Reproductive sink enhanced drought induced senescence in wheat fertile line is associated with loss of antioxidant competence compared to its CMS line

Vimal Kumar Semwal^{1,2} · Renu Khanna-Chopra¹

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Abstract Reproductive sinks regulate monocarpic senescence in wheat as desinking delayed flag leaf senescence under irrigated condition. In this study, wheat cv. HW 2041 and its isonuclear male sterile line (CMS) were subjected to post-anthesis water deficit stress to understand the association between sink strength, senescence and drought response in relation to oxidative stress and antioxidant defense at cellular and sub-cellular level. CMS plants maintained better water relations and exhibited delayed onset and progression of flag leaf senescence in terms of green leaf area, chlorophyll and protein content than fertile plants under water deficit stress (WDS). Delayed senescence in CMS plants under water deficit stress was associated with less reactive oxygen species generation, lower damage to membranes and better antioxidant defense both in terms of antioxidant enzyme activities and metabolite content compared to fertile plants. Expression of some senescence associated genes (SAGs) such as WRKY transcription factor (*WRKY53*), glutamine synthetase1 (*GS1*), wheat cysteine protease (*WCP2*) and wheat serine protease (*WSP*) was lower while catalase 2 (*CAT2*) transcript levels were higher in the CMS plants compared to HW2041

during senescence under water deficit stress. Antioxidant defense in chloroplasts was better in CMS line under water deficit stress compared to HW2041. This is the first report showing that reproductive sink enhanced drought induced senescence in flag leaf of wheat fertile line is associated with higher oxidative stress and damage and loss of antioxidant competence compared to its sterile line under water deficit stress. Higher expression of some SAGs and decline in superoxide dismutase and ascorbate peroxidase activity in the chloroplasts also contributed to the accelerated senescence in fertile line compared to its CMS line under WDS.

Keywords Reproductive sink · Senescence · Oxidative stress · Antioxidant defense · Drought resistance · Senescence associated genes

Abbreviations

AsA	Ascorbic acid reduced
APX	Ascorbate peroxidase
CAT	Catalase
DHA	Ascorbic acid oxidized
DAA	Days after anthesis
GS1	Glutamine synthetase
GSH	Glutathione reduced
GSSG	Glutathione oxidized
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SAGs	Senescence associated genes
WCP	Wheat cysteine protease
WSP	Wheat serine protease
WDS	Water deficit stress

Renu Khanna-Chopra is INSA Senior Scientist.

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✉ Renu Khanna-Chopra
renu.chopra3@gmail.com; renu_wtc@rediffmail.com

¹ Stress Physiology Lab, Water Technology Centre, Indian Agricultural Research Institute, New Delhi 110012, India

² Present Address: Africa Rice Center (AfricaRice), C/O IITA, PMB 5320, Oyo Road, Ibadan, Nigeria

Introduction

In monocarpic crops, whole plant senescence is observed during development of reproductive sinks i.e. grains/pods and is characterized by yellowing of leaves followed by death of the plants. Leaf senescence is a genetically controlled developmental process involving catabolism of chlorophyll, proteins, lipids and nucleic acids associated with nutrient remobilization to the developing grains/pods leading to cell death (Lim et al. 2007). Leaf senescence is influenced by internal factors such as reproductive sinks and environmental cues including abiotic stresses and others.

The development of reproductive sinks regulates leaf senescence in monocarpic crops including wheat (Nooden 1988; Semwal et al. 2014). Removal of spikelets delayed flag leaf senescence in wheat (Biswas and Mandal 1986; MacKown et al. 1992; Srivalli and Khanna-Chopra 2004, 2009). Cytoplasmic male sterile (CMS) wheat plants exhibited longevity, longer green flag leaf area duration, slower deceleration in chlorophyll, protein content, photosynthesis rate and Rubisco content, coupled with lower protease activities than fertile plants (Semwal et al. 2014). Hence, CMS wheat line exhibited functional stay green character compared to its fertile line under irrigated environment. Under water deficit stress also, CMS lines of wheat and sorghum showed slower senescence by maintaining green flag leaf area and chlorophyll content for a longer duration during post-anthesis phase compared to its fertile plants (Khanna-Chopra and Sinha 1988).

Crop yield is influenced by the rate of leaf senescence during grain development in crops. In crop plants, the functional stay green trait is associated with longer duration of greenness and photosynthesis that increases carbon filling into seeds and improved grain yield and quality (Thomas and Howarth 2000; Derkx et al. 2012; Uauy et al. 2006). Prematurely induced senescence caused by abiotic stresses can reduce crop yield (Gregersen et al. 2013). Many studies have shown that functional stay green crops also have increased tolerance to both biotic and abiotic stresses (Joshi et al. 2007; Tian et al. 2013; Verma et al. 2004; Thomas and Ougham 2014). Hence, stay-green phenomenon is a desirable trait as it enhanced biomass and grain yield under drought stress in wheat (Araus et al. 2002; Verma et al. 2004; Christopher et al. 2016) and tobacco (Rivero et al. 2007). Infact stay green trait, a reliable indicator of delayed senescence is often used by breeders to improve drought tolerance in sorghum and wheat (Borrell et al. 2014; Christopher et al. 2016).

Reactive oxygen species (ROS) are common output of abiotic stresses and developmental senescence (Khanna-Chopra 2012). ROS are known to have multifaceted roles

in plants as besides their damaging impacts on cellular membranes and macromolecules, they also serve as secondary messengers to induce abiotic stress response and senescence (Foyer and Noctor 2005; Thomas and Ougham 2014). ROS are managed by antioxidant metabolites and enzymes in the cell. Antioxidant defense declines during senescence leading to oxidative stress and damage (Prochazkova and Wilhelmova 2007; Khanna-Chopra et al. 2013). Effective antioxidant defense in wheat contributed to delayed senescence under irrigated conditions (De Simone et al. 2014) and also under drought and heat stress (Huseynova 2012; Khanna-Chopra and Chauhan 2015). NAC transcription factors play an important regulatory role in senescence and abiotic stress responses (Lim et al. 2007; Shao et al. 2015). Studies on NAC transcription factors in *Arabidopsis* have shown that they also modulate ROS metabolism. Thus JUB1, a NAC transcription factor when overexpressed in *Arabidopsis* lowered cellular H₂O₂ levels, delayed leaf senescence and increased tolerance to abiotic stresses. JUB1 is H₂O₂ responsive and enhanced the expression of DREB2A and several ROS responsive genes (Wu et al. 2012). Similarly NTL4, a drought responsive NAC transcription factor promoted ROS production by binding directly to the promoters of genes that encode enzymes involved in ROS biosynthesis and thus linked ROS metabolism to drought induced leaf senescence in *Arabidopsis* (Lee et al. 2012).

Chloroplasts and mitochondria are the major sites of ROS generation in plants (Apel and Hirt 2004). Chloroplasts are the major targets of oxidative stress during drought stress and senescence and are degraded earlier than mitochondria. Under oxidative stress, membranes and proteins of chloroplast and mitochondria are inevitably subjected to oxidative damage through carbonylation. Chloroplastic proteins are carbonylated, degraded and amino N mobilized to the developing seeds in monocarpic plants such as wheat and rice (Møller et al. 2007). Protein damage was higher in mitochondria compared to chloroplasts during drought stress and senescence in wheat (Bartoli et al. 2004; Srivalli and Khanna-Chopra 2009). During dark induced senescence also, mitochondria are the first organelle to be damaged followed by peroxisomes and chloroplasts in *Arabidopsis* (Rosenwasser et al. 2011). Chloroplasts and mitochondria are endowed with antioxidants such as ascorbate, glutathione and enzymatic defense in terms of superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities. In addition, chloroplasts contain other antioxidant metabolites like carotenoids and tocopherols. In wheat, SOD and APX activities were much higher in chloroplasts compared to mitochondria under drought stress and monocarpic senescence (Selote and Khanna-Chopra 2006; Srivalli and Khanna-Chopra 2009). Hence, higher damage to mitochondrial proteins

was attributed to relatively poor ROS detoxification capacity compared to chloroplasts. Limited information is available on protein oxidation and antioxidant defense in chloroplasts and mitochondria under combination of drought stress and senescence under field conditions in crop plants.

Microarray studies in *Arabidopsis* and wheat have shown that significant changes in gene expression occur during leaf senescence and water deficit stress (Woo et al. 2013; Shinozaki and Yamaguchi-Shinozaki 2007; Gregersen and Holm 2007). Many senescence associated genes (SAGs) have been identified in plants which mainly encode for proteins responsible for degradation of cellular components and mobilization related functions. These include genes for cysteine protease, glutamine synthetase and catalase involved in protein degradation, nitrogen mobilization and antioxidant defense respectively. Glutamine synthetase (*GSI*) is also upregulated in leaves of tomato plants during drought as nitrogen is mobilized to developing fruits (Bauer et al. 1997). Cysteine protease was upregulated both during monocarpic senescence and drought induced senescence while excision of flowers resulted in delay in senescence and upregulation of cysteine protease in pea (Pic et al. 2002). Transcription factors such as NAC and WRKY control the expression of several SAGs and hence regulate senescence (Woo et al. 2013).

It has been shown earlier that delayed monocarpic senescence in desinked wheat plants was associated with lesser oxidative stress and damage coupled with an efficient antioxidant defense under irrigated conditions compared to the plants with sink (Srivalli and Khanna-Chopra 2004, 2009). In the present study, an attempt was made to understand the role of ROS metabolism in the regulation of monocarpic senescence by reproductive sink under post-anthesis drought stress in wheat under field conditions using a fertile wheat cultivar and its cytoplasmic male sterile line. The oxidative damage and antioxidant defense was also examined in the chloroplasts and mitochondria along with expression analysis of some SAGs at various stages between anthesis to maturity in irrigated and drought stressed plants.

Materials and methods

Plant material and sampling

Wheat cv. HW2041 and its CMS line used in this study were also used in our previous study (Semwal et al. 2014). This material was sown in separate plots in the fields of Water Technology Center, IARI, New Delhi, India on 24th November, 2012. The details of sowing and growing conditions are given in Semwal et al. (2014). Control plants

were well-watered throughout the experiment. In the stress treatment, irrigation was withheld at anthesis in both fertile and sterile plants. There was negligible rainfall between anthesis to maturity enabling development of water deficit stress (WDS). The treatments were HW2041 control, CMS control, HW2041 drought and CMS drought respectively. Sampling of flag leaf was done for various biochemical analysis at anthesis and 7, 12, 17, 22, 25, 28 and 33 days after anthesis (DAA) in the morning hours from 10 to 11 AM. The parameters studied included water relations, senescence, oxidative stress, oxidative damage and antioxidant defense both in terms of enzymes and metabolites. The leaf samples were cut into small pieces after measuring fresh weight, frozen in liquid nitrogen and stored at -80°C in triplicate. Three independent biological replicates were maintained for all parameters while for H_2O_2 content, five replicates were maintained.

Water relations

Water relations were measured both in terms of water potential and relative water content (RWC). Water potential (Ψ_w) of the flag leaf was measured using the pressure chamber (Model 3005, Soil Moisture Equipment Corporation, USA, Scholander et al. 1964). RWC was measured by following the method of Barrs and Weatherley (1962).

Senescence

Senescence was characterized as the loss in green flag leaf area (GFLA), chlorophyll and protein content between anthesis to maturity. Green flag-leaf area was measured following Aggarwal and Sinha (1987). Chlorophyll content was determined according to Lichtenthaler (1987). For isolating total soluble proteins, frozen leaf samples were extracted in 30 mM Tris buffer, pH 7.8 (Srivalli and Khanna-Chopra 2004). The homogenate was centrifuged at 10,000g for 20 min. The supernatant was used for measuring total soluble proteins using BSA as a standard (Lowry et al. 1951).

Oxidative stress and damage

Oxidative stress was quantified as H_2O_2 content and damage in terms of lipid peroxidation and protein oxidation in the flag leaf using the procedures of Veljovic-Jovanovic et al. (2002), Heath and Packer (1968), and Levine et al. (1994) respectively. The details of the procedures for measurement of H_2O_2 , lipid peroxidation and protein carbonylation are given in Srivalli and Khanna-Chopra (2004).

Antioxidant enzymes activities and metabolite content

Frozen leaf samples were processed as described in Srivalli and Khanna-Chopra (2009). The supernatant was used for measuring soluble proteins (Lowry et al. 1951) and enzyme assays. Superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR) activities were measured spectrophotometrically following the methods of Beauchamp and Fridovich (1971), Nakano and Asada (1981), Aebi (1984), Schaedle and Bassham (1977), (Asada 1984), and Navari-Izzo et al. (1998) respectively. Ascorbic acid and glutathione content was measured using the procedures described by Wang et al. (1991) and Loggini et al. (1999) respectively. The details of the procedures are given in Selote and Khanna-Chopra (2006).

Isolation of chloroplasts and mitochondria

The procedure for isolation of chloroplast and mitochondria is described in Srivalli and Khanna-Chopra (2001). The percentage intactness of chloroplast as measured by ferricyanide reduction test (Lilley et al. 1975) was 80–84% from anthesis up to 21 DAA and 65–70% at 25 DAA. Fumarase (EC 4.2.1.2) activity, a marker for mitochondrial intactness (Hatch 1978) revealed the percentage intactness to be in the range of 81–86% from anthesis up to 21 DAA and 72–78% at 25 DAA. Oxidative damage to proteins, SOD and APX activity was studied in protein fraction from chloroplast and mitochondria as described for crude leaf extracts.

Isolation of RNA and gene expression analysis of senescence associated genes

Leaf samples were collected at anthesis and 7, 12, 17 and 25 DAA for gene expression analysis. RNA isolation was done using TRIZOL reagent as per manufacturer's instructions (Invitrogen). Isolated RNA samples were treated with RNase-free DNaseI (Qiagen, USA) to eliminate contaminating DNA.

Reverse transcription polymerase chain reaction (RT-PCR) was used for the expression analysis of senescence associated genes *WCP2* (cysteine protease), *WSP* (serine protease), *GSI* (glutamine synthetase), *CAT2* (catalase) and *WRKY53* (transcription factor). The Tm, sequences of gene specific primers used and the details of first strand cDNAs synthesis using Superscript III reverse transcriptase (Invitrogen) and PCR conditions are given in Semwal et al. (2014). 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 µg/mL ethidium bromide. Three separate gels for each gene under study were scanned using gel doc. system (Biosystematica, Wales, UK) and the intensity of the bands on the gels were quantified by Total Lab software.

Statistical analysis

The results are presented as means of three independent replicates with standard error ($\pm$ se). ANOVA and mean comparisons (CRD, three-factor analysis, genotypes $\times$ treatments $\times$ stages) were done using the MSTAT software (CIMMYT, Mexico, Version 1.00/EM 1988). Student's *t* test was used for calculating critical difference (CD) at $P < 0.05$.

Results

Senescence and water relations

Withholding irrigation resulted in significant decline ($P < 0.05$) in leaf RWC and water potential (Ψ) from 12 DAA in both wheat cv. HW2041 and its CMS line compared to control (Fig. 1). Decline in leaf Ψ and RWC was significantly higher ($P < 0.05$) in HW2041 plants compared to its CMS line at all stages of senescence.

Water deficit stress (WDS) enhanced senescence rate in both HW2041 and its CMS line compared to control from 12 DAA and 17 DAA respectively (Fig. 2). CMS line maintained higher green flag leaf area, chlorophyll and protein content than HW2041 at all stages under WDS and survived up to 33 DAA while HW2041 was fully senesced by 28 DAA. HW2041 matured fully at 33 DAA while CMS line matured at 40 DAA under WDS. Hence cv.HW 2041 exhibited faster flag leaf senescence compared to its CMS line under WDS.

Oxidative stress and damage

WDS enhanced H_2O_2 content and lipid peroxidation in HW2041 and its CMS line significantly ($P < 0.05$) from 12 DAA and 17 DAA respectively compared to the respective control (Fig. 3). HW2041 exhibited significantly higher ($P < 0.05$) H_2O_2 content and lipid peroxidation compared to its CMS line at almost all stages of flag leaf senescence. In control plants, protein damage increased gradually up to 22 DAA in both HW2041 and its CMS line but declined subsequently in fertile line only. WDS enhanced protein damage in both HW2041 and its CMS line initially but CMS line exhibited higher protein damage compared to HW2041 at later stages of senescence.

Fig. 1 Effect of water deficit stress on water relations **a** water potential and **b** relative water content in flag leaf of wheat cv. HW2041 and its isonuclear male sterile (CMS) line during monocarpic senescence. HW2041 control (square), HW2041 drought (square), CMS control (triangle) and CMS drought (triangle). Error bars indicate mean $\pm$ SE (n = 3). In some cases error bars are smaller than the symbol. Critical difference (cd) value at error degrees of freedom for water potential and RWC (genotype $\times$ treatment $\times$ stages) = 0.25 and 3.1 respectively, $P < 0.05$

Antioxidant defense

SOD and CAT activity were significantly higher ($P < 0.05$) under WDS at all stages while APX activity was higher only up to 22 DAA compared to control in both HW 2041 and its CMS line (Fig. 4). CMS line maintained significantly higher ($P < 0.05$) SOD activity at all stages while APX and CAT activity were higher only at later stages of senescence than HW2041 under WDS.

HW2041 and its CMS line maintained significantly higher ($P < 0.05$) GR, DHAR and MDHAR activities at all stages of senescence under WDS compared to control (Fig. 5). CMS line maintained significantly higher ($P < 0.05$) GR and DHAR activity at all stages while MDHAR activity was higher only at later stages of

Fig. 2 Effect of water deficit stress on **a** green flag leaf area, **b** protein content, and **c** chlorophyll content activity in flag leaf of wheat cv. HW2041 and its CMS line during monocarpic senescence. HW2041 control (square), HW2041 drought (square), CMS control (triangle) and CMS drought (triangle). Error bars indicate mean $\pm$ SE (n = 3). In some cases, error bars are smaller than the symbol. Critical difference (cd) value for GFLA, chlorophyll content and protein content (genotype $\times$ treatment $\times$ stages) = 3.1, 2.2 and 6.2 respectively, $P < 0.05$

senescence compared to HW2041 during senescence under WDS (Fig. 5).

Ascorbate (AsA) and glutathione (GSH) content, reduced/oxidized ascorbic acid (AsA/DHA) and reduced/oxidized glutathione (GSH/GSSG) ratios declined significantly ($P < 0.05$) during senescence under WDS in both

Fig. 3 Effect of water deficit stress on **a** H₂O₂ content, **b** lipid peroxidation and **c** oxidative damage to leaf proteins in flag leaf of wheat cv. HW2041 and its CMS line during monocarpic senescence. HW2041 control (square), HW2041 drought (square), CMS control (triangle) and CMS drought (triangle). Error bars indicate mean $\pm$ SE ($n = 5$ for H₂O₂ content and $n = 3$ for other parameters). In some cases, error bars are smaller than the symbol. Critical difference (cd) value for H₂O₂ Content, lipid peroxidation and oxidative damage to proteins (genotype $\times$ treatment $\times$ stages) = 0.41, 5.3 and 39.9 respectively, $P < 0.05$

HW2041 and its CMS line compared to control (Fig. 6). CMS plants maintained significantly higher ($P < 0.05$) AsA and GSH content, AsA/DHA and GSH/GSSG ratios under WDS compared to HW2041 during senescence (Fig. 6).

Fig. 4 Effect of water deficit stress on activities of **a** SOD, **b** APX and **c** CAT in flag leaf of wheat cv. HW2041 and its CMS line during monocarpic senescence. HW2041 control (square), HW2041 drought (square), CMS control (triangle) and CMS drought (triangle). Error bars indicate mean $\pm$ SE ($n = 3$). In some cases, error bars are smaller than the symbol. Critical difference (cd) value for SOD, APX CAT activity (genotype $\times$ treatment $\times$ stages) = 0.23, 0.03 and 3.11 respectively, $P < 0.05$

Oxidative damage and antioxidant defense in chloroplasts and mitochondria

Protein damage in chloroplast and mitochondria increased significantly ($P < 0.05$) during senescence under WDS in both HW2041 and its CMS line compared to control (Fig. 7). At later stages of senescence, chloroplast and

Fig. 5 Effect of water deficit stress on activities of **a** GR, **b** DHAR and **c** MDHAR in flag leaf of wheat cv. HW2041 and its CMS line during monocarpic senescence. HW2041 control (square), HW2041 drought (square), CMS control (triangle) and CMS drought (triangle). Error bars indicate mean $\pm$ SE ($n = 3$). In some cases, error bars are smaller than the symbol. Critical difference (cd) value for GR, DHAR and MDHAR activity (genotype $\times$ treatment $\times$ stages) = 0.014, 0.01 and 0.01 respectively, $P < 0.05$

mitochondrial protein damage declined in HW2041 while its CMS line exhibited higher level of protein damage under WDS. Chloroplastic SOD and APX activities increased significantly ($P < 0.05$) under WDS compared to control up to 17 DAA and 22 DAA in HW2041 and its CMS line respectively and then declined. CMS plants maintained significantly higher ($P < 0.05$) chloroplastic SOD and APX activities compared to HW2041 plants during senescence under WDS. Mitochondrial SOD activity did not change significantly ($P < 0.05$) under WDS in both HW2041 and its CMS line at any stage compared to

control. Under WDS, mitochondrial APX activity was significantly higher ($P < 0.05$) up to 17 DAA and 22 DAA in HW2041 and its CMS line respectively compared to control. CMS line maintained significantly higher ($P < 0.05$) mitochondrial APX activity compared to HW2041 only at 22 DAA under WDS.

Expression analysis of senescence associated genes

WRKY transcription factor (*WRKY53*), glutamine synthetase (*GSI*), wheat cysteine protease (*WCP2*) and wheat serine protease (*WSP*) expression enhanced early on in HW2041 compared to its CMS line under WDS (Fig. 8, supplementary Fig. 1). CMS plants exhibited lower *WRKY53*, *GSI*, *WCP2* and *WSP* and higher catalase (*CAT2*) mRNA levels than HW2041 during senescence under WDS (Fig. 8, supplementary Fig. 1).

Discussion

Monocarpic senescence is a complex process involving nutrient remobilization and culminating in cell death (Lim et al. 2007). It is regulated by internal factors including reproductive development, ROS and others, while external factors include environmental stresses. In wheat, the rate of flag leaf senescence was slower in CMS plants and also after spikelet removal compared to plants with developing grains (Srivalli and Khanna-Chopra 2004, 2009; Semwal et al. 2014). Under water deficit stress, CMS lines of wheat and sorghum exhibited slower rate of senescence during post-anthesis phase than its fertile line (Khanna-Chopra and Sinha 1988). Similarly, in the present study, senescence of flag leaf and the whole plant was earlier in wheat cv. HW2041 compared to its CMS line under WDS and hence was in consonance with our earlier results (Fig. 2). Reproductive sink intensity also influences water status and root growth in wheat. Degraining caused an increase in flag leaf water potential, final root weight and flag leaf longevity under drought stress compared to plants with developing grains and thus improved drought resistance (Blum et al. 1988). In the present study also, sterile wheat plants maintained better water relations than fertile plants during senescence under WDS (Fig. 1). It has been observed earlier that drought tolerant wheat cultivar maintained higher flag leaf RWC compared to the drought susceptible cultivar under drought stress during post-anthesis period (Khanna-Chopra and Selote 2007). Source-sink studies in wheat have clearly shown that on removal of ears, roots become the alternate sinks as root biomass increased during grain ripening compared to plants with developing grains (Koide and Ishihara 1992). Similar results have been obtained on root/shoot partitioning studies with fertile and sterile rice plants

Fig. 6 Effect of water deficit stress on **a** AsA, **b** GSH, **c** AsA/DHA ratio and **d** GSH/GSSG ratio in flag leaf of wheat cv. HW2041 and its CMS line during monocarpic senescence. HW2041 control (square), HW2041 drought (square), CMS control (triangle) and CMS drought (triangle). Error bars indicate mean $\pm$ SE ($n = 3$). In some cases,

error bars are smaller than the symbol. Critical difference (cd) value for AsA content, GSH content, AsA/DHA ratio and GSH/GSSG ratio (genotype $\times$ treatment $\times$ stages) = 1.31, 0.71, 1.47 and 1.11 respectively, $P < 0.05$

between anthesis to maturity (Kato et al. 2004). It is likely that in the present study, the wheat sterile line maintained better water relations than its fertile line under stress by investing in root growth and drawing water from deeper layers of soil and thus improved drought resistance.

ROS production is increased leading to oxidative stress and membrane damage during drought stress and senescence (Lim et al. 2007; Khanna-Chopra 2012). Developing grains enhanced senescence, oxidative stress and damage in wheat compared to desinked plants (Srivalli and Khanna-Chopra 2004; Semwal et al. 2014). Wheat stay green mutant *tasg1* exhibited delayed senescence associated with lesser ROS accumulation, membrane damage and protein oxidation compared to wild type during senescence under drought (Tian et al. 2012, 2013). Drought susceptible wheat cultivars also showed higher ROS content and membrane damage than the tolerant ones during senescence under post-anthesis drought stress in the field (Simova-Stoilova et al. 2009; Hameed et al. 2011; Khanna-Chopra and Selote 2007). In the present study, HW2041

plants showed higher H_2O_2 content and membrane damage than its CMS line under WDS between anthesis and maturity (Fig. 3). Hence, lesser oxidative stress and membrane damage in CMS wheat plants may have contributed to delayed leaf senescence under drought stress than HW2041 plants.

Protein oxidation increased under drought stress in both HW2041 and its CMS line (Fig. 3). CMS line showed higher levels of oxidized proteins than HW2041 both under irrigated and WDS during later stages of senescence. ROS catalyze the oxidative modification of cellular proteins and mark them for degradation by proteases (Møller et al. 2007). In the senescing wheat flag leaves, protein carbonylation increased concomitantly with a stimulation of endoproteolytic activity and decrease in protein content (Havé et al. 2015). As damaged proteins are degraded and mobilized to developing grains, the protein carbonyl level declined sharply by 28 DAA in HW2041 under irrigated and WDS while in CMS plants, higher protein carbonyls were observed at these stages but declined subsequently,

Fig. 7 Oxidative damage to proteins as protein carbonyls (**a, d**), SOD activity (**b, e**) and APX (**c, f**) activity in chloroplast (**a–c**) and mitochondria (**d–f**) from the flag leaf of wheat cv. HW2041 and its CMS line during monocarpic senescence under water deficit stress. HW2041 control (square), HW2041 drought (square), CMS control (triangle) and CMS drought (triangle). Error bars indicate mean $\pm$ SE ($n = 3$). In some cases error bars are smaller than the symbol. Critical

difference (cd) value for oxidative damage to proteins, SOD activity and APX activity in chloroplast (genotype $\times$ treatment $\times$ stages) = 19, 0.9, and 0.02 respectively, $P < 0.05$. Critical difference (cd) value for oxidative damage to proteins and APX activity in mitochondria (genotype $\times$ treatment $\times$ stages) = 14.5, not significant, and not significant respectively, $P < 0.05$

perhaps indicative of N mobilization to other plant parts (Fig. 3).

During senescence and stress, disruption of cellular homeostasis is accompanied by increase in production of ROS and the extent of oxidative stress induced damage can be attenuated depending on the response of the cell's antioxidant system. The cellular ascorbate and glutathione pools shift towards their oxidized forms, the redox state of ascorbate and glutathione and the activity of the antioxidant enzymes is decreased during senescence (Rivero et al. 2007; Srivalli and Khanna-Chopra 2004, 2009; Jiménez

et al. 1998; Zimmermann et al. 2006). Efficient antioxidant defense contributed towards delayed senescence in desinked wheat plants compared to plants with developing grains under irrigated condition (Srivalli and Khanna-Chopra 2009) and in wheat mutant *tasg1* relative to the wild type under drought stress respectively (Tian et al. 2012). Higher concentrations of AsA and GSH and/or higher activities of antioxidant enzymes including SOD, APX and GR have been observed in transgenic plants harboring the *P_{SAG}-IPT* autoregulated IPT expression system showing delayed senescence under drought stress

Fig. 8 Effect of water deficit stress on expression of *WRKY53*, *GSI*, *WCP2*, *WSP* and *CAT2* genes in flag leaf of wheat cv. HW2041 and its CMS line during monocarpic senescence under WDS. The gels were scanned using gel documentation system to calculate relative transcript level. HW 2041 control (red bar), HW 2041 drought (black bar), CMS control (green bar) and CMS drought (blue bar). Three separate gels were scanned. Error bars indicate mean $\pm$ SE ($n = 3$). Asterisks (*) above the bars indicate significant differences between HW2041 and its CMS line under control and drought stress ($P < 0.05$) (color figure online)

(Rivero et al. 2007; Merewitz et al. 2011). Previous research has shown that delayed leaf senescence confers extreme drought resistance (Guo and Gan 2014). Also

drought tolerant wheat cultivar maintained higher activities of antioxidant defense enzymes, higher AsA, GSH content and higher AsA/DHA and GSH/GSSG ratio compared to

the drought susceptible cultivar under post-anthesis water deficit stress in the field (Khanna-Chopra and Selote 2007; Huseynova 2012). The drought tolerant wheat cultivar exhibiting delayed senescence also preserved photosynthetic apparatus better by maintaining higher chlorophyll content and photosynthetic efficiency than the drought susceptible cultivar at all stages between anthesis and maturity in drought stressed plants (Huseynova 2012). In the current study also, CMS wheat line maintained higher activities of SOD, CAT, APX, GR, DHAR and MDHAR and higher AsA and GSH content and AsA/DHA and GSH/GSSG ratios than its fertile HW2041 line during monocarpic senescence under WDS (Figs. 5, 6). It is likely that the efficient antioxidant defense leading to better ROS management contributed to delayed senescence under drought stress in the CMS line compared to HW2041 under field conditions.

Chloroplasts are the major sites of ROS generation and nutrient mobilization during senescence and abiotic stresses (Munné-Bosch and Alegre 2002; Khanna-Chopra 2012). Elevated ROS levels in chloroplasts damage key photosynthetic proteins which are subsequently degraded by proteases and mobilized to developing grains during senescence (Møller et al. 2007). It is known that ROS more damaging than H_2O_2 like superoxide and singlet oxygen are responsible for deterioration of chloroplast functions as loss of antioxidant defense occurs during drought induced senescence (Munné-Bosch et al. 2001; Noctor et al. 2014). In wheat, Cu/Zn SOD and Fe SOD activity declined in chloroplast during senescence due to inactivation of SOD isozymes by H_2O_2 followed by SOD protein degradation while in barley, senescent leaves were unable to modulate SOD mRNA in response to increasing ROS formation (Casano et al. 1994, 1997). Decline in chloroplast APX activity was linked to depletion of AsA in chloroplast and decline in chloroplast APX gene expression during senescence in pea and *Arabidopsis* respectively (Palma et al. 2006; Panchuk et al. 2005). In the present study, chloroplast protein oxidation increased under drought stress in both HW2041 and its CMS line compared to controls (Fig. 7). However, HW2041 plants exhibited lesser damage in chloroplasts than its CMS line under WDS. It is likely that in the presence of active reproductive sink HW2041 plants showed higher mobilization compared to its CMS line and thus maintained lower carbonyls in chloroplasts during senescence. Higher SOD and APX activities in chloroplasts of CMS line during senescence under WDS resulted in better antioxidant defense compared to HW2041 (Fig. 7). Efficient ROS management in chloroplasts of CMS plants may contribute to prolonged chloroplast stability associated with delayed senescence compared to HW2041 under WDS.

Oxidative damage to mitochondrial proteins was observed in wheat leaves during senescence and drought stress (Srivalli and Khanna-Chopra 2009; Bartoli et al. 2004; Rosenwasser et al. 2011; Semwal et al. 2014). Mitochondrial AAA proteases *m*-AAA and *i*-AAA degrade carbonylated proteins especially under stress condition (Smakowska et al. 2014). In pea, SOD and APX activity in mitochondria declined during leaf senescence (Jiménez et al. 1998). Proteomic analysis of protein oxidation in matrix of rice leaf mitochondria and apple fruit mitochondria revealed that mitochondrial MnSOD was inactivated due to carbonylation and degraded subsequently during senescence (Kristensen et al. 2004; Qin et al. 2009). In the present study also, higher oxidative damage to mitochondrial proteins during early stages of senescence may be attributed to lesser induction of SOD and APX activities in mitochondria compared to chloroplast in both HW2041 and its CMS line under WDS (Fig. 7). It is likely that at later stages of senescence, the damaged mitochondrial proteins were degraded by the mitochondrial proteases under WDS in both wheat lines.

Transcription factors (TFs) play an important role in initiation and progression of senescence in plants (Woo et al. 2013). *WRKY53*, a member of WRKY family has a positive role in senescence as it targets SAGs involved in nutrient mobilization and cell death (Miao et al. 2004). *WRKY53* gene knockout plants showed delayed senescence while overexpression caused early senescence in *Arabidopsis*. In the present study also, the expression of *WRKY53* was higher and increased early on in HW2041 exhibiting faster senescence compared to its CMS line showing delayed senescence under WDS (Fig. 8, supplementary Fig. 1). Hence, our results are in consonance with the observations in the literature.

Protein degradation and nitrogen mobilization from senescing leaves to developing grains is an important component of senescence in wheat (Hörtensteiner and Feller 2002). Genes encoding for cysteine and serine proteases showed enhanced expression during leaf senescence in *Arabidopsis* and wheat (Buchanan-Wollaston et al. 2005; Gregersen and Holm 2007). SAG12, a vacuolar cysteine protease is a molecular marker of senescence and especially ABA induced senescence in *Arabidopsis* (Weaver et al. 1998). SAG12 induction was higher in transgenic *Arabidopsis* plants overexpressing ABA receptor and showing faster senescence under drought and ABA application compared to wild type plants (Zhao et al. 2016). Cytokinins delayed senescence and caused decline in SAG12 transcripts in senescing *Arabidopsis* leaves (Noh and Amasino 1999). Glutamine is the major amino acid mobilizing N from the senescing wheat leaves to the grains, and cytosolic glutamine synthetase (*GS1*) plays a major role in glutamine synthesis during senescence

(Gegersen and Holm 2007). Gene expression of *GS1* in ear leaves was lower in stay green maize variety showing lower nitrogen harvest index compared to the maize variety showing faster leaf senescence and higher nitrogen harvest index during post-silking drought stress (Li et al. 2016). Catalase activity and *CAT2* transcript expression declined during progression of senescence in *Arabidopsis* (Zimmermann et al. 2006). Catalase plays an important role in managing H_2O_2 levels and maintaining glutathione and ascorbate levels in plants during oxidative stress and thus helps in stress tolerance in tobacco (Willekens et al. 1997). Cytokinin overexpressing tobacco plants showing delayed senescence under drought also exhibited higher *CAT2* mRNA levels than wild type plants (Rivero et al. 2007). In the present study also, gene expression of *WCP2* and *GS1* was higher while *CAT2* expression was lower in HW2041 showing accelerated senescence compared to its CMS line during WDS (Fig. 8, supplementary Fig. 1).

In conclusion, this is the first report showing that developing grains influenced oxidative stress metabolism which provides a link between senescence and drought stress response in wheat. Reproductive sink regulated senescence under water deficit stress as fertile wheat cv. HW2041 exhibited faster monocarpic senescence than its CMS line. CMS line maintained better water relations, lesser oxidative stress and better antioxidant defense than HW2041 under WDS. Co-ordinated antioxidant defense both in terms of higher AsA and GSH content and higher activities of SOD, APX, CAT and Halliwell-Asada pathway enzymes in CMS line compared to HW2041 under WDS helped in mitigating drought induced oxidative stress resulting in slower rate of flag leaf senescence. HW2041 showed early and higher induction of *WRKY53*, glutamine synthetase (*GS1*), cysteine protease (*WCP2*), serine protease (*WSP*) expression while *CAT2* expression was downregulated compared to CMS plants under WDS. Delayed senescence in CMS line was also accompanied by better antioxidant defense in the chloroplasts compared to HW2041 under WDS which may help in maintaining chloroplast integrity associated with delayed senescence. Hence, our study has shown that reproductive sink enhanced drought induced senescence in flag leaf of wheat fertile line is associated with lower RWC, higher oxidative stress and damage and loss of antioxidant competence compared to its sterile line under water deficit stress. Higher expression of some SAGs and decline in SOD and APX activity in the chloroplasts also contributed to the accelerated senescence in fertile line compared to its CMS line under water deficit stress.

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Compliance with ethical standards

Conflict of interest Dr. Khanna-Chopra has nothing to disclose.

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