



Ethylene induced stay-green gene expression regulates drought stress in wheat

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Stay-green is an integrated drought adaptation trait characterized by a green leaf phenotype during grain filling under terminal drought. Ethylene is the key hormone for regulating the leaf senescence pathway under natural and stress conditions. The present study was conducted to assess the associative function of ethylene in regulating chlorophyll degrading enzymes viz., chlorophyllase (*TaCHLase*) and pheophorbide a oxygenase (*TaPaO*) in wheat (*Triticum aestivum* L.) under drought stress. Three wheat genotypes (HW 4059, HW 4022 and HW 2078) differing in drought tolerance efficiency were subjected to drought stress for ten days at the reproductive stage. A decline in stay-green traits was found in susceptible genotypes (HW 4059) with yield losses compared to tolerant ones (HW 4022 and HW 2078). The expression level of *TaCHLase1* and *TaPaO* was higher in susceptible genotypes than tolerant ones under drought/osmotic stress. Ethylene upregulated, while ethylene inhibitors downregulated the gene expression. In this study, a novel gene annotated as *TaCHLase1* was cloned. The complete cDNA sequence of *TaCHLase1* is composed of 1054 bp nucleotides containing an open reading frame of 960 bp encoding 319 amino acids. The encoded protein contained conserved residues such as lipase motif GX SXGG at position 143-148 and putative active site Ser145. Sequence alignment showed *TaCHLase1* shares a higher degree of identity with other species. The result suggested that ethylene upregulates the expression of *TaCHLase1* gene, inducing chlorophyll degradation. The study further helps in understanding the mechanism of stay-green trait-induced drought tolerance mechanism in wheat.

Keywords: Abiotic stress, cDNA, Cloning, *TaPaO*, *TaCHLase1*, *Triticum aestivum* L., Water deficit stress

Impacts from climate related extremes in the recent years make our planet earth limited for natural resources particularly, water and nutrients¹. Water scarcity threatens the crop production in many part of the world, because agriculture alone consumes about two-thirds of the total amount of fresh water available on earth. Drought a serious abiotic stress, which greatly affects the growth and development of economically important crops. As per the report of FAO, over 1.5 billion people relying on agriculture as the main source of income, drought is putting the livelihood of many at risk, often halting and reversing gains in food security and poverty reduction. Overall drought-driven annual yield loss is estimated between 3.4-26.6% in different crops². Plants use various morphological, anatomical and physiological mechanisms to cope with environmental stress. Some plant species exhibit stress tolerance,

while others employ escape or avoidance strategies involving highly plastic adaptation and acclimation mechanisms, in which plant hormones are key factor³.

Wheat is one of the important cereal crops that feed about one-fifth of the world's population⁴. Moreover, the most important constraints for agriculture are declining water resources with frequent drought episode is nowadays becoming the major challenge in wheat production worldwide^{5,6}. Drought stress affects wheat performance throughout the growth stages but most critically during anthesis and grain filling stage which results in tremendous yield losses⁷. In order to mitigate the drought stress in wheat, there is a need to develop an improved knowledge of the various plant mechanisms and tolerant genotypes with improved traits and high water use efficiency⁸. In wheat, cultivar with stay-green trait and delayed leaf senescence is an important drought adaptive strategy as photosynthesis is the most sensitive process to drought stress^{9,10}. In plants, leaf senescence is a highly

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regulated and developmental process occurred during crop maturation or under the influence of various abiotic stresses. If drought occurs during reproductive stage, leaf senescence rate accelerates resulting in substantial yield reduction¹¹. Chlorophyll degradation is one of the earliest symptoms of leaf senescence resulting in subsequent loss of leaf greenness and photosynthesis activity¹². Chlorophyll degradation pathway have been resolved by several researchers involving a multistep pathway involving dephytylation reaction of chlorophyll molecules into specific chlorophyllide followed by removal of the central magnesium ion by Mg-dechelataase¹³⁻¹⁵. During leaf senescence process levels of many plant hormones are altered. Phytohormones like ethylene, jasmonic acid, abscisic acid and salicylic acid promote leaf senescence, whereas cytokinin, auxin, gibberellic acid, nitric oxide, and polyamines suppress the process¹⁶. Among them ethylene is the key hormone for regulating leaf senescence by accelerating the chlorophyll degradation pathway under natural as well as in stress condition¹⁷. It has been reported that during drought stress leaf senescence rate is triggered for maintaining water status and nutrient balance in plant but under severe condition it leads to tremendous reduction in photosynthesis activity and yield¹⁸.

Thus, delayed leaf senescence rate has become a desirable trait for drought stress and the knowledge of chlorophyll degradation pathway is particularly important for developing stay-green wheat genotype. In the present study, we hypothesized that drought induced chlorophyllase gene may be regulated by ethylene in wheat crop. Similarly, cloning and expression of genes encoding chlorophyll degrading enzymes *viz.*, chlorophyllase (*TaCHLase1*) and pheophorbide a oxygenase (*TaPaO*) in the presence of ethylene inducer, help us to understand the mechanisms regulating the process of drought tolerance in wheat.

Materials and Methods

Plant materials and treatments

Pot experiments were conducted with three wheat genotypes differing in their drought tolerance efficiency *viz.*, HW 4022 (drought tolerant), HW 2078 (relatively drought tolerant) and HW 4059 (drought susceptible). These genotypes were grown with recommended package of practices and subjected to water deficit stress at 50% anthesis stage by

withholding irrigation for 10 days. Scheduled routine of irrigation was practiced for control plants throughout the crop growth period. For biochemical and molecular analysis, flag leaf sampling was done from both control Relative Water Content (RWC) (RWC; 80-85%) and stressed (RWC; 65-75%) plants. For *In vitro* analysis, seeds of the above mentioned genotypes were surface sterilized and then germinated in Petri dishes. After 48 h of incubation, uniformly germinated seeds were selected and raised in 50 mL test tubes containing half-strength Hoagland solution. The hydroponically raised seedlings were grown for 10 days in growth chamber at 25°C day/20°C night, light intensity 1200 $\mu\text{mol m}^{-2}\text{s}^{-1}$ and 90% relative humidity. Osmotic stress was imposed to 10 days old seedlings using 20% polyethylene glycol (PEG 6000) solution giving osmotic potential of -4.91 MPa in presence of ethylene inducer (Ethrel; 10 ppm), ethylene biosynthesis inhibitor (Aminoethoxy vinylglycine; AVG, 2 ppm) and ethylene signalling inhibitor (1-methylcyclopropene; 1 MCP, 10 ppm) for 6 h whereas, double distilled water served as control.

Photosynthesis traits

Chlorophyll and carotenoid content were estimated by extracting 0.5 g fresh leaf sample in 10 mL dimethyl sulfoxide (DMSO)¹⁹. Chlorophyll content was calculated as per Arnon²⁰, while carotenoid content was determined using the formula given by Lichtenthaler & Welburn²¹. The values were expressed in mg/g dry wt. of the sample.

Total chlorophyll content (mg gDW^{-1}) =

$$[20.2 \times A_{645} + 8.02 \times A_{663}] \times (\text{Vol. of extract/Dry wt.}) \times 1000$$

Total carotenoid content (mg gDW^{-1}) =

$$[A_{470} + (0.114 \times A_{663}) - (0.638 \times A_{645})] \times (\text{Vol. of extract/Dry wt.}) \times 1000$$

Gas exchange parameters like photosynthesis, transpiration and stomatal conductance were measured using portable Infrared Gas Analyzer (IRGA), LI-6400XT Model (Li-COR Ltd., Lincoln, Nebraska, USA) by operating the IRGA in the closed mode between 10.00-11.00 a.m. when relative humidity, temperature, photosynthetic photon flux density and CO₂ conc. ranged from 50-60%, 30 to 35°C, 120°C $\mu\text{mol m}^{-2}\text{s}^{-1}$ and 350 to 360 $\mu\text{mol mol}^{-1}$, respectively. Fifteen flag leaves per treatment were selected at random for measurement.

Yield and associated parameters

Measurements on 1000 seed weight, grain yield plant⁻¹ were recorded in control and water deficit stressed plants as economic yield. The whole plant dry weight was measured as biological yield. Three

replications were taken for each parameter. Drought Tolerance Efficiency (DTE) percent was calculated using the formula given by Fischer and Wood (1981)²² and harvest index (HI; %) was calculated as the ratio of the economic yield to biological yield and was expressed in percentage.

$$\text{DTE (\%)} = \frac{(\text{Yield under water deficit stress})}{(\text{Yield under irrigated condition})} \times 100$$

$$\text{HI (\%)} = \frac{(\text{Economic yield})}{(\text{Biological yield})} \times 100$$

RNA isolation and cDNA synthesis

In order to determine the semiquantitative gene expression analysis of chlorophyll degrading genes (*TaCHLase1* and *TaPAO*); the total RNA was extracted from the flag leaf after 10 days of water deficit stress in pot culture and leaves from 10 days old seedlings after above mentioned treatment combinations. Isolation of total RNA was carried out by TRIzol® reagent (Invitrogen, USA). By running appropriate amount of formamide denaturing gel RNA quality and integrity were resolute while quantity of total RNA was determined using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, USA). The first-strand cDNA was synthesized according to the instructions of the cDNA Synthesis Superscript® III First- Strand Synthesis System (Invitrogen, USA). Resulting cDNA was stored at -20°C and employed as template for two-step RT-PCR reactions following recommended conditions provided in user's manual.

Primer designing

Reported nucleotide sequence of pheophorbide a oxygenase (PaO) gene from National Centre for Biotechnology Information (NCBI) database *viz.* Accession No. JN689384 was selected for designing gene specific primers for semi-quantitative gene expression analysis (RT-PCR) in wheat. Search for nucleotide sequence in NCBI database for chlorophyllase (*CHLase1*) gene in wheat failed to retrieve any results. Hence we selected the protein sequence of *CHLase1* from *Arabidopsis* (NCBI Accession No. NP_564094) and performed TBLASTn analysis with nucleotide sequence database of NCBI in wheat. We obtained a cDNA sequence

from wheat (GenBank accession No. BT009214) having 95% coverage and 48% identity with *Arabidopsis CHLase1*. The complete open reading frame (ORF) coverage was confirmed by ORF finder tool of NCBI. The deduced amino acid sequence using ExpAsy translate tool of the putative *CHLase1* gene showed significant homology with rice, *Arabidopsis* and *Brachypodium* protein sequences by BLASTp analysis. This putative sequence of *TaCHLase1* was used for designing gene specific primers for semi-quantitative expression analysis (RT-PCR) and cloning in wheat. The gene specific primers were designed manually and the quality parameters were confirmed using Integrated DNA Technologies (IDT) Oligo analyzer tool (<https://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/>). The primers used for semiquantitative RT-PCR analysis and cloning are listed in Table 1. Every RT-PCR measurement was performed at least thrice. Expression of *TaActin* was used as an internal standard for normalization.

Cloning and sequencing of the *TaCHLase1* cDNA

PCR amplification with gene specific primers and proofreading enzyme Platinum Hi-fidelity *Taq* DNA polymerase (Invitrogen, USA) gave approximately 1 kb fragment that was cloned in TA cloning vector pTZ57R/T (Thermo Scientific, USA). The construct was transformed into *E. coli* strain XL1-Blue and confirmed by blue-white screening in a media containing IPTG (Isopropyl β-D-1-thiogalactopyranoside), X-gal and kanamycin. Positive clones were sub cultured in medium having same composition and further confirmed by colony PCR, restriction digestion and sequencing.

In silico analysis of *TaCHLase1* gene sequence

BLAST online software (<http://www.ncbi.nlm.gov/blast>) was used to analyze the cDNA and protein sequences. Prediction of putative subcellular localization was carried out using the WoLF PSORT, SMART program and TargetP 1.1. Its isoelectric point (pI) and molecular weight (MW) were analyzed by ExpAsy ProtParam tool. The multiple sequence alignment of *TaCHLase1* and its homologs from other species was carried by ClustalW2 tool of BioEdit

Table 1 — Primers sequence with GC%, TM and Product size of expression analysis of *TaPaO* and *CHLase1* genes

Gene	Primer	Primer sequence	% GC	TM	Product size (Bp)
Pheophorbide a oxidase (<i>TaPaO</i>)	PAO_F	ACCGGTAATCCTCGTATCACTG	50.0	56	360
	PAO_R	AGTGAGCTTTGTGTACTGCTG	47.6		
Chlorophyllase (<i>CHLase1</i>)	CASE_F	ATCTGGACATGCTGGACGAC	55.0	55.1	300
	CASE_R	GCTCAGAGGTACAGTACATCTG	50.0		

software and represented using BoxShade server of ExPASy. The phylogenetic tree was constructed with the neighbor joining clustering method using MEGA 6.06 software with 500 bootstrap replicates for ClustalW alignment and the evolutionary distances were estimated using Poisson correction model.

Statistical analysis

Statistical results are expressed as means with standard error (S.E.). Significance difference (at $P < 0.05$) between control and stressed samples were determined by Duncan's multiple range tests at a significance level of 0.05 for all biochemical parameters and was evaluated by analysis of variance (ANOVA). ANOVA and critical difference value were calculated using SPSS 10.0 (SPSS Inc., Chicago, IL, USA), OPSTAT (hau.ernet.in/opstat.html) and Microsoft Excel.

Results and Discussion

Photosynthesis is one of the most drought sensitive physiological process while, chloroplast injury is commonly the first sign of abiotic stress in plants²³. Drought stress damages the chloroplast thylakoid membrane, disturbing its functions and ultimately decreasing the rate of assimilation and crop yield⁸. Changes in photosynthetic pigments like chlorophyll and carotenoids contents provide further insight into the modification processes taking place in the photosynthetic apparatus during drought stress²⁴. Thus, drought-induced disorganization of chloroplast

structure and changes in photosynthetic pigments *viz.*, chlorophyll and carotenoid content is considered to be a good indicator of plant water status under drought stress²⁵.

Water deficit stress and gas exchange characteristics

Water deficit stress induced reduction in the gas exchange parameters differ significantly amongst the genotypes as well as between the treatments (Fig. 1). Reduction in photosynthesis rate, transpiration rate and stomatal conductance was more in susceptible non stay-green genotype (HW 4059) as compared to stay-green tolerant genotypes (HW 4022 and HW 2078). In addition, susceptible genotype recorded the maximum reduction in chlorophyll and carotenoid pigments while, it was comparatively higher in tolerant genotypes under water deficit stress (Fig. 1 A & B). Many researchers reported water stress reduced the stay-green traits like transpiration, stomatal conductance and chlorophyll in wheat^{26,27}, sorghum²⁸, chickpea²⁹, pearl millet³⁰ and maize³¹.

Water deficit stress and yield attributes

Water deficit stress showed significant decline in 1000 seed weight and harvest index in all the three genotypes (HW 4022, HW 2078 and HW 4059) (Fig. 2 A & B). Decline in terms of 1000 seed weight was more in susceptible genotype (HW 4059) as compared to tolerant ones (HW 4022 and HW 2078) under water deficit stress (Fig. 2A). Similarly, HW 4022 maintained highest drought tolerance efficiency

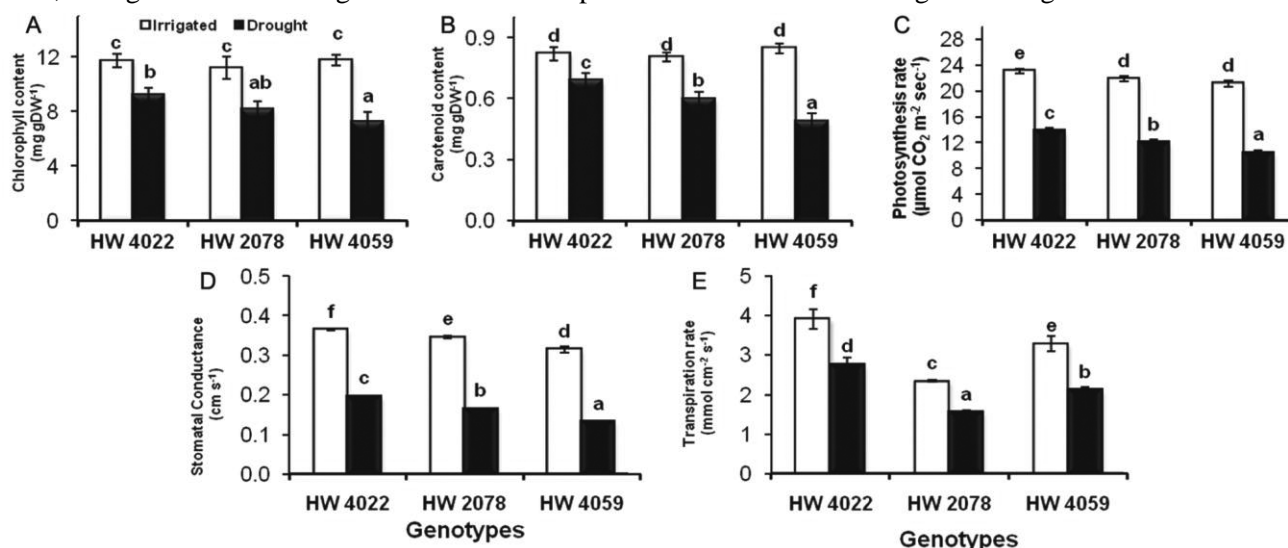


Fig. 1 — Stay-green trait indicators (A) Total chlorophyll content; (B) Carotenoid content; (C) Photosynthesis rate; (D) Stomatal conductance; and (E) Transpiration rate in three wheat genotypes *viz.*, (HW 4022; drought tolerant, HW 2078; relatively drought tolerant and HW 4059; drought susceptible) subjected to water deficit stress by withholding irrigation for 10 days at anthesis stage. [Error bars indicate \pm Standard Error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$). DW, Dry weight; and CO₂, Carbon dioxide]

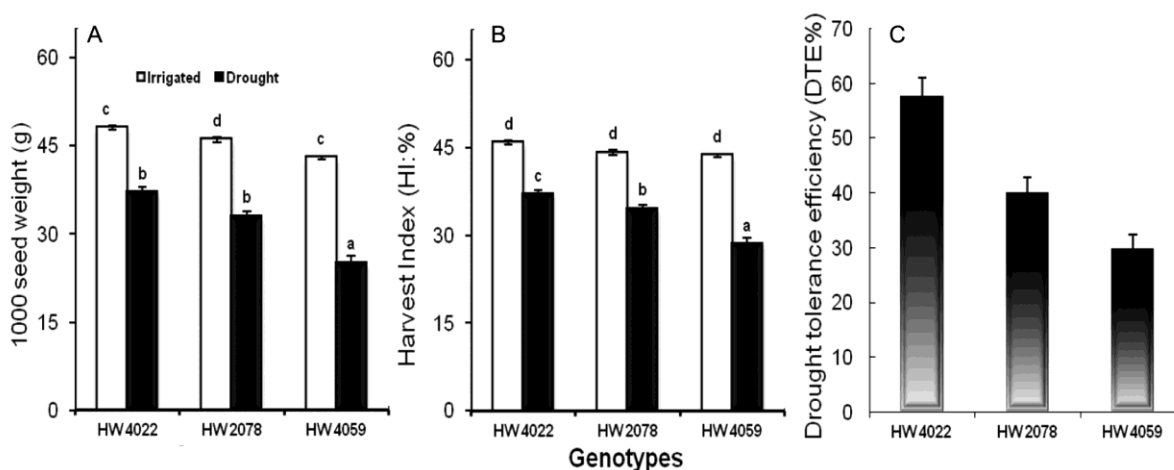


Fig. 2 — Yield traits attribute (A) 1000 seed weight; (B) Harvest Index; and (C) Drought tolerance efficiency in three wheat genotypes *viz.*, (HW 4022; drought tolerant, HW 2078; relatively drought tolerant and HW 4059; drought susceptible) subjected to water deficit stress by withholding irrigation for 10 days at anthesis stage. [Error bars indicates \pm Standard Error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$). DTE, Drought tolerance efficiency; and HI, Harvest Index]

(DTE; %) followed by HW 2078 while, HW 4059 found to have lowest DTE% value (Fig. 2C). Results also showed that increase in yield traits in tolerant genotypes is closely associated with higher photosynthesis rate and stomatal conductance under drought stress (Figs 1C, D & Fig. 2C) Statistical analysis revealed highly significant and positive correlation between grain yield, harvest index and drought tolerance efficiency when compared with photosynthesis rate in the studied genotypes and proved that positive correlation exist between stay-green traits and yield in wheat under drought stress. Earlier findings reported in crop like maize³² and rice³³ suggesting that, delayed leaf senescence rate by maintaining the photosynthesis characteristics can be positively correlated with higher grain yield when stress occurred at drought sensitive stage in plants.

Expression Profiles of *TaCHLase1* and *TaPaO* under osmotic/ water deficit stress

The amount of chlorophyll content directly determines the photosynthetic potential of crop plants. Studies on transcript level of *TaPaO* and *TaCHLase1* genes using gene specific primers resulted in 360 and 300 bp amplicon size (Suppl. Plates 1 & 3. *All supplementary data are available only online along with the respective paper at NOPR repository at <http://nopr.res.in>*), respectively and further confirmed by sequencing (Table 1) in all the three wheat genotypes in presence of various chemical regulators (ethylene inducers/ inhibitors) (Fig. 3 A & B). Significant differences were observed in gene

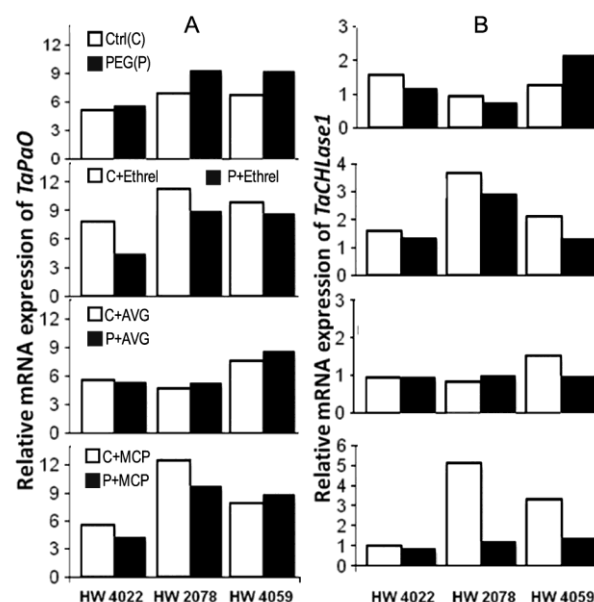


Fig. 3 — Expression analysis of (A) *TaPaO* and (B) *TaCHLase1* gene encoding enzyme involved in chlorophyll degrading pathway in 10 days old wheat seedlings of genotypes HW 4022 (drought tolerant), HW 2078 (relatively drought tolerant) and HW 4059 (drought susceptible). Different panels showing relative mRNA expression (IDV) under various treatment combinations of (A) *TaPaO*; and (B) *TaCHLase1* gene expression. Control and osmotic stress (20% PEG 6000, 4.91MPa) conditions; Ethylene inducer (Ethrel, 10 ppm) under control and osmotic stress; Ethylene biosynthesis inhibitor (AVG, 2 ppm) under control and osmotic stress; Ethylene action inhibitor (1 MCP, 10 ppm) under control and osmotic stress; Ethylene action inhibitor (1-MCP, 10 ppm) under control osmotic stress. [Each value represents the mean (\pm SE) with three replicates each. PaO, Pheophorbide a Oxygense; PEG, Polyethylene Glycol; AVG, Aminoethoxy Vinyl Glycine; 1 MCP, 1 Methyl Cyclopropene; and CHLase, Chlorophyllase]

expression level of *TaPaO* and *TaCHLase1* under control (RWC; 80-90%) and osmotic stress (RWC; 70-80%) in 10 days old leaf sample. Amongst the three genotypes studied, susceptible genotype (HW 4059) showed higher expression level of both chlorophyll degrading genes *TaPaO* and *TaCHLase1* followed by relatively drought tolerant genotype (HW 2078), while lowest expression was observed in case of tolerant genotype (HW 4022) under both water regimes. Ethrel application (ethylene inducer) slightly induced the expression level of *TaPaO* under osmotic stress in HW 4059 and HW 2078 but the genotype HW 4022 maintained the lower expression level, while in case of *TaCHLase1* gene, HW 2078 showed higher transcript level under both control and osmotic stress. In presence of AVG (Ethylene biosynthesis inhibitor, Aminoethoxy vinylglycine) and 1-MCP (Ethylene signalling inhibitor, 1-Methylcyclopropene) the transcript of *TaPaO* and *TaCHLase1* decreased significantly under both osmotic regimes while, in case of HW 2078 the expression of *TaPaO* was comparatively high under control and osmotic stress condition. In pot culture condition, after imposing drought stress for 10 days at reproductive stage the expression level of *TaPaO* drastically reduced in HW 4022 and HW 2078 but in HW 4059 the expression level is high under both water regimes (Suppl. Plate 2 & Fig. 4A). In case of *TaCHLase1* expression level was maintained to be high in HW 4059 and HW 2078, while tolerant genotype HW 4022 showed comparatively reduced expression level of *TaCHLase1* under irrigated as well as water deficit stress condition (Suppl. Plate 4 & Fig. 4B). Similar to above finding, earlier studies have shown the over-expressing of *OsPaO* gene in rice with enhanced leaf senescence rate and cell death^{34,35}. Treatment of ethylene perception inhibitor (1-MCP) resulted in higher level of chlorophyll content which can be positively correlated with lower expression of chlorophyll degrading enzymes in tomato, citrus and cabbage³⁶⁻³⁸.

Cloning and *In silico* characterization of *TaCHLase1* gene from wheat

The molecular mechanism governing the function of *TaPaO* gene in wheat³⁹ and perennial ryegrass⁴⁰ is already known, but little information is available on the molecular characteristics of *TaCHLase1* gene in wheat. Here, we identified the novel and putative chlorophyllase gene from hexaploid wheat (*Triticum aestivum* L.) and annotated as *TaCHLase1* owing to its homology with *AtCLH1*.

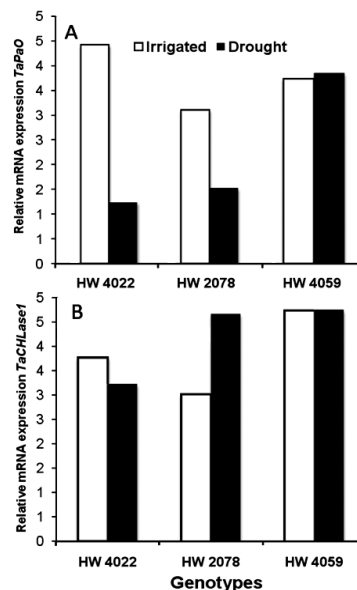


Fig. 4 — Expression analysis of *TaPaO* and *TaCHLase1* gene encoding enzyme involved in chlorophyll degrading pathway in flag leaf of wheat genotypes HW 4022 (drought tolerant), HW 2078 (relatively drought tolerant) and HW 4059 (drought susceptible) under control (normal irrigation) and water deficit stress condition in wheat. (A) Relative mRNA expression of *TaPaO* gene; and (B) Relative mRNA expression of *TaCHLase1* gene. [Each value represents the mean (\pm SE) with three replicates each. PaO, Pheophorbide a Oxygenase; and CHLase, Chlorophyllase]

A full length cDNA encoding a polypeptide with sequence similarity to *Arabidopsis* chlorophyllase (*AtCHLase1*) was amplified by PCR of cDNA using gene specific primers and cloned from drought susceptible non-stay-green wheat genotype HW 4059. Through the bioinformatical analysis, obtained cDNA sequence contained an open-reading frame for a 319 amino acid polypeptide with a calculated molecular mass of 33.84 kDa and isoelectric point of 5.71. The multiple protein sequence alignment (ClustalW2) with *Arabidopsis* and *Brachypodium* showed considerable homology between the sequences by 45% and 81% with *Arabidopsis* and *Brachypodium* *CHLase1* genes, respectively. Multiple sequence alignment also showed conserved residues such as lipase motif (GX₂SXGG) i.e., GHSRGG of wheat at amino acid position 143-148 and the putative active site serine (Ser145) (Fig. 5). Cloning of *TaCHLase1* from wheat is a significant breakthrough in studying the stay-green trait phenotype in crop plants. Multiple sequence alignment showed that *CHLase* is widely distributed in plant kingdom.

The phylogeny tree has been constructed (MEGA 6.06 software) using Neighbor joining method for

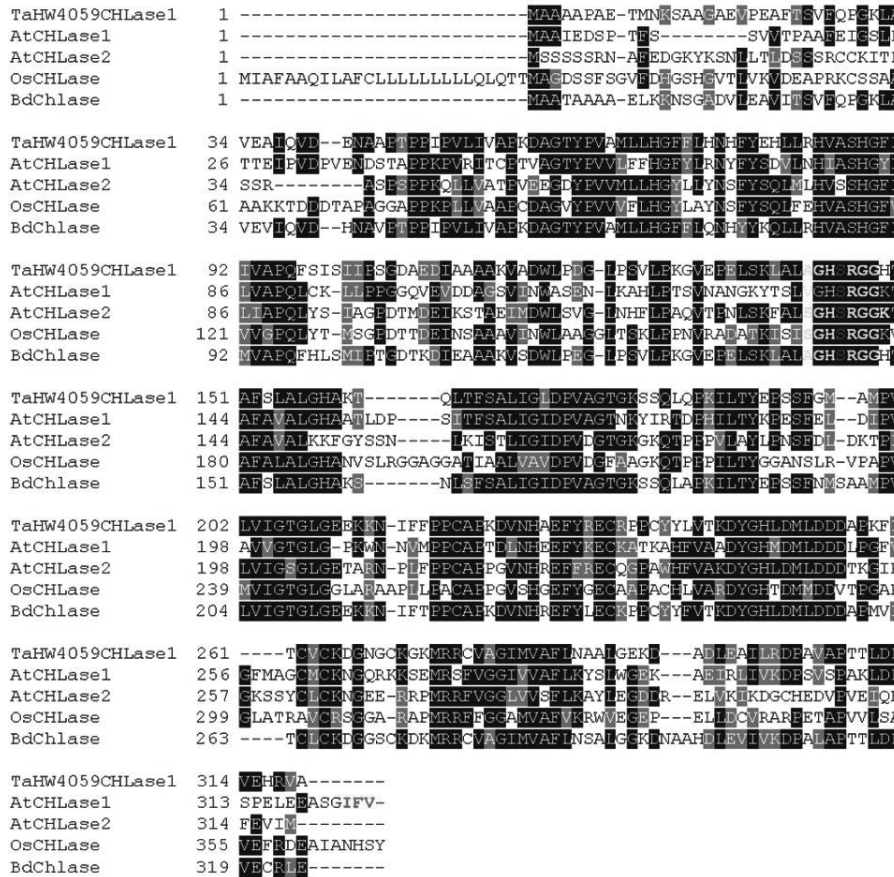


Fig. 5 — Alignment of *TaCHLase1* and other homologous *CHLase* proteins using ClustalW2 and represented by Box Shade. Dark shaded regions show amino acid residues conserved in all five sequences and grey shaded regions represent similar residues. Conserved lipase motifs are indicated by yellow shade, while catalytically active serine is highlighted in red colour. The post-transcriptionally removed motif at the N and C- terminus are highlighted in green colour. [Sequences used in addition to *TaCHLase1* are: *Arabidopsis thaliana* (*AtChlase1*, NCBI GenBank accession no. NP_564094); *Arabidopsis thaliana* (*AtChlase2*, NCBI GenBank accession no. NP_199199); *Oryza sativa* (*OsChlase*, NCBI GenBank accession no. NP_001064620); and *Brachypodium distachyon* (*BdChlase*, NCBI GenBank accession no. XP_003576870)]

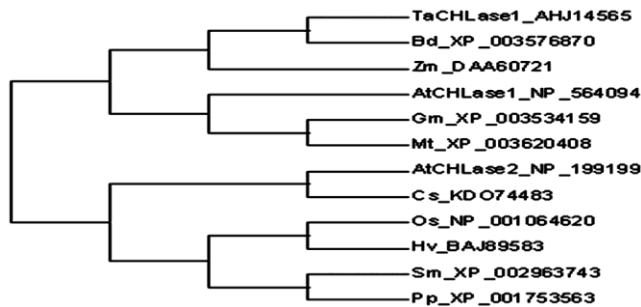


Fig. 6 — Phylogenetic tree constructed by neighbor-joining method of MEGA 6.06 with 500 bootstrap replicates of ClustalW alignment. Evolutionary distances were estimated using poisson correction model. Phylogenetic tree of *CHLase* proteins from different plant species along with their accession numbers are provided. [Sequences used: Ta, *Triticum aestivum*; At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Bd, *Brachypodium distachyon*; Hv, *Hordeum vulgare*; Sm, *Selaginella moellendorffii*; Zm, *Zea mays*; Gm, *Glycine max*; Mt, *Medicago truncatula*; Ct, *Citrus sinensis*; and Pp, *Physcomitrella patens*]

TaCHLase1 using the amino acid sequence from the selected plant species (<http://www.phytozome.net/>), which gave light into the evolution of the *TaCHLase1* (Fig. 6). The evolution of chlorophyllase can be dated back from the fern (*Selaginella mollendorffii*) and bryophyte (*Physcomitrella patens*) to higher plants

like dicots and monocots. The result showed that the available *CHLase* proteins from various plant species were classified into two groups. The first group mainly included *PpCHLase* (*Physcomitrella patens*), *SmCHLase* (*Selaginella mollendorffii*), *OsCHLase* (*Oryza sativa*), *HvCHLase* (*Hordeum vulgare*), *CsCHLase* (*Citrus sinensis*) and *AtCHLase2* (*Arabidopsis thaliana*), while the second group included *MtCHLase* (*Medicago truncatula*), *GmCHLase* (*Glycine max*), *ZmCHLase* (*Zea mays*), *BdCHLase* (*Brachypodium distachyon*), *AtCHLase1* (*Arabidopsis thaliana*) and *TaCHLase1* (*Triticum aestivum*). Interestingly, the phylogeny tree showed higher identity between *TaCHLase1* and *AtCHLase1* (45%) than with *OsCHLase* (40%). Consensus with the previous reports⁴¹⁻⁴³, the current study on comparative analysis of protein sequences revealed significant homology between wheat and other higher plants.

Conclusion

Results of the above study conclude that stay-green is an integrated drought adaptation trait in crops and ethylene is involved in regulating chlorophyll

degrading enzymes under drought stress. Molecular mechanism of chlorophyll degrading enzymes chlorophyllase and pheophorbide a oxygenase during drought stress provide us with the strong sign that chlorophyllase gene might be the important gene regulating chlorophyll catabolic pathway and its expression is directly or indirectly regulated by certain environmental clues like drought stress and plant growth regulators. Overall, the ethylene regulates the chlorophyll catabolic gene *TaCHLase* at transcriptional level during drought induced leaf senescence. The sequence information of *TaCHLase1* deserves further investigation by reverse genetic approaches and functional validation recognizing chlorophyllase gene as a candidate gene for developing stay-green drought tolerant wheat variety.

Conflict of Interest

Authors declare no competing interests.

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