BIOLOGIA PLANTARUM 57 (2): 370-374, 2013 DOI: 10.1007/s10535-012-0286-9

BRIEF COMMUNICATION

Drought induced programmed cell death and associated changes in antioxidants, proteases, and lipid peroxidation in wheat leaves

A. HAMEED*, M. GOHER, and N. IQBAL

Nuclear Institute for Agriculture and Biology, P.O. Box 128, Jhang Road, Faisalabad, Pakistan

Abstract

Two wheat (*Triticum aestivum* L.) genotypes with varying degree of drought tolerance were used to analyze programmed cell death (PCD) and related biochemical changes under drought stress. Drought induced PCD in leaves, as evident by internucleosomal nDNA fragmentation, was observed in sensitive genotype Nesser. Drought tolerant genotype (FD-83) showed higher peroxidase, superoxide dismutase, and catalase activities and ascorbate content under drought stress compared to sensitive genotype. Total phenolic content increased whereas lipid peroxidation remained un-changed under drought in FD-83. In contrast, drought enhanced the proteases and ascorbate peroxidase activities and lipid peroxidation (MDA content) in Nesser.

Additional key words: MDA, phenolics, proteases, *Triticum aestivum*.

Drought stress is one of the most important abiotic stresses seriously influencing crop productivity (Akhter *et al.* 2008). Generation of reactive oxygen species (ROS) are common events during drought (Farooq *et al.* 2009). ROS can damage chlorophyll, protein, DNA, lipids, and other important macromolecules, thus fatally affecting plant metabolism and limiting growth and yield (Sairam and Tyagi 2004). Recently, overproduction of superoxide anions by salt stress induced programmed cell death (PCD) in primary roots of wheat which was consistent with the progressive DNA laddering (Ling *et al.* 2009). Induction of ROS and necrotic death-like destruction in strawberry leaves has been reported (Tanou *et al.* 2009). Cell death has also been reported to occur in plants in response to other factors such as heat shock (Fan and Xing 2004), leaf senescence (Munne-Bosch and Alegre 2004), and recently by water stress in *Arabidopsis* root tips (Duan *et al.* 2010).

Plants have evolved both enzymatic and non-enzymatic systems to scavenge the ROS. Enzymes, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), non-specific (guaiacol) peroxidases (POD), glutathione reductase, *etc*., work in concert with non-enzymatic antioxidants such as glutathione and ascorbate to detoxify ROS (Kranner *et al.* 2006). Antioxidant defense system may have a crucial role in signaling and execution of plant PCD. An increased antioxidant defense contributed towards the delayed flag leaf senescence in wheat (Srivalli and Khanna-Chopra 2009). Moreover, plant stress generally becomes lethal when reduction potential of glutathione decreased considerably (Kranner *et al.* 2006).

To our knowledge, there is no report concerning drought induced cell death evident by DNA laddering and parallel changes in antioxidants in wheat. In this view, present study was conducted to analyze the drought stress induced PCD and to find out the differences in cell death induction and antioxidant response in drought tolerant and sensitive genotypes.

The experiment was conducted under controlled conditions using two wheat (*Triticum aestivum* L.) genotypes differing in drought sensitivity: FD-83 (relatively tolerant) and Nesser (relatively sensitive) (Hameed *et al.* 2010). Seeds were germinated (three replicates) in plastic pots filled with soil and sufficiently irrigated by water. Seedlings were grown in an incubator (*FISONS*, Loughborough, UK) at day/night temperature of 25/22 °C, a 16-h photoperiod, irradiance of 120 μmol m^{-2} s^{-1},

Received 7 March 2012, *accepted* 9 August 2012.

Abbreviations: APX - ascorbate peroxidase, AsA - ascorbate; CAT - catalase; MDA - malondialdehyde; PCD - programmed cell death; POD - quiacol peroxidase; PRO - protease; SOD - superoxide dismutase; SP - soluble protein; TPC - total phenolic content.

Acknowledgement: The authors are thankful to Pakistan Science Foundation for financial support through project P-NIAB-Bio (353).

* Corresponding author, fax: (+92) 41 9201776, e-mail: amjad46pk@yahoo.com

and relative humidity of 60 - 70 %. In control pots, water was maintained at soil water holding capacity. No irrigation was provided to drought treated plants and water in these pots dropped to approximately 10 - 20 % of soil water holding capacity. Leaf samples for biochemical analysis were collected at 19th day after sowing. Fresh leaves (0.5 g) were ground in a cold extraction buffer specific for different enzymes (Dixit *et al*. 2001). Samples were centrifuged at 15 000 *g* and 4 °C for 20 min. The supernatant was separated and used for the determination of different enzyme activities. SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) following the method of Giannopolitis and Ries (1977). One unit was defined as the amount of enzyme that caused 50 % inhibition of photochemical reduction of NBT. POD activity was measured using the method of Chance and Maehly (1955). Catalase (CAT) activity was estimated by the method described by Beers *et al.* (1952) One unit of POD and CAT was defined as an absorbance change of 0.01 min^{-1}. Protease activity was determined by the casein digestion assay described by Drapeau (1974) One unit was defined as the amount of enzyme that releases acid soluble fragments equivalent to A_{280} change of 0.001 min^{-1} at 37 °C and pH 7.8. APX activity was determined following the decrease in absorbance (Chen and Asada 1989). One unit was defined as A_{290} change of 0.01 min^{-1}. Total soluble protein content was measured as described by Bradford (1976). The lipid peroxidation was measured in terms of malondialdehyde (MDA) content (a product of lipid peroxidation) determined by the thiobarbituric acid reaction using method of Heath and Packer (1968) with minor modifications as described by Dhindsa *et al.* (1981) and Zhang and Kirkham (1994). For ascorbic acid determination, 2,6-dichloroindophenol (DCIP) method that measures only reduced ascorbic acid was used (Hameed *et al.* 2005). A micro colorimetric method as described by Ainsworth and Gillespie (2007) was used for total phenolics assay which utilizes Folin-Ciocalteu (F-C) reagent. The DNA from leaves was extracted as described by Hameed *et al.* (2004). To check cell death induced internucleosomal nDNA fragmentation, equal amounts of DNA were subjected to electrophoresis in 1.5 % (m/v) agarose gel at 100 V for 3 - 4 h. The gel was stained with ethidium bromide and observed under UV transilluminator. Stained gels were photographed using UVI pro-platinum gel documentation system (*UVItec*, Cambridge, UK). Significance of data was tested by analysis of variance and Duncan's multiple range test at $P < 0.05$ using *MSTAT* software. Values presented in the table are mean ± SD of triplicate data with 30 plants per replication.

PCD was evident by internucleosomal nDNA fragmentation that is a biochemical hall-mark of PCD and is detectable at advanced stages of water stress. This biochemical marker of cell death was clearly seen in relatively sensitive genotype Nesser (Fig. 1). In FD-83, apoptotic DNA fragmentation was not observed indicating maintained cell homeostasis and relative drought tolerance. Results thus provide evidence that drought stress at seedling stage induces PCD in wheat leaves through a pathway involving internucleosomal nDNA fragmentation. In connection to this observation, several morphological and biochemical similarities have been found between animal cells undergoing apoptosis and dying plant cells which include cytoplasm and nuclei condensation and shrinkage, the formation of DNA-containing (apoptosis-like) bodies, and genomic DNA degradation (Ryerson and Heath 1996, Stein and Hanson 1999, Wang *et al.* 1999, Young and Gallie 1999, Kawai and Uchimiya 2000, Xu and Hanson 2000). Moreover, cell death has been reported to occur in plants in response to drought (Munne-Bosch and Alegre 2004), heat (Fan and Xing 2004), and salt stress (Katsuhara 1997, Huh *et al.* 2002, Li *et al.* 2007, Ling *et al.* 2009, Tanou *et al.* 2009).

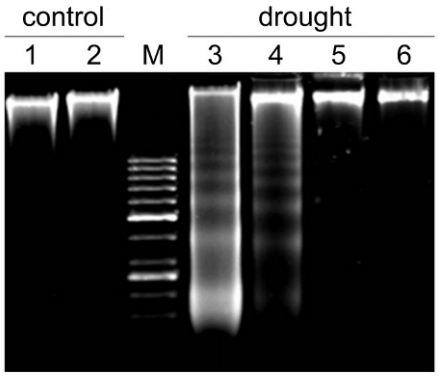

Fig. 1. Electrophoregram of DNA isolated from leaves of wheat seedlings grown under control conditions (1, 2) and drought stress (3 - 6). 1 - Nesser, 2 - FD-83, 3, 4 - Nesser, 5, 6 - FD-83, M - DNA molecular mass marker (50 - 1 000 bp).

In the present study, drought induced oligo-nucleosomal DNA fragmentation or DNA ladder formation at seedling stage is quite unique, as previously reported cell death by senescence in plants did not appear to involve accumulation of distinct oligonucleosomal DNA fragments. Nuclear DNA fragmentation was detected during leaf senescence of *Ornithogalum virens*, tobacco (Simeonova *et al.* 2000), and rice (Lee and Chen 2002) but no DNA laddering was observed. All these previous reports point out that leaf senescence did not involve cleavage of nuclear DNA into oligonucleosomal fragments and this marker is typical for apoptosis induced by drought stress at seedling stage in the present study. DNA laddering phenomenon is a very specific character of apoptosis in animals and plants (Danon *et al.* 2000). Previously in plants, DNA laddering phenomenon have been observed in different developmental processes (Danon *et al.* 2000), in wheat roots after D-mannose treatment (Hameed *et al.* 2008), under cold (Koukalova *et al.* 1997), UV radiation (Danon and Gallois 1998), salinity (Katsuhara 1997, Ling *et al.* 2009), and heat stress (Balk *et al.* 1999). Therefore it is important to point out that the cell death resulting from the drought stress at

Table 1. Content of malondialdehyde (MDA) and total phenolics (TPC), activities of catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), ascorbate peroxidase (APX), and protease (PRO), content of soluble proteins (SP) and ascorbate (AsA) in wheat leaves under control and drought stress. Means ± SE, $n = 3$. In each row, the values followed by the same letter did not differ at $P \leq 0.05$.

Parameters	Nesser (sensitive) control	drought	FD-83 (tolerant) control	drought
MDA [μmol g^{-1}(f. m)]	69.26±1.69a	88.77±4.37b	59.77±2.88c	59.77±2.17c
TPC [μmol g^{-1}(f. m)]	26295.65±1007.24a	33418.84±1657.19b	26897.10±1232.22a	31237.68±2153.28b
CAT [U g^{-1}(f. m)]	650.67±13.33a	714.67±23.25b	856.00±24.87c	949.33±27.33d
POD [U g^{-1}(f. m)]	45571.50±4885.03a	35551.10±3362.79b	31520.00±5245.95b	89224.20±4230.56c
SOD [U g^{-1}(f. m)]	166.41±4.04a	197.03±7.40b	155.82±6.61a	246.30±5.44c
APX [U g^{-1}(f. m)]	1300.00±120a	1650.00±115b	1150.00±102ac	1010.00±105c
PRO [U g^{-1}(f. m)]	2074.67±103.83a	2927.17±115.15b	4952.00±107.41c	5080.00±113.42c
SP [mg g^{-1}(f. m)]	5.76±0.27a	3.79±0.17b	6.60±0.20a	8.52±0.09c
AsA [μg g^{-1}(f. m)]	205.00±19.8a	160.00±11.2b	275.00±27.36c	300.00±30.12c

seedling stage in present study is different from the cell death resulting from drought induced senescence reported earlier (Rosa *et al.* 2007).

Second important observation is the difference in induction of cell death features in wheat genotypes. As DNA fragmentation was observed in drought sensitive genotype, it seems to relate with degree of drought tolerance of genotype. Previously, based on coincidence of the difference in drought tolerance between the two rice ecotypes and their differences in the osmotic/salt induced cell death, it has been postulated that cell death may have played an important role contributing to rice tolerances to different abiotic stresses (Liu *et al.* 2007). Similar seems to be true for wheat genotypes. This means wheat genotypes vary in induction of cell death program relative to degree of drought tolerance. Therefore, this biochemical marker proves to be useful for screening wheat genotypes relatively sensitive or tolerant to drought stress.

Application of drought stress significantly enhanced the membrane deterioration as reflected by increased MDA content. In Nesser (Table 1), MDA content was 28 % over the control while in FD-83 it remained unchanged under drought. Drought caused an increase in CAT activity in both genotypes, however, magnitude of increase was slightly higher in Nesser (Table 1). POD activity considerably increased under the stress in FD-83 while decreased in Nesser. A sharp increase in SOD activity was observed in FD-83 where increase over the control was 58 %. In Nesser, increase over the control was only 18 %. Furthermore, drought significantly raised the APX activity in Nesser (Table 1) but APX activity was not affected in FD-83. Among non-enzymatic antioxidants, total phenolic content was elevated under drought stress irrespective of genotype (Table 1). FD-83 had higher ascorbate content compared to sensitive genotype under stressed and non-stress conditions. This higher accumulation of ascorbate by the tolerant genotype may be in part responsible for its better performance under drought stress. Ascorbate content decreased in Nesser under drought while remained unchanged in FD-83. Proteolytic activity significantly increased in Nesser as a result of drought stress (Table 1). This increase in protease activity over the control was 41 %, while in FD-83 the activity was not affected. Drought stress significantly decreased the total soluble protein content in Nesser while increased it in FD-83 (Table 1).

Present finding pointed out that antioxidants like POD and ascorbate were repressed while lipid peroxidation and protease activity were enhanced under drought in Nesser in which drought induced apoptosis was also observed. Decreased ascorbate content in the sensitive genotype may be due to its conversion to dehydroascorbate by APX to quench H_2O_2 produced as a result of oxidative stress induced by drought. Observed higher APX activity in the sensitive genotype further strengthen this justification. Similarly, drought induced oxidative stress in rice anthers leading to a PCD and pollen abortion along with down-regulation of antioxidants has been reported (Nguyen *et al.* 2009). Possibly, the enhanced lipid peroxidation due to drought induced oxidative stress and increased proteolysis by raised protease activities may have compelled the sensitive genotype to trigger DNA degradation and cell death. In this connection, massive protein degradation that was largely due to cysteine protease activity has been reported recently in *Nicotiana tabacum* cell suspension during PCD induced by Yariv reagent (Chaves *et al.* 2011).

In conclusion, drought stress can induce PCD by apoptotic pathway in wheat leaves at seedling stage. To our knowledge, this is the first report of cell death involving apoptotic internucleosomal nDNA fragmentation induced by drought stress in wheat leaves during early seedling stage. Compromised antioxidant activities and enhanced protein, DNA, and lipid degradation play central role in drought induced cell death in drought sensitive genotype. On the contrary, antioxidative enzymes like CAT, POD, and SOD play a significant role in conferring drought tolerance in wheat.

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