein components were measured immunochemically after isolation of the thylakoid membranes. Intact chloroplasts were isolated from leaves by homogenization (Robinson and Barnett 1988). Thylakoid membranes were resuspended in 10 mM Tricine-NaOH (pH 7.0), 300 mM sucrose, and 5 mM MgCl$_2$. For protein gel blots, the membrane proteins were solubilized in 60 mM Tris-HCl (pH 7.8), 12% (w/v) sucrose, 2% (w/v) SDS, 1 mM EDTA, and 58 mM DTT. Protein gel electrophoresis was performed according to Laemmli (1970). The separated proteins were electrophoretically transferred to Immobilon-P membrane (Millipore). Chemiluminescence detection using antibodies was performed according to the manufacturer’s instructions (Amersham Pharmacia Biotech.: ECL + Plus). Polyclonal antibodies raised against specific photosynthetic components were purchased from Agrisera Co (Vännäs, Sweden) (Table 1). The soluble protein contents were measured using BioRad protein assay reagents according to the manufacturer’s instructions.

Results

Morphology of Chinese cabbage F$_1$ hybrids derived from SI or Ogura-CMS

Since cultivated Chinese cabbage varieties are F$_1$ hybrids, we have focused on leaves of F$_1$ hybrids derived from SI and Ogura-CMS. As shown in Figure 1, CMS Chinese cabbage exhibited slight pale green before the exposure to LT for long period of time (the mid-September in Daejeon, Korea), but severe chlorosis in young developing leaves after the exposure to LT (the mid-October). These phenomena appear to be similar to that of previous work (Pelletier et al. 1983) and imply defective in photosynthetic efficiency or assembly of photosynthetic electron transport. All experiments were performed with slight pale green leaves (minor chlorosis).
| Br_SEQ_ID | At_Locus | Gene Description | Fold Change (CMS/SI) |
|-----------|----------|------------------|---------------------|
| BRAS0001S00026533 AT2G01520 | MLP328 (MLP-like protein 328) | 21.04 |
| BRAS0001S00022245 No_Hit | A09 sequence (3'UTR) | 13.67 |
| BRAS0001S00003192 AT3G08610 | Unknown (mitochondrial respiratory chain complex I) | 13.56 |
| BRAS0001S00008086 AT4G24420 | RNA-binding (RRM/RBD/RNP motifs) family protein | 9.38 |
| BRAS0001S00010278 AT2G07707 | Plant mitochondrial ATPase, F0 complex, subunit 8 protein | 7.36 |
| BRAS0001S00022734 No_Hit | Bra002978: Brassicarapa putative beta-glucosidase 41 (LOC103844910) | 7.09 |
| BRAS0001S00024268 AT5G56010 | Bra035593: HSP90.3 (heat shock protein 81-3) | 6.44 |
| BRAS0001S00015630 AT4G24450 | GWD2; PWD (phosphoglucan, water dikinase) (involved in phosphorylation) | 6.29 |
| BRAS0001S00017181 AT3G48000 | Aldehyde dehydrogenase 2 | 5.69 |
| BRAS0001S00017773 AT2G07708 | Unknown protein (mitochondrion) | 5.40 |
| BRAS0001S00004814 AT1G70850 | MLP-LIKE PROTEIN 34 (MLP34) | 5.02 |
| BRAS0001S00002171 AT2G29460 | GST4 (Glutathione S-transferase 22) | 4.90 |
| BRAS0001S00016599 AT2G25140 | CLPB-M (Casein lytic proteinase B4)/HSP98.7 | 4.90 |
| BRAS0001S00017434 AT1G66130 | NAD(P)-binding Rossmann-fold superfamily protein | 4.64 |
| BRAS0001S00018422 AT3G49620 | DIN11 (Dark inducible 11) | 4.54 |
| BRAS0001S00022560 No_Hit | Bra030240 (no_hit_found) | 4.51 |
| BRAS0001S00019384 AT4G11890 | ARCK1 (ABA- AND OSMOTIC-STRESS-INDUCIBLE RECEPTOR-LIKE CYTOSOLIC KINASE1) | 4.43 |
| BRAS0001S00023080 No_Hit | Bra015764 (Brassicarapa nucleolin2-like:LOC103832086) | 4.41 |
| BRAS0001S00021743 AT3G12580 | HSP70 | 4.20 |
| BRAS0001S00006263 AT5G56010 | HSP90.3/HSP81-3 | 4.16 |
| BRAS0001S00006395 AT2G40280 | S-adenosyl-L-methionine-dependent methyltransferases superfamily protein | 4.04 |
| BRAS0001S00006011 AT3G09350 | FES1A (Encodes one of the Arabidopsis orthologs of HspBP-1 and yeast Fes1p:Fes1A) | 4.01 |
| BRAS0001S00014904 AT2G24150 | SQE5/SQP1 (SQUALENE MONOOXYGENASE 5) | 3.91 |
| BRAS0001S00004940 AT2G46650 | CB5-C/CYTB5C (CYTOCHROME B5 ISOFORM C) | 3.73 |
| BRAS0001S00010715 AT3G12580 | HSP70 | 3.70 |
| BRAS0001S00003420 AT3G56060 | Glucose-methanol-choline (GMC) oxidoreductase family protein | 3.66 |
| BRAS0001S00019692 AT2G18860 | Bra038827; Syntaxin/t-SNARE family protein | 3.64 |
| BRAS0001S00019491 AT3G15210 | EFR4 (ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 4) | 3.61 |
| BRAS0001S00019052 AT3G12580 | HSP70 | 3.60 |
| BRAS0001S00003941 AT4G35160 | ASMT (N-ACETYLSEROTONIN O-METHYLTRANSFERASE) | 3.52 |
| BRAS0001S00019993 AT4G19840 | PP2-A1 (PHLOEM PROTEIN 2-A1) | 3.52 |
| BRAS0001S00018407 AT4G19645 | TRAM, LAG1 and CLN8 (TLC) lipid-sensing domain containing protein | 3.52 |
| BRAS0001S00017767 AT5G65070 | Agamous-like 69 (AGL69, FCL4, MAF4) | 3.48 |
| BRAS0001S00013824 AT3G56010 | Hsp90.3 (Hsp81.3) | 3.46 |
| BRAS0001S00029134 AT5G60200 | Dof-type transcription factor (DOF5.3) | 3.40 |
| BRAS0001S00017206 AT3G06880 | Transducin/WD40 repeat-like superfamily protein | 3.37 |
| BRAS0001S00006213 AT5G40240 | Nodulin Mn21-like transporter family protein | 3.17 |
| BRAS0001S00026154 AT4G01995 | Unknown | 3.16 |
| BRAS0001S00029341 AT4G17910 | Acyl transferase | 3.06 |
| BRAS0001S00018291 AT4G36350 | SAS10/C1D family protein (Embryodfective 2777) | 3.04 |
| BRAS0001S00016302 AT1G02820 | Late embryogenesis abundant 3 (LEA3) | 3.02 |
| BRAS0001S00028858 AT5G64040 | Encodes the only subunit of photosystem 1 located entirely in the thylakoid lumen | 2.99 |
| BRAS0001S00023256 AT3G22380 | Time for Coffee | 2.98 |
| BRAS0001S00025328 AT5G6230 | ARM repeat superfamily protein | 2.92 |
| BRAS0001S0001224 AT1G23260 | MMZ1/UEV1A (DNA damage response) | 2.88 |
| BRAS0001S00019193 AT5G02490 | HSP70-2 | 2.87 |
| BRAS0001S0005028 AT1G49600 | RNA-binding protein 47A (RBP47A) | 2.82 |
| BRAS0001S0010541 AT1G70830 | MLP-like protein 28 (MLP28) | 2.79 |
| BRAS0001S0001764 AT2G28000 | Chaperonin-60 alpha | 2.77 |
Table 2 Continued

| Br_SEQ_ID       | At_Locus     | Gene Description                              | Fold Change (CMS/SI) |
|-----------------|--------------|-----------------------------------------------|----------------------|
| BRAS0001S00013300 | AT1G07790    | Histone 2B (HTB21)                           | 2.75                 |
| BRAS0001S00015262 | AT5G59480    | Haloacid dehalogenase-like hydrolase (HAD) superfamily protein | 2.72                 |
| BRAS0001S00010375 | AT5G09590    | Heat shock protein 70 (Hsc70-5)              | 2.71                 |
| BRAS0001S00014652 | AT1G76860    | Small nuclear ribonucleoprotein family protein (LSM3B) | 2.66                 |
| BRAS0001S0006795 | AT1G75750    | GASAI                                         | 2.65                 |
| BRAS0001S00011885 | AT3G61620    | Exonuclease RRP41                            | 2.65                 |
| BRAS0001S00011316 | AT5G40160    | Ankryin repeat protein EMB506               | 2.63                 |
| BRAS0001S0008586 | AT1G06720    | P-loop containing nucleoside triphosphate hydrolases superfamily protein | 2.63                 |
| BRAS0001S00018549 | AT3G04870    | PIGMENT DEFECTIVE EMBRYO 181                 | 2.61                 |
| BRAS0001S0004433 | AT3G19170    | Presencequence protease 1                    | 2.57                 |
| BRAS0001S0002521 | AT2G37990    | Ribosome biogenesis regulatory protein (RRS1) family protein | 2.56                 |
| BRAS0001S0009108 | AT3G29200    | Chorismate mutase 1, chloroplast (CM1)        | 2.56                 |
| BRAS0001S00017553 | AT4G31210    | DNA topoisomerase family protein              | 2.56                 |
| BRAS0001S00011599 | AT4G23760    | COX19-like CHCG family protein               | 2.51                 |
| BRAS0001S00017175 | AT1G19730    | Thioredoxin-type r (TRX4)                    | 2.50                 |
| BRAS0001S00003312 | AT3G48000    | Aldehyde dehydrogenase 2                     | 2.50                 |

Table 3 Genes down-regulated in CMS over 2.5 fold

| Br_SEQ_ID       | At_Locus     | Gene Description                              | Fold Change (CMS/SI) |
|-----------------|--------------|-----------------------------------------------|----------------------|
| BRAS0001S00017904 | AT2G45790    | Cytoplasmic phosphomannomutase (ascorbate biosynthesis) | -4.97                |
| BRAS0001S00017830 | ATCG00520    | YCF4 (Encodes a protein required for photosystem I assembly and stability) | -4.53                |
| BRAS0001S00010846 | AT3G14210    | EPITHIOSPECIFIER MODIFIER 1, ESM1             | -4.51                |
| BRAS0001S00000039 | AT3G27690    | LHC2 (LIGHT-HARVESTING CHLOROPHYLL B-BINDING 2) | -4.13                |
| BRAS0001S00013286 | AT3G14210    | ESM1 (epithiospecifier modifier 1)           | -4.06                |
| BRAS0001S00000044 | AT5G48850    | SDH1 (sulphur deficiency-induced 1)          | -3.30                |
| BRAS0001S00009785 | AT1G52190    | NPF1.1 (nitrate transporter 1.1)             | -3.29                |
| BRAS0001S00024200 | No_Hit       | Unknown                                       | -3.26                |
| BRAS0001S00005206 | AT1G75900    | Unknown                                       | -3.25                |
| BRAS0001S0001705 | AT1G15860    | Unknown                                       | -3.19                |
| BRAS0001S00022937 | AT1G74670    | GASA6 (GA-stimulated arabidopsis 6)          | -2.94                |
| BRAS0001S00018403 | AT1G25440    | BBX15 (B-box type zinc finger protein with CCT domain) | -2.89                |
| BRAS0001S00002525 | AT5G02580    | Unknown                                       | -2.81                |
| BRAS0001S00006406 | AT2G44080    | ARL (ARGOS-LIKE)                             | -2.80                |
| BRAS0001S00000344 | AT2G45960    | Aquaporin                                     | -2.79                |
| BRAS0001S00003340 | AT1G65310    | XTH17 (Xyloglucan-endotransglycosylase/hydrolase 17) | -2.77                |
| BRAS0001S00006587 | AT5G37300    | WSD1 (wax ester synthase(WS) and diacylglycerol acyltransferase (DGAT) | -2.72                |
| BRAS0001S00003214 | ATCG00530    | AYCF16                                        | -2.67                |
| BRAS0001S00017873 | AT3G20370    | TRAF-like protein                             | -2.56                |
| BRAS0001S00019530 | AT2G34620    | Mitochondrial transcription termination factor family protein | -2.55                |
| BRAS0001S00000993 | AT5G14030    | Transien-associated protein beta (TRAPB) family protein | -2.54                |
| BRAS0001S00010320 | AT4G03560    | CCH1 (Calcium channel 1)                     | -2.53                |
| BRAS0001S00008089 | AT5G19530    | ACL5 (Acalulis 5)                            | -2.53                |
| BRAS0001S00021018 | AT1G02335    | GERMIN-LIKE PROTEIN SUBFAMILY 2              | -2.53                |
| BRAS0001S0004281 | AT5G57800    | CER3 (Ecriferum 3)(similar to sterol desaturase family) | -2.51                |
| BRAS0001S00019903 | AT1G28290    | AGP31 (Arabinogalactan protein 31)(vascular tissue function) | -2.50                |

Analysis of differentially expressed genes (DEGs)

To identify DEGs in leaves of Ogura-CMS Chinese cabbage, transcriptomics experiment was carried out with KBGP24 oligomeric chips (Supplementary Table 1). Out of 24,000 genes, 66 genes and 26 genes were up-regulated and down-regulated over 2.5 fold in the CMS, respectively (Table 2 and 3). Many up-regulated genes, such as HSP70s and
HSP90s, are stress-related genes. Interestingly, genes encoding mitochondrial components were also up-regulated in CMS: mitochondrial respiratory chain complex I (BRAS0001S00003192), mitochondrial ATPase subunit 8 (BRAS0001S000010278) (Table 2). The highest up-regulated gene was BRAS0001S00002653, which is related to a cis-cinnamic acid responsive gene (AT2G01520) in Arabidopsis thaliana. AT2G01520 is a member of the major latex protein-like gene family, and plays a role in promoting vegetative growth or delaying flowering. On the other hand, down-regulated genes in CMS included a cytoplasmic phosphomannomutase-like gene (BRAS0001S00017904) and putative components for photosynthesis light reaction, such as YCF4 (BRAS0001S00017830) and LHC2 (BRAS0001S00000039) (Table 3). These results suggest that protection for photosystems and light reaction efficiency could be greatly reduced in CMS Chinese cabbage. One more interesting finding was EPI-THIOSPECIFIER MODIFIER1 (ESM10) genes (BRAS0001S00010846 and BRAS0001S00013286) were highly down-regulated in CMS, altering glucosinolate hydrolysis and increasing insect feeding (Zhang et al. 2006).

Expression of photosynthesis-related genes

Expression of photosynthesis-related genes (encoding proteins for photosystem, electron transport and CO2 fixation) was strongly expressed in general, but there was no significant difference detected between SI and CMS Chinese cabbage at the transcript level (Table 4). Among LIGHT HARVESTING COMPLEX B (LHCB) 2.4 (= CYCLIN-DEPENDENT KINASE E1; CDKE1) paralogs, only BRAS0001S00000039 was highly up-regulated in SI compared to that of CMS.

To answer whether mitochondrial signal in Ogura-CMS affects expression of chloroplast and nucleus-encoded thylakoid proteins, expression levels of 8 proteins listed in Table 1 were examined by western blot analysis (Fig. 2). Except PsaA and PsaC, expressions of all other proteins showed a great reduction in CMS, suggesting that expression of these genes are regulated at the post-transcriptional levels. This result also revealed that mitochondria in Ogura-CMS affect plastid gene expression, along with nuclear gene expression.

Photosynthesis efficiency and chlorophyll content

Since protein levels associated with light reaction of photosynthesis were greatly reduced in CMS leaves (Fig. 2), we suspected that the pigment contents for photosynthetic reaction center would also be low in CMS-leaves. As shown in Figure 3, both chlorophyll a and b levels were low in CMS-leaves, possibly related to the observation that Ogura-CMS Chinese cabbage develops chlorosis in LT (Fig. 1). Reduction in thylakoid proteins and chlorophyll a/b has caused a lower photosynthetic efficiency in CMS-leaves (Fig. 4). It was found that both chlorophyll fluorescence parameter and O2 evolution were low in CMS-leaves. Yield expressed as electron flux through PSII was also low in CMS-leaves (Fig. 4A) and these results were consistent with the rate of oxygen evolution under the high light (Fig. 4B). The higher excitation pressure combined with lower non-photochemical quenching detected in CMS-leaves may be responsible for less resistance to high light in certain stress conditions, such as LT.

Discussion

CMS is important for hybrid breeding of crop plants and Ogura-CMS from wild radish can be an option for Chinese cabbage, which is an important leafy vegetable in Korea. However, F1 hybrids B. rapa derived from Ogura-CMS could not be widely used because F1 plants did not show
Table 4: Expression of photosynthesis-related genes from SI and CMS F1 Brassica rapa

| Classification | At_Locus | Gene Annotation | Br/SEQ_ID | Probe Intensity | SI/CMS | CMS/SI |
|----------------|----------|-----------------|-----------|----------------|--------|--------|
| Photosystem    |          |                 |           | SI/CMs         |        |        |
|                |          |                 |           | CMS/CMs        |        |        |
|                |          |                 |           | CMS/SI         |        |        |
| Electron       |          |                 |           |                |        |        |
| Transport      |          |                 |           |                |        |        |

**Light Harvesting Complex B (LHCB) 2**: ORF of AtLHCb2.

**Cyclin-dependent kinase E1 (CDKE1)**: ORF of AtCDKE1.

**Photosystem II chlorophyll-binding protein PsbS**: ORF of AtPsbS.

**Photosystem II chlorophyll-binding protein PsbS**: ORF of AtPsbS.

**Chlorophyll a/b-binding protein CP26 in PS II**: ORF of AtCP26.

**Chlorophyll a/b-binding protein CP26 in PS II**: ORF of AtCP26.

**Putative cytochrome b561**: ORF of AtCytochrome b561.
heterosis but instead developed severe chlorosis under low temperature (Ke et al. 1992). This undesirable effect is due to the incompatibility between chloroplast and nucleus, and the problem could be overcome by chloroplast substitution, which involved somatic hybridization and repeated backcrossing to cabbage (Dey et al. 2013). With similar approaches being tried, more detailed understanding of the mechanism by which leaf chlorosis is induced can accelerate breeding efforts for Chinese cabbage.

From transcriptome analysis, it was found that DEGs in Ogura-CMS leaves (Table 2 and 3) are less obvious in gene numbers and fold changes in expression compared to those observed with male gametophyte (Dong et al. 2013; Wei et al. 2015). At the transcript level, most genes involved in photosynthesis were highly expressed in leaves of both SI- and CMS-derived F1 hybrid (Table 4 and Supplementary Table 1). However, accumulation of the proteins showed clear difference between two genotypes (Fig. 2), implying that expression of these genes are regulated at the post-transcriptional level. With reduced levels of thylakoid components, the amount of photosynthetic pigments and photosynthesis efficiency were also decreased (Fig. 3 and 4). In addition, heat stress-related protein genes were highly up-regulated in CMS-leaves (Table 2), suggesting the CMS mimics the effects of oxidative stress conditions. Particularly, down-regulation of phosphomannomutase gene in Ogura-CMS leaves implied that the protective capability of photosystem under oxidative stress is decreased. This gene is involved in ascorbate biosynthesis, which is related to high temperature tolerance (Hoeberichts et al. 2008). Ogura-CMS chloroplasts appear to be impaired in removal of excess energy absorbed by photosystems under high light (Fig. 4A), leading to the loss of chlorophyll pigments (Fig. 4B).

Both mitochondria and chloroplasts are important to maintain metabolic and energy homeostasis in the plant cell. Therefore, extensive researches on the interaction between

| Classification | At_Locus | Gene Annotation | BSEQ_ID | Probe Intensity | Fold Change |
|----------------|----------|-----------------|---------|----------------|-------------|
|                |          |                 |         | SI             | CMS         |
| CO2 Fixation   | AT1G20620| Ribulose bisphosphate carboxylase/oxygenase small subunit | BRAS0001S00019581 | 64284 | 63188 | 1.0 | 1.0 |
|                | AT5G38420| Ribulose bisphosphate carboxylase/oxygenase small subunit | BRAS0001S00013358 | 59841 | 60723 | 1.0 | 1.0 |
|                | AT4G29350| Ribulose bisphosphate carboxylase/oxygenase small subunit | BRAS0001S00013535 | 59037 | 61972 | 1.0 | 1.0 |
|                | AT1G08380| Ribulose bisphosphate carboxylase | BRAS0001S00000087 | 1152 | 1373 | 0.8 | 1.2 |
|                | AT1G07920| Ribulose bisphosphate carboxylase/oxygenase small subunit | BRAS0001S00000188 | 57299 | 60560 | 0.9 | 1.1 |

**Fig. 4** Photosynthetic efficiencies of SI- and CMS-derived F1 hybrids: Chlorophyll fluorescence parameters (A) and O₂ evolution (B). A, Electron flux through PSII (Yield), excitation pressure (1-qP), and non-photochemical quenching parameter (NPQ) in Chinese cabbage leaves under irradiance of 800 μmol photons m⁻² s⁻¹. B, Light-response curves of photosynthetic O₂ evolution in Chinese cabbage leaves.
Table 5 Expression of key retrograde signaling genes for chloroplast and mitochondria

| *At* Locus | Gene Annotation | *Br*-SEQ_ID | PI (Probe intensity) |
|-----------|-----------------|-------------|---------------------|
| AT3G27690 | LIGHT HARVESTING COMPLEX B (LHCB) 2.4 | BRAS0001S00000036 | 57,498 (SI) 48,062 (CMS) |
|           |                  | BRAS0001S00013452 | 49,690 (SI) 35,900 (CMS) |
|           |                  | BRAS0001S00000079 | 49,312 (SI) 41,730 (CMS) |
|           |                  | BRAS0001S00023696 | 34,142 (SI) 21,332 (CMS) |
|           |                  | BRAS0001S00000039 | 4,343 (SI) 1,051 (CMS) |
|           |                  | BRAS0001S00013388 | 1,329 (SI) 1,195 (CMS) |
| AT3G22370 | ALTERNATIVE OXIDASE1a | BRAS0001S00000062 | 1,378 (SI) 1,372 (CMS) |
|           |                  | BRAS0001S00000137 | 1,364 (SI) 1,375 (CMS) |
| AT5G63610 | CYCLIN-DEPENDENT KINASE E1 (CDKE1) | BRAS0001S00027374 | 3,788 (SI) 3,714 (CMS) |
|           |                  | BRAS0001S00004511 | 858 (SI) 831 (CMS) |
| AT2G40220 | ABSCISIC ACID INSENSITIVE 4 (ABI4) | . | . |

these organelles have been carried out with respect to photosynthesis and respiration at physiological levels. But, only one paper (Liao et al. 2016) has mentioned that dysfunctional chloroplast is related to the up-regulation of mitochondrial gene expression in *Arabidopsis*. Our results is the first report for the chloroplast gene expression change by dysfunctional mitochondria, showing accumulation of chloroplast proteins can be regulated by mitochondrial signal.

Plant mutations responsible for mitochondrial dysfunction result in change of nuclear gene expression (Newton et al. 2004; Dong et al. 2013), and several candidate signals have suggested: redox sensors and signals, kinases/phosphatases, hormones, and other sensors (Rhoads 2011). Recently, key genes for retrograde signaling for chloroplast and mitochondria have been identified (Giraud et al. 2009; Blanco et al. 2014; Saha et al. 2016). The expression of these four key genes in our experiments using Chinese cabbage was not similar to *Arabidopsis* data (Table 5). Only one paralog (*BRAS0001S00000039* corresponding to *Arabidopsis* *AT3G27690* (LHCB)) was differentially expressed between SI- and CMS leaves. These results may suggest that retrograde signaling or organelle interaction is regulated at the protein level or different signaling components unique to species are used. Considering that finding of the best combination between nucleus and organelles is prerequisite for CMS-based breeding, molecular mechanisms associated with CMS need to be further elucidated.

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