Delayed expression of SAGs correlates with longevity in CMS wheat plants compared to its fertile plants

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Abstract Reproductive sinks regulate monocarpic senescence in crop plants. Monocarpic senescence was studied in wheat fertile (cv. HW 2041) and its isonuclear cytoplasmic male sterile (CMS) line. CMS plants exhibited slower rate of senescence accompanied by longer green leaf area duration and slower deceleration in chlorophyll, protein content, P_N and rubisco content coupled with lower protease activities than fertile (F) plants. CMS plants also exhibited lower ROS levels and less membrane damage than F plants. CMS plants maintained better antioxidant defense, less oxidative damage in chloroplast and higher transcript levels of both *rbcl* and *rbcs* genes during senescence than F plants. F plants exhibited early induction and higher expression of SAGs like serine and cysteine proteases, glutamine synthetases *GS1* and *GS2*, *WRKY53* transcription factor and decline in transcript levels of *CAT1* and *CAT2* genes than CMS plants. Hence, using genetically fertile and its CMS line of wheat it is confirmed that delayed senescence in the absence of reproductive sinks is linked with slower protein oxidation, rubisco degradation and delayed activation of SAGs. Better antioxidant defense in chloroplasts at later stages of senescence was able to mitigate the deleterious effects of ROS in CMS plants. We propose that delayed increase in ROS in cytoplasmic male sterile wheat plants resulted in delayed activation of *WRKY53*, SAGs and the associated biochemical changes than fertile plants.

Keywords Senescence · Oxidative stress · Protein carbonylation · CMS wheat

Abbreviations

AsA	Ascorbate
APX	Ascorbate peroxidase
CP	Cysteine protease
CAT	Catalase
DAA	Days after anthesis
GS	Glutamine synthetase
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SAGs	Senescence associated genes
SP	Serine protease

Introduction

Senescence is an orderly genetically controlled process of nutrient relocation and cell death. It is regulated by both endogenous and environmental factors. Endogenous factors include reproductive development, hormones, reactive oxygen species (ROS) and environmental factors include biotic and abiotic stresses. Senescence involves the active turnover and recapture of cellular materials for use in the growing parts such as developing seeds (Lim et al. 2007). Leaf senescence is an important determinant of crop productivity. Delaying leaf senescence resulted in prolongation of greenness and photosynthesis that increased carbon filling into seeds thus improving grain yield and quality (Derkx et al. 2012; Uauy et al. 2006).

Reactive oxygen species levels increase during leaf senescence resulting in higher lipid peroxidation and protein oxidation (Zimmermann and Zentgraf 2005). Antioxidants and ROS scavenging enzymes play an important role in ROS detoxification in the cell (Foyer and Noctor 2009). Balance

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between production and scavenging of ROS, which normally is tightly regulated, appears to be disrupted during the progression of senescence in different cellular compartments due to depletion of antioxidants and/or decline in activities of ROS scavenging enzymes (Zimmermann and Zentgraf 2005; Srivalli and Khanna-Chopra 2009).

Chloroplasts are the main site of ROS production due to the photooxidative nature of their components (Asada 2006). ROS produced in the chloroplasts are scavenged by an efficient antioxidant defense system including both enzymes and metabolites. In chloroplasts both stromal and thylakoid bound isozymes of superoxide dismutase (SOD) and ascorbate peroxidase (APX) are present to detoxify ROS. However, the equilibrium between production and scavenging of ROS may be perturbed during senescence as the activity of stromal and thylakoidal SOD and APX declined during senescence in wheat (Srivalli and Khanna-Chopra 2009). High ROS accumulation in chloroplasts resulted in protein oxidation and degradation especially of large subunit of rubisco (Feller et al. 2008).

Senescence process requires up-regulation and/or activation of many different transcription factors (TFs) including NAM, NAC, and WRKY (Guo et al. 2004). WRKY53 was identified as a key player in the regulation of leaf senescence in *Arabidopsis* and is proposed to regulate expression of downstream senescence associated genes SAGs (Miao et al. 2004). Expression of many SAGs is induced when accumulation of ROS increases (Bieker et al. 2012). All catalase (CAT) genes are known to be down regulated during senescence however *CAT2* is the major CAT isozyme downregulated (Zimmermann et al. 2006).

In wheat, salvaged nitrogen (N) from the leaves accounts for up to 90 % of the total grain N content (Kichey et al. 2007). rubisco is the major source of N in leaves and both rubisco subunit genes *rbcL* and *rbcS* are known to show altered expression during senescence (Suzuki et al. 2010). Nitrogen is mainly mobilized to grains in the form of glutamine. Hence the extent of glutamine synthetase (GS) activity which is enhanced during senescence is considered an important determinant of total grain N in crops (Kawakami and Watanabe 1988; Goodall et al. 2013). Both *GS1* and *GS2* are upregulated during senescence.

We have shown earlier that flag leaf senescence is delayed on removal of spikelets in wheat (Srivalli and Khanna-Chopra 2004, 2009). Physical removal of spikelets may cause ROS accumulation as wounding in plants transiently produces ROS in the damaged tissue (Leon et al. 2000). Hence, in the present study we used wheat fertile and its isonuclear cytoplasmic male sterile line to examine the regulation of senescence through reproductive sink and its influence on oxidative stress, damage and expression of SAGs in the flag leaf. Here we show that longevity in cytoplasmic male sterile wheat plants is associated with delayed increase in ROS, *WRKY53*,

senescence associated genes and better antioxidant defense in chloroplasts than fertile plants.

Materials and methods

Wheat cv. HW2041 (fertile, F) and its isonuclear CMS line obtained from the Division of Genetics, IARI, New Delhi, were sown in the fields of Water Technology Center, IARI, New Delhi, India in the last week of November, 2011. The CMS lines of HW2041 has been developed by crossing between wheat *Triticum aestivum* cv. HW2041 and *Triticum timopheevi*. *T. timopheevi* was used as female parent and HW2041 was used as male parent. This cross results in cytoplasmic male sterile F1 hybrids as described in Tomar and Anbalagan (2004). The CMS line was developed through repeated backcross breeding with *T. timopheevi* (Sinha et al. 2013).

The plant-to-plant and row-to-row distances were 10 cm and 25 cm, respectively in a plot size of 3×2 m. Fertilizers were applied at the rate of 100 N:40P:40 K kg/ha as urea: single super phosphate: muriate of potash respectively. Plants were well-watered throughout the experiment. The ears of CMS plants were covered with paper bags in both the main shoot and the tillers to avoid any external pollination. Flag leaf was sampled from anthesis to grain maturity at 7 days interval. For the biochemical assays, the leaves were sampled and processed as described in Srivalli and Khanna-Chopra (2009).

Green flag-leaf area was determined by using nondestructive method based on the formula: leaf area = length × maximum breadth × 0.7 (Aggarwal and Sinha 1987). Chlorophyll (Chl) content was measured according to Lichtenthaler (1987). Total soluble proteins were measured according to Lowry et al. (1951) using BSA as a standard.

Rubisco protein content was measured by western blot analysis as described in Chauhan et al. (2009). Leaf samples frozen in liquid nitrogen were ground in a mortar and a pestle with liquid nitrogen and then extracted [3 ml per 0.25 g(FW)] in 30 mM Tris buffer, pH 7.8, containing 1 mM ascorbic acid, 1 mM EDTA, 5 mM MgCl₂, 1 mM DTT, and 0.5 mM PMSF (Zivy et al. 1983). PVPP [4 % (w/w)] was added at the time of grinding. The extracted samples were passed through four layers of cheesecloth and centrifuged at 10,000×g for 20 min. To the supernatant, eight volumes of acetone were added and the protein precipitated overnight. The samples were again centrifuged at 10,000×g for 20 min. To the pellet, electrophoresis sample solution [125 mM Tris–HCl buffer, pH 6.8, 10 % glycerol, 5 % 2-mercaptoethanol (β-ME), and 2 % SDS] was added and boiled for 4 min. Aliquots of the protein samples were subjected to electrophoresis on a 10 % SDS PAGE (Laemmli 1970). In all cases, 10 μg of total soluble protein was loaded. Western blot analysis of rubisco large subunit (LSU) was done according to Towbin et al. (1979). After electrophoresis, the separated polypeptides were

electrotransferred at 4 °C onto a nitrocellulose membrane (0.45 µm, *Bio-Rad*, Richmond, CA, USA) at 50 V for 1 h in transfer buffer using a transblot unit (*Bio-Rad*, Richmond, CA, USA). The membrane was probed with 1:2,000 dilution of polyclonal antibodies against rubisco LSU. The antibodies were prepared as reported earlier (Srivalli et al. 2001). The immunoreactive protein bands were visualized using anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate antiserum in 1:20,000 dilution with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) as substrate. Rubisco LSU was quantified by scanning the blots using a gel documentation system (*GelDocMega system*, *Biosystematica*, Wales, UK).

Endopeptidase activity was measured following the modified version of Peoples et al. (1983). Samples (0.5 g) frozen in liquid nitrogen were ground using a mortar and pestle in liquid nitrogen and after that suspended in 2 ml of 250 mM Tris–HCl buffer, pH 7.0, containing 10 mM β-ME. PVP [2 % (w/v)] was added during homogenization and the extract was centrifuged at 10,000×g, 4 °C for 20 min. The supernatant was collected and passed through three layers of cheesecloth. The supernatant was dialysed overnight against 25 mM Tris–HCl buffer, pH 7.0, containing 10 mM β-ME. The reaction mixture contained 50 µl of crude extract, 125 µl of 250 mM sodium acetate buffer, pH 4.8, containing 10 mM β-ME and 75 µl of rubisco [0.45 % (w/v)] (*Sigma-Aldrich*) as a substrate. After incubation at 50 °C for 1 h, the reaction was stopped by adding 1 ml of 10 % TCA solution and incubated at 4 °C for 1 h. After centrifugation at 25,000×g for 10 min, the absorbance by TCA-soluble peptides generated during the reaction were estimated of the supernatants at 340 nm using a spectrophotometer (*Lambda 2S UV/VIS spectrophotometer*, *Perkin Elmer*, Massachusetts, USA). Controls were kept for zero time, i.e. the reaction was stopped immediately without further incubation and without substrate. One unit of proteolytic activity in 1 cm cuvette was defined as an increment of 0.01 in A340 in 1 h. For inhibitor studies, the reaction mixture containing 50 µl of the crude extract was preincubated for 10 min with one of the following inhibitors –3.8 µl of 2 mM PMSF, 18.8 µl of 10 mM EDTA, 9.4 µl of 25 µM DL-norleucine and 3.8 µl of 10 mM iodoacetamide for serine-, metallo-, aspartate-, and cysteine proteases, respectively (Ye and Varner 1996). The remaining procedure was the same as for the endopeptidase activity. All the inhibitors were dissolved in water except PMSF which was dissolved in isopropanol.

Net photosynthetic rate (P_N) was measured in flag leaves from anthesis to maturity in the morning between 10:00 and 11:00 using *LI-COR-6400* portable photosynthesis instrument (*LI-COR Inc.*, Lincoln, NE, USA). The measurements were done on the adaxial surface of the flag leaf in the field under saturating photosynthetic photon flux density (PPFD) 1,200–1,500 µmol m⁻² s⁻¹, CO₂ concentration ~380 ppm, air temperature 24–29 °C and air humidity 50±5 %.

Chloroplasts were isolated from flag leaf using a 40–80 % discontinuous Percoll density gradient method as previously described in Khanna-Chopra and Sabarinath (2004). Two green bands were generated. The upper band contained broken chloroplasts and the lower intact. The intact chloroplasts were transferred to a clean Corex tube and diluted with a cold buffer containing 50 mM Hepes–KOH pH 7.5, 330 mM sorbitol, 4 mM ascorbate, 2 mM EDTA, 1 mM MgCl₂ and 1 mM MnCl₂ and then centrifuged at 4,300 g for 3 min. Resuspended the chloroplast pellets in a smaller volume of the above buffer. Intactness of purified chloroplasts was measured according to ferricyanide reduction test (Lilley et al. 1975) using Clark-type O₂ electrode (Hansatech Instruments Ltd., England). The percentage intactness of chloroplasts was 80–85 % up to 21 DAA and 68–74 % at 28 DAA. The remaining chloroplasts were again centrifuged at 4,300 g for 3 min and the chloroplast pellets were resuspended in a hypotonic medium containing 50 mM Hepes–KOH, pH 7.5 and 4 mM ascorbate to lyse them. The lysed chloroplasts were used for carbonyl assay, SOD activity and APX activity assay.

H₂O₂ was extracted according to the procedure of Veljovic-Jovanovic et al. (2002). 0.2 g fresh leaf material was ground in liquid nitrogen and extracted with 2 ml of 5 % trichloroacetic acid. Four percentage of insoluble PVPP was added at the time of grinding. The homogenate was centrifuged at 18,000 g for 5 min, and the supernatant was passed through a Dowex anion exchange resin (1×8–400, *Sigma-Aldrich Co.*, St. Louis, MO, USA) equilibrated with 2 ml of 5 % TCA to remove the coloured compounds. After centrifugation for 5 min at 1,000 g, the extracts were neutralized to pH 5.6 with K₂CO₃. One unit of ascorbate oxidase was added to the samples, which were then incubated for 10 min at room temperature. The reaction mixture contained 50 µL test solution, 50 µL of 0.5 mM luminol in 0.2 M NH₄OH (pH 9.5), and 800 µL of 0.2 M NH₄OH in 1 mL test tubes which were placed in Luminoskan TL Plus luminometer (Labsystems). Chemiluminescence was initiated by the addition of 100 µL of 0.5 mM K₃Fe(CN)₆ in 0.2 M NH₄OH and the photons emitted were counted over 5 s. H₂O₂ content was determined using a calibration curve.

Lipid peroxidation was estimated by measuring the content of 2-thiobarbituric acid-reactive substances in leaf homogenates, prepared in 20 % TCA containing 0.5 % of 2-thiobarbituric acid, and heated at 95 °C for 25 min (Heath and Packer 1968). Malondialdehyde (MDA) ($\epsilon=155 \text{ mM}^{-1} \text{ cm}^{-1}$) content was determined spectrophotometrically at 532 nm and corrected for non-specific turbidity at 600 nm. Carbonyl groups in the proteins of chloroplasts fractions were determined by spectrophotometric assay according to the method of (Levine et al. 1994). Samples with a concentration of 10 µg of total soluble proteins were mixed with an equal volume of 12 % SDS and then with two volumes of 20 mM dinitrophenylhydrazine dissolved in 10 % trifluoroacetic acid (TFA). As controls,

samples were treated only with 10 % TFA. The mixture was incubated for 25 min at room temperature and the reaction was stopped by adding 1.5 sample volumes of 2 M Tris/30 % (v/v) glycerol. The volumes mentioned above always refer to the volume of the sample, before the addition of the derivatizing agent. The absorbance at 370 nm was measured. The carbonyl content was calculated using molar absorption coefficient of $22,000 \text{ M}^{-1} \text{ cm}^{-1}$.

For gene expression study leaf samples were collected at three stages i.e. 7, 21 and 28 DAA for RNA. Total RNA was isolated using TRIzol reagent in an RNase-free centrifuge tubes as per manufacturers protocol (Invitrogen). To eliminate DNA from aqueous RNA extractions, samples were treated with 10 units of RNase-free DNaseI (Qiagen, USA). Total RNA was quantified spectrophotometrically by measuring absorbance at 260 nm. RNA was fractionated on 1 % agarose gel to check the quantity and integrity.

Reverse transcription polymerase chain reaction (RT-PCR) was used to study the expression of genes related to senescence namely *rbcL*, *rbcS*, *WCP2*, *WSP*, *GS1*, *GS2*, *CAT1*, *CAT2* and *WRKY53* (Table 1). To ensure that equal amounts of RNA template were added to each RT-PCR reaction, amplification of *Actin* gene was performed (Fig. 2). Primers used for different genes were taken from previously reported papers (Table 1). For RT-PCR expression analysis, the first-strand cDNAs were synthesized using Superscript III reverse transcriptase following the manufacturer's protocol (Invitrogen). Second strand PCRs were performed in 50 μL reaction mixture with 1:5 times diluted first strand reaction (cDNA) as template. The reaction mix contained cDNA template, 200 μM dNTPs, 2.5 mM MgCl_2 , 0.5 μM gene-specific primers, and 2 unit Taq DNA polymerase in $1\times$ PCR buffer. The temperature profile for PCR was as follows: 94 $^{\circ}\text{C}$ for 3 min followed by 30 cycles of 94 $^{\circ}\text{C}$ for 30 s, 44–61 $^{\circ}\text{C}$ (specific for different primer sets, Table 1) for 30 s and 72 $^{\circ}\text{C}$ for 1 min. The final extension was for 10 min at 72 $^{\circ}\text{C}$. For all the RT-PCR reactions, actin was used as an internal control. PCR products were fractionated in a 3 % agarose gel containing 0.5 $\mu\text{g/mL}$ ethidium bromide. The gels were scanned using gel documentation system (Biosystematica, Wales, UK) and the bands obtained on the gels were quantified by TotalLab software.

ANOVA and mean comparisons (CRD, two-factor analysis) were conducted using the MSTAT-C (CIMMYT, Mexico, Version 1.00/EM 1988). Critical difference (CD) was calculated using students 't' test ($P 0.05$).

Results

In the present experiment, senescence was monitored in the flag leaf of wheat cv. HW2041 and its isonuclear CMS line. CMS plants maintained greener canopy for longer period of

time than F plants which was also apparent visually (Table 2). Chlorophyll content and P_N was higher in CMS plants upto 28 DAA than F plants. P_N declined after 14 DAA and 21 DAA in F and CMS plants respectively. Hence, CMS plants exhibited delayed senescence than F plants.

Protein and rubisco content declined sharply after 14 DAA in F plants (Table 2). CMS plants maintained higher levels of protein and rubisco content at 21 and 28 DAA than F plants. The decline in leaf protein and rubisco content was parallel with increase in endopeptidase activity in both the lines. F plants exhibited significantly higher endopeptidase activity than CMS plants at all stages studied. Cysteine proteases (CP) followed by serine proteases (SP) contributed to the increase in protease activity at 21 DAA and 28 DAA in both isolines (Table 2).

Reactive oxygen species (ROS) content increased significantly during senescence in both wheat isolines (Fig. 1). H_2O_2 content increased earlier in F plants reaching a peak at 21 DAA while CMS plants exhibited maximum H_2O_2 content at 28 DAA. F plants exhibited higher membrane damage than CMS plants at all stages studied. At 21 DAA lipid peroxidation increased by 7 fold and 5 fold in F and its CMS line respectively. F plants showed early and higher induction of the transcription factor *WRKY53* and maximum *WRKY53* expression was observed at the same time when H_2O_2 content was at the peak in both F and CMS plants i.e. 21 and 28 DAA respectively (Fig. 1).

The expression of SAGS was also different in the F and its CMS line. CMS plants had higher mRNA levels of both *rbcL* and *rbcS* throughout the study (Figs. 2 and 3). No significant change in *rbcS* and *rbcL* mRNA levels was observed during senescence in both F and CMS plants. Expression of both *WCP2* and *WSP* increased earlier in F than CMS plants. F plants exhibited about 4 fold and 6 fold increase in mRNA levels of *WCP2* and *WSP* at 21 and 28 DAA respectively while CMS plants showed significant increase in mRNA levels of *WCP2* and *WSP* only at 28 DAA. *GS1* and *GS2* mRNA levels increased in both F and CMS plants. However, increase was prominent in F plants. Both *CAT1* and *CAT2* mRNA levels were higher during senescence in CMS plants compared to F plants (Figs. 2 and 3).

Chloroplasts in both F and CMS plants showed increase in SOD and APX activity with increase in age up to 21 DAA. However, at 28 DAA CMS plants exhibited significantly higher SOD and APX activities than F plants (Fig. 4). At 28 DAA protein carbonyls in chloroplasts declined sharply in F plants but remained higher in CMS plants.

Discussion

Reproductive sinks regulate leaf senescence in monocarpic plants and manipulation of sink strength can accelerate/delay

Table 1 Primers sequences used and PCR conditions for quantitative reverse transcriptase analysis of different SAGs

Gene	Primer sequence	Tm	Reference
<i>WRKY53</i>	F-5'-GACTCTCGAAAAATCTCGTGCTC-3' R-5'-ACATGTAAACGCCACAGGGGAAC-3'	54 °C	Wu et al. 2008
<i>rbcl</i>	F-5'-CGCCTCATGGTATCCAAGTTG-3' R-5'-CGATTAGCTGCTGCACCAGGTG-3'	60 °C	Almeselmani et al. 2012
<i>rbcs</i>	F-5'-CTGTGATGGCTTCCTCGG-3' R-5'-TTAGGCCTTGCCGGACTC-3'	61 °C	Rampino et al. 2006
<i>WCP2</i>	F-5'-TTCCGCTCGTTGGCTCTCCTC-3' R-5'-CCGCCCCCTCGACAACATCTC-3'	61 °C	Simova et al. 2010
<i>WSP</i>	F-5'-CAGCGGAAGCAACATATCATT-3' R-5'-GGGTACTTCCGTCTGACCAT-3'	50 °C	Simova et al. 2010
<i>GS 1</i>	F-5'-TCCCCTGTCCCGCATTTCCAGAGA-3' R-5'-GGAGCTTGCTGGGGTCATCAACGGG-3'	51 °C	Grabowska et al. 2012
<i>GS 2</i>	F-5'-AGGTCGCCCCGCCCCCTTCCCTCCTC-3' R-5'-GGCCAGCACCTTGAAGCCGGAGGT-3'	60 °C	Grabowska et al. 2012
<i>CAT 1</i>	F-5'-ACTACGACGGGCTCATG-3' R-5'-GGAGCTGAGACGGCTTC-3'	46 °C	Luna et al. 2005
<i>CAT 2</i>	F-5'-CCTTAATCAGCAGGGATG-3' R-5'-AGATAGAACACGCGGAG-3'	46 °C	Luna et al. 2005
<i>Actin</i>	F-5'-GAGAAGATGACTCAGATC-3' R-5'-ATCCTTCCTGATATCGAC-3'	44 °C	Luna et al. 2005

the rate of senescence (Nooden 1988). CMS plants exhibited delayed senescence (Table 2). It has been reported earlier that de-sinking results in delayed leaf senescence in wheat and soybean (Srivalli and Khanna-Chopra 2004, 2009; Crafts-Brandner and Egli 1987).

In monocarpic plants such as wheat senescence is associated with nutrient mobilization (including N) from leaves to developing grains (Khanna-Chopra 2012). CMS plants maintained higher levels of protein and rubisco content and less protease activity than F plants during senescence. In soybean also leaf N and rubisco protein content declined faster in plants with sinks compared to de-sinked plants (Crafts-Brandner and Egli 1987). A net degradation of rubisco and other chloroplast proteins can be observed during endogenously initiated leaf

senescence as well as during or after abiotic stresses which allows the reutilization of N in other organs (Feller et al. 2008). During early stages of senescence, rubisco accounts for about 90 % of the degraded proteins (Miller and Huffaker 1985).

Despite the sharp decline in rubisco LSU protein content after 14 DAA in F and 21 DAA in CMS plants both isolines maintained mRNA levels of both *rbcl* and *rbcs* at all stages (Figs. 2 and 3). CMS plants had higher expression of *rbcl* and *rbcs* than F plants at all stages (Table 2, Figs. 2 and 3). This suggests that post-transcriptional regulation of rubisco content may occur in senescing wheat leaves. It was reported recently that rubisco content in rice was regulated at transcription level in young and post-transcriptional level in old senescent leaves

Table 2 Senescence related parameters in flag leaf of wheat fertile and sterile isolines during monocarpic senescence

Days after anthesis →	14		21		28		CD
Parameters↓	Fertile	Sterile	Fertile	Sterile	Fertile	Sterile	
Green flag leaf area (% of anthesis)	94	92	79	76	5	40	9.8
Chlorophyll content (mgg ⁻¹ FW)	4.28	4.91	3.76	4.54*	0.58	2.95*	0.88
Photosynthesis rate (μmolCO ₂ m ⁻¹ s ⁻¹)	19.6	20.4	12.3	19.1*	1.7	11.8*	3.12
Protein content (mgg ⁻¹ FW)	21.7	20.42	8.84	15.68*	2.3	8*	2.11
Rubisco LSU content (relative units)	19.3	20.3	12.5	19.1*	2.2	11.1*	2.12
Endopeptidase activity (units mg ⁻¹ protein)	1164	902*	1485	944*	2359	1968*	95
Endopeptidase characterization (% of the activity)	Cysteine	–	67	84	57	68	–
	Serine	–	25	10	29	29	–

Values represent means of three independent replicates. Asterisks indicate significant differences between fertile and sterile plants ($P<0.05$). Critical difference (CD) value for all enzymes/metabolites (treatment × stages) was calculated at 5 % level ($P<0.05$)

Fig. 1 Effect of reproductive sinks on expression of *WRKY53* transcript gel analysis (**a**) and relative transcript level as calculated using gel documentation (**b**), H₂O₂ content (**c**) and lipid peroxidation (**d**). Fertile (crossed bar) and CMS (open bar) for (**b**) and fertile (filled box), CMS (open box) for (**c**) and (**d**). Error bars indicate mean $\pm$ SE ($n=3$). In some cases error bars are smaller than the symbol. Vertical bars indicate critical difference (CD) treatments $\times$ stages ($P<0.05$)

(Suzuki and Makino 2013). Expression of *rbcl* and *rbcs* generally declines during senescence (Breeze et al. 2011) and decline in *rbcl* mRNA levels was lesser and slower than *rbcs* (Suzuki et al. 2010).

It is known that both cysteine and serine proteases help in protein mobilization in wheat leaves during monocarpic senescence (Table 2, Martínez et al. 2007; Chauhan et al. 2009). F plants showed higher expression of *WCP2* and *WSP* than CMS plants during senescence (Figs. 2 and 3). Transcriptome study of *Arabidopsis* senescent leaves also revealed that cysteine and serine proteases are predominantly induced during natural and/or abiotic stress induced senescence (Buchanan-

Fig. 2 Expression pattern of *rbcl*, *rbcs*, *WCP2*, *WSP*, *GS1*, *GS2*, *CAT1* and *CAT2* genes in flag leaf of wheat during monocarpic senescence

Wollaston et al. 2005). Roberts et al. (2003) reported a serine protease activity in senescing wheat leaves for which rubisco was a target protein.

GS is involved in the synthesis of glutamine which is the major form of mobilized N from senescing tissues to grains/pods (Finnemann and Schjoerring 2000). *GS1* and *GS2* in wheat and its homologue in maize, barley and *Arabidopsis* showed upregulation during senescence which was associated with high grain protein (Buchanan-Wollaston et al. 2005; Martin et al. 2006; Goodall et al. 2013). Increased mRNA levels of *GS1* and *GS2* may lead to higher activity of respective proteins in F plants than CMS plants which is needed for N mobilization to developing grains. In CMS plants stem and other vegetative parts acted as alternate sinks, which was evident from their better vegetative growth than F plants. In fact, in the absence of reproductive sinks vegetative parts act as alternate sinks in plants (del Molino et al. 1995; Srivalli and Khanna-Chopra 2004).

WRKY53 expression peaked with accumulation of H₂O₂ in both F and CMS plants which was at 21 and 28 DAA in F plants and CMS plants respectively (Figs. 2 and 3). *WRKY53* transcription factor is known to regulate leaf senescence in plants (Miao et al. 2004). Induction of *WRKY53* expression was observed at early stages of senescence before expression of many SAGs (Hinderhofer and Zentgraf 2001; Miao et al. 2004). At later stages of senescence *WRKY53* expression was

Fig. 3 Expression pattern of *rbcL*, *rbcS*, *WCP2*, *WSP*, *GS1*, *GS2*, *CAT1* and *CAT2* genes in flag leaf of wheat during monocarpic senescence. The gels were scanned using gel documentation system to calculate relative transcript level. Fertile (filled bar) and CMS (open bar). Three separate gels were scanned. Error bars indicate mean $\pm$ SE ($n=3$)

required for the continued expression of SAGs and was controlled by ethylene responsive factors (Koyama et al. 2013). Regulator of *WRKY53* expression and DNA binding of corresponding protein itself is involved in signal transduction from H_2O_2 to *WRKY53* promoter (Miao et al. 2007). Expression of *WRKY53* induced rapidly in *Arabidopsis* on treatment with H_2O_2 . *WRKY53* is known to regulate expression of other members of *WRKY* family genes, ROS defense related genes i.e. *CAT1* and *CAT2*, SAGs including *SAG12*, and receptor kinases involved in senescence induced downstream signaling in *Arabidopsis* (Miao et al. 2004). It is likely that the early oxidative burst in F plants compared to CMS plants may serve as a signal triggering early senescence as observed in other PCD processes (Fig. 1, Zentgraf et al. 2012).

Senescence like other PCD processes is associated with enhanced ROS accumulation culminating in death in plants (Foyer and Noctor 2009). Early ROS accumulation in F plants than CMS plants may be due to reproductive sinks, which act as a stress in F plants and serve as a signal for start of the

Fig. 4 Oxidative damage to proteins as protein carbonyls (a), SOD activity (b) and APX activity (c) in chloroplast from the flag leaf of fertile and its CMS isoline of wheat during monocarpic senescence. Fertile (crossed bar) and CMS (open bar). Error bars indicate mean $\pm$ SE ($n=3$). In some cases error bars are smaller than the symbol. Vertical bars indicate critical difference (CD) treatments $\times$ stages ($P < 0.05$)

senescence program (Srivalli and Khanna-Chopra 2004; Zentgraf et al. 2012). ROS accumulation increases during and is associated with increased expression of senescence associated genes (Bieker et al. 2012; Navabpour et al. 2003). In *Arabidopsis*, 15 senescence-associated NAC domain TFs are upregulated by H_2O_2 and an oxidative burst played a role in *ANAC092*-mediated senescence (Balazadeh et al. 2010; Petrov and Van Breusegem 2012).

Membrane damage is the key signal of cellular deterioration and death as maintaining cell membrane integrity is essential for survival in plants (Khanna-Chopra 2012). It was clear that CMS plants could maintain membrane integrity for a longer period during monocarpic senescence than F plants. The increase in the leaf H_2O_2 level occurs in parallel with increase in lipid peroxidation and protein oxidation in senescent leaves (Vanacker et al. 2006). CMS plants also showed higher activities of antioxidant enzymes, better redox state than F plants (data not shown). This also contributed to

the lesser membrane damage in CMS plants. Similar results have been observed in our studies in wheat wherein spikelets were removed and plants without spikelets showed better antioxidant defense and less oxidative damage than the control (Srivalli and Khanna-Chopra 2004, 2009).

CMS chloroplasts exhibited better antioxidant defense than F plants while protein carbonyls in chloroplasts declined sharply in F plants but remained higher in CMS plants during senescence (Fig. 4). Chloroplasts are also the major source of ROS in plants because of photosynthesis in an aerobic environment, and are the first organelles to show visible symptoms of degradation processes during senescence (Munné and Alegre 2002; Foyer and Noctor 2009). It is likely that damaged proteins in chloroplasts are mobilized quickly in F plants while CMS plants accumulate oxidized proteins in absence of reproductive sinks. Chloroplastic proteins are major source of nitrogen in leaves for mobilization towards the developing seeds/plant parts (Wada et al. 2009) and reproductive sinks drive N mobilization from leaves to grains (Srivalli and Khanna-Chopra 2004, 2009; del Molino et al. 1995; Möller et al. 2007). It is tempting to speculate that the better antioxidant defense in chloroplasts of the CMS plants may help in maintaining chloroplasts integrity for a longer period of time than F plants during senescence.

This study using wheat genetically fertile and its cytoplasmic male sterile line shows that reproductive sinks regulate monocarpic senescence through enhanced ROS, rubisco degradation and proteolysis in wheat. Delayed increase in ROS in cytoplasmic male sterile plants resulted in delayed activation of senescence events such as cellular damage, *WRKY53* and other senescence associated genes than F plants. Better antioxidant defense in chloroplasts at later stages of senescence was able to mitigate the deleterious effects of ROS in cytoplasmic male sterile plants, which may contribute towards longevity. Hence, we propose that longevity in cytoplasmic male sterile wheat plants is associated with delayed increase in ROS, *WRKY53*, senescence associated genes and better antioxidant defense in chloroplasts than fertile plants.

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Conflict of interest The authors declare that they have no conflict of interest.

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