J Plant Biotechnol (2017) 44:115–124
DOI:https://doi.org/10.5010/JPB.2017.44.2.115

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

Research Article

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

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Received: 13 June 2017 / Revised: 19 June 2017 / Accepted: 20 June 2017

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 organellar 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.

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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. *pekinensis*) 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. *pekinensis*) 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) (the 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.

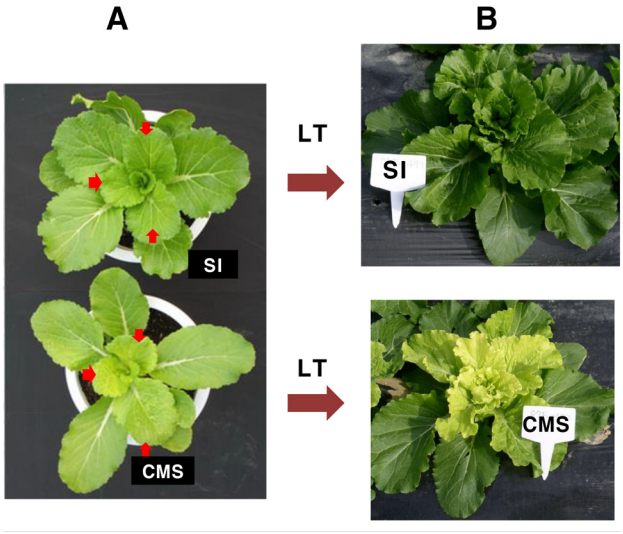

Fig. 1 Morphology of Chinese cabbage F_1 hybrids derived from SI or Ogura-CMS. A, Plants before low-temperature (LT) exposure (mid-September); CMS shows light chlorosis on 7^{th} to 9^{th} leaf, which are indicated by red arrows. B, Plants after LT exposure (mid-October); CMS shows severe chlorosis in young developing leaves

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	PSII
	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 $(F_m-F_0)/F_m$, where F_v is the dark-adapted variable fluorescence, F_m is the maximum fluorescence and F_0 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: (Fm/Fm' − 1) during illumination at 800 μmol 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 μmol 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 Hepes, 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).

Table 2 Genes up-regulated in CMS by over 2.5-fold

*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
BRAS0001S00000806	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*putativebeta-glucosidase41(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	GSTU4 (Glutathione S-transferase 22)	4.90
BRAS0001S00016599	AT2G25140	CLPB-M (Casein lytic proteinase B4)/HSP98.7	4.90
BRAS0001S00017434	AT1G66130	NAD(P)-binding Rossamann-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
BRAS0001S00000623	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	AT5G24150	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	AT3G22200	GABA-T (GAMMA-AMINOBUTYRATE TRANSAMINASE)	3.61
BRAS0001S00000758	AT1G18540	Ribosomal protein L6 family protein	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	AT5G56010	Hsp90.3 (Hsp81.3)	3.46
BRAS0001S00029134	AT5G60200	Dof-type transcription factor (DOF5.3)	3.40
BRAS0001S00017206	AT3G06880	Transducin/WD40 repeat-like superfamily protein	3.27
BRAS0001S00006213	AT5G40240	Nodulin MtN21-like transporter family protein	3.17
BRAS0001S00026154	AT4G01995	Unknown	3.16
BRAS0001S00029341	AT4G17910	Acyl transferase	3.06
BRAS0001S00018291	AT2G43650	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 I located entirely in the thylakoid lumen	2.99
BRAS0001S00023256	AT3G22380	Time for Coffee	2.98
BRAS0001S00025328	AT5G36230	ARM repeat superfamily protein	2.92
BRAS0001S00001224	AT1G23260	MMZ1/UEV1A (DNA damage response)	2.88
BRAS0001S00001913	AT5G02490	HSP70-2	2.87
BRAS0001S00005028	AT1G49600	RNA-binding protein 47A (RBP47A)	2.82
BRAS0001S00010541	AT1G70830	MLP-like protein 28 (MLP28)	2.79
BRAS0001S00001764	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
BRAS0001S00006795	AT1G75750	GASA1	2.65
BRAS0001S00011885	AT3G61620	Exonuclease RRP41	2.65
BRAS0001S00011316	AT5G40160	Ankryin repeat protein EMB506	2.63
BRAS0001S00008586	AT1G06720	P-loop containing nucleoside triphosphate hydrolases superfamily protein	2.63
BRAS0001S00018549	AT3G04870	PIGMENT DEFECTIVE EMBRYO 181	2.61
BRAS0001S00004433	AT3G19170	Preseqeunce protease 1	2.57
BRAS0001S00002521	AT2G37990	Ribosome biogenesis regulatory protein (RRS1) family protein	2.56
BRAS0001S00009108	AT3G29200	Chorismate mutase 1, chloroplast (CM1)	2.56
BRAS0001S00017531	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	SDI1 (sulphur deficincy-induced 1)	-3.30
BRAS0001S00009785	AT1G52190	NPF1.1 (nitrate transporter 1.11)	-3.29
BRAS0001S00024200	No_Hit	Unknown	-3.26
BRAS0001S00005206	AT1G75900	Unknown	-3.25
BRAS0001S00001705	AT1G15860	Unknown	-3.19
BRAS0001S00022937	AT1G74670	GASA6 (GA-stimulated arabidopsis 6)	-2.94
BRAS0001S00018403	AT1G25440	BBX15 (B-box type zinc finger protein with CCT damain)	-2.89
BRAS0001S00002525	AT5G02580	Unknown	-2.81
BRAS0001S00006406	AT2G44080	ARL (ARGOS-LIKE)	-2.80
BRAS0001S00000344	AT2G45960	Aquaporin	-2.79
BRAS0001S00003430	AT1G65310	XTH17 (Xyloglucan-endotransglucosylase/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 tranascription termination factor family protein	-2.55
BRAS0001S00000993	AT5G14030	Transiocon-associated protein beta (TRAPB) family protein	-2.54
BRAS0001S00010320	AT4G03560	CCH1 (Calcium channel 1)	-2.53
BRAS0001S00008089	AT5G19530	ACL5 (Acaulis 5)	-2.53
BRAS0001S00021018	AT1G02335	GERMIN-LIKE PROTEIN SUBFAMILY 2	-2.53
BRAS0001S00004281	AT5G57800	CER3 (Eceriferum 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 *HSP70*s and

*HSP90*s, 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* (BRAS0001S00010278) (Table 2). The highest up-regulated gene was *BRAS0001S00026533*, 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 *EPITHIOSPECIFIER 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 CO_2 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

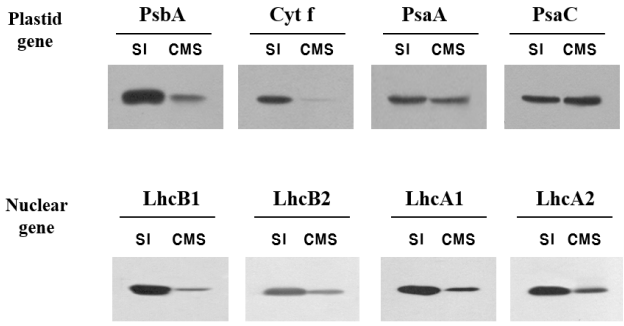

Fig. 2 Western blot analysis of thylakoid membrane components involved in the light reactions of photosynthesis

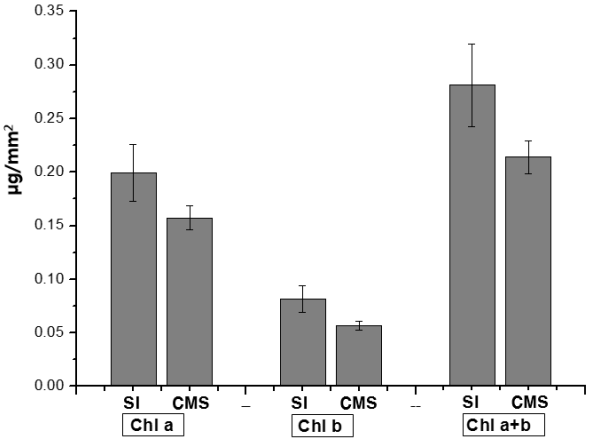

Fig. 3 Chlorophyll content of leaves from SI- and CMS-derived F_1 hybrids of Chinese cabbage

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 O_2 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, F_1 hybrids *B. rapa* derived from Ogura-CMS could not be widely used because F_1 plants did not show

Table 4 Expression of photosynthesis-related genes from SI and CMS F_1 *Brassica rapa*

Classification	*At*_Locus	Gene Annotation	*Br*SEQ_ID	Probe Intensity		Fold Change	
				SI	CMS	SI/CMS	CMS/SI
Photosystem	AT3G27690	LIGHT HARVESTING COMPLEX B (LHCB) 2.4 :CYCLIN-DEPENDENT KINASEE1(CDKE1)(LHCb2)	BRAS0001S00000039	4343	1051	4.1	0.2
			BRAS0001S00000036	57498	48062	1.2	0.8
			BRAS0001S00004511	858	831	1.0	1.0
			BRAS0001S00000149	58245	49668	1.2	0.9
			BRAS0001S00000425	50249	51813	1.0	1.0
			BRAS0001S00011198	45858	46917	1.0	1.0
			BRAS0001S00024982	45072	44977	1.0	1.0
	AT3G22370	LHCb6 protein	BRAS0001S00013783	37311	28983	1.3	0.8
			BRAS0001S00000558	33950	32016	1.1	0.9
	AT5G63610	LHCa2 protein 2	BRAS0001S00000423	33626	32622	1.0	1.0
			BRAS0001S00000424	23529	21477	1.1	0.9
	AT1G60950	Putative LHCa2 protein	BRAS0001S00010799	1602	1452	1.1	0.9
	AT4G30650	Chlorophyll a/b-binding protein CP26 in PS II	BRAS0001S00011564	55770	53536	1.0	1.0
	AT1G29910	Chlorophyll a/b binding protein	BRAS0001S00021229	55707	55473	1.0	1.0
	AT5G38420	Putative chlorophyll a/b-binding protein	BRAS0001S00000056	55233	52843	1.0	1.0
	ATCG00740	Chlorophyll a/b binding protein	BRAS0001S00000031	54957	55997	1.0	1.0
	AT3G63160	Photosystem II chlorophyll-binding protein PsbS	BRAS0001S00013419	54853	48328	1.1	0.9
	AT1G29930	Chlorophyll a/b binding protein	BRAS0001S00013518	50797	53893	0.9	1.1
	AT2G39730	Chlorophyll a/b-binding protein CP26 in PS II	BRAS0001S00013654	49470	43664	1.1	0.9
	AT1G44575	PSI type III chlorophyll a/b-binding protein	BRAS0001S00000421	49460	41222	1.2	0.8
	ATCG00530	Chlorophyll a/b binding protein	BRAS0001S00000217	45484	49544	0.9	1.1
	AT3G22120	Chlorophyll a/b-binding protein-like	BRAS0001S00000385	43766	33051	1.3	0.8
	ATCG01100	Chlorophyll A-B binding protein / LHCI type I (CAB)	BRAS0001S00011375	41492	31280	1.3	0.8
	AT1G29910	Putative chlorophyll a/b-binding protein	BRAS0001S00000686	41411	26674	1.6	0.6
	AT2G07727	LHCb3 chlorophyll a/b binding protein	BRAS0001S00013469	41059	32760	1.3	0.8
	AT4G29350	Chlorophyll a/b binding protein	BRAS0001S00013494	39981	40065	1.0	1.0
	AT5G54770	Chlorophyll a/b-binding protein-like	BRAS0001S00026135	38668	41453	0.9	1.1
	AT2G06520	Photosystem II chlorophyll-binding protein PsbS	BRAS0001S00000186	37576	47164	0.8	1.3
	AT3G04120	Chlorophyll a/b binding protein	BRAS0001S00000064	33412	32756	1.0	1.0
	AT2G15970	Chlorophyll a/b-binding protein CP29	BRAS0001S00000685	30857	28607	1.1	0.9
	AT1G54780	PSI type III chlorophyll a/b-binding protein	BRAS0001S00000422	25078	26156	1.0	1.0
	AT1G08380	Putative chlorophyll a/b binding protein	BRAS0001S00014191	19691	22435	0.9	1.1
	AT3G62030	Chlorophyll A-B binding family protein	BRAS0001S00015167	1523	2727	0.6	1.8
	AT1G55670	Chlorophyll a/b-binding protein	BRAS0001S00023979	794	723	1.1	0.9
	AT5G54160	Putative chlorophyll a/b binding protein	BRAS0001S00010384	376	612	0.6	1.6
	AT4G10340	Chlorophyll a/b-binding protein (cab-12)	BRAS0001S00000180	336	313	1.1	0.9
	AT1G61520	PSI type III chlorophyll a/b-binding protein	BRAS0001S00013679	289	298	1.0	1.0
Electron Transport	AT3G16670	Cytochrome b561	BRAS0001S00001778	27306	28235	1.0	1.0
	AT5G38420	Cytochrome b559	BRAS0001S00007312	11509	15551	0.7	1.4
	AT5G17870	Cytochrome b5	BRAS0001S00013366	10695	10876	1.0	1.0
	ATCG00480	Cytochrome b5	BRAS0001S00000053	5748	5853	1.0	1.0
	AT2G43560	Cytochrome b6	BRAS0001S00008540	4356	5801	0.8	1.3
	ATCG00630	Putative cytochrome b5	BRAS0001S00000068	4168	5772	0.7	1.4
	AT5G02380	Cytochrome b5	BRAS0001S00018351	4101	6001	0.7	1.5
	ATCG00140	Cytochrome b5	BRAS0001S00018217	3803	4762	0.8	1.3
	AT5G38410	Cytochrome b-561D	BRAS0001S00009511	2633	1904	1.4	0.7
	AT2G02100	Cytochrome b-561D	BRAS0001S00017536	2085	1718	1.2	0.8
	AT1G12090	Cytochrome b5 domain-containing protein	BRAS0001S00010516	1252	1514	0.8	1.2
	AT1G08380	Putative cytochrome b5	BRAS0001S00005558	844	665	1.3	0.8
	AT4G10340	Putative cytochrome b561	BRAS0001S00005335	725	631	1.1	0.9
	AT1G29910	Cytochrome b apoenzyme	BRAS0001S00022368	368	361	1.0	1.0
	AT3G56940	Putative cytochrome b5	BRAS0001S00011929	237	268	0.9	1.1
	AT5G18070	Plastocyanin-like domain-containing protein	BRAS0001S00019135	3842	3333	1.2	0.9
	AT5G24340	Cu^{2+}plastocyanin-likeprotein	BRAS0001S00010386	454	420	1.1	0.9

Table 4 Continued

Classification	*At*_Locus	Gene Annotation	*Br*SEQ_ID	Probe Intensity		Fold Change	
				SI	CMS	SI/CMS	CMS/SI
CO2 Fixation	AT1G20620	Ribulose bisphosphate carboxylase /oxygenase small subunit	BRAS0001S00019581	64284	63188	1.0	1.0
			BRAS0001S00019914	460	492	0.9	1.1
	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
			BRAS0001S00000087	1152	1373	0.8	1.2
	AT1G08380	Ribulose bisphosphate carboxylase	BRAS0001S00000188	57299	60560	0.9	1.1
	AT1G07920	Ribulose bisphosphate carboxylase /oxygenase small subunit	BRAS0001S00000032	49140	46726	1.1	1.0
	AT4G37210	Ribulose bisphosphate carboxylase	BRAS0001S00013364	9254	9598	1.0	1.0

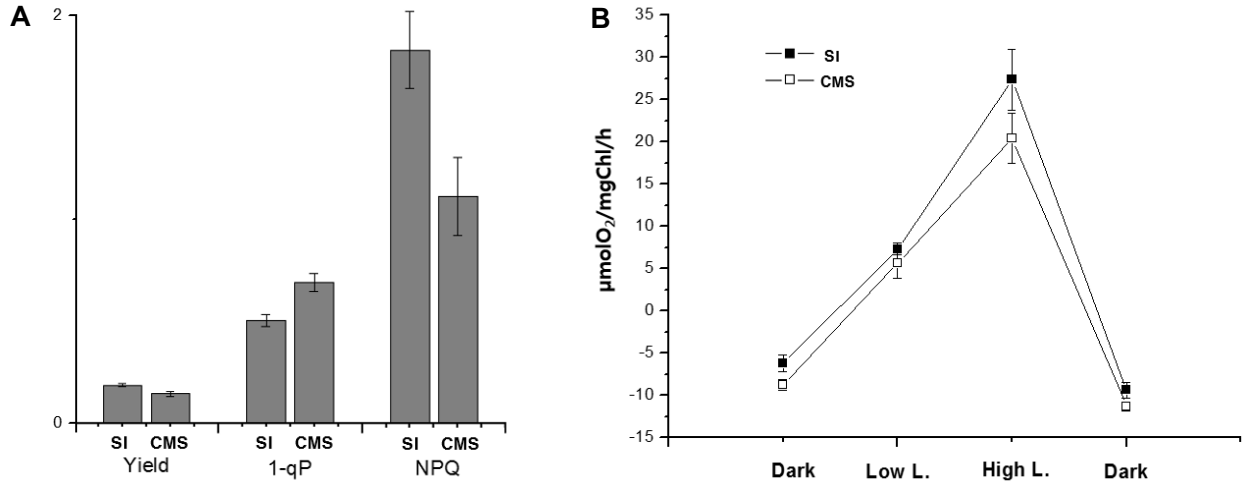

Fig. 4 Photosynthetic efficiencies of SI- and CMS-derived F_1 hybrids: Chlorophyll fluorescence parameters (A) and O_2 evolution (B). **A**, Electron flux through PSII (Yield), excitation pressure (1- qP), and non-photochemical quenching parameter (NPQ) in Chinese cabbage leaves under irradiance of 800 µmol photons m^{-2} s^{-1}. **B**, Light-response curves of photosynthetic O_2 evolution in Chinese cabbage leaves.

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

Table 5 Expression of key retrograde signaling genes for chloroplast and mitochondria

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

Acknowledgements

This work was supported by Research Fund of Chungnam National University (CNU), Daejeon, Korea, to Yoonkang Hur (2016).

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