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Functional analysis of Candidate Genes Associated with Stay Green Trait in Wheat

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Abstract—*The delayed senescence (stay green) has been frequently attributed to significant yield gains in different crops including wheat. This study aims to check the expression profiling of orthologous stay green genes reported from rice, sorghum in wheat genotypes. A total of 100 wheat genotypes were evaluated and four new lines with stay green phenotype were identified through field screening and chlorophyll content analysis. All the data recording and chlorophyll content estimation was done in four growth stages (GS69, GS73, GS77 and GS83). The chlorophyll content in stay green and non-stay green genotypes was similar during initial stages of growth. During later stages, the chlorophyll content in stay green was clearly higher than that of non-stay green genotypes. Over 167 primers were designed from known sequences of other crops and screened for amplification and polymorphism. Five of these primers produced clear profile with single band. These primers were used for QRT PCR using six lines including five stay green and one non-stay green line. Three of these primers 4540 F1R2, 4540 F, 5563 F showed differential expression between stay green and non-stay green genotypes. The expression was more in stay green genotypes than in non-stay green genotypes. All the three primers were originally designed from different regions of the same gene sequence from Oryza sativa var. japonica NYC3 mRNA. These results indicating that this region (sequence) of the genome may be highly correlated with stay green trait in wheat. While other two primers could not show differential expression within the contrasting genotypes. There expression was low and found almost consistent in all growth stages. The results are quit expected because insertion/deletion within the gene is very rare most of the distinct phenotypes arise due to singe base substitution so the quantitate estimation of gene expression is prerequisite. Further investigation on expression and regulation of this gene may provide information on the mechanism of this trait and its potential relationship with drought tolerance in wheat.*

1. INTRODUCTION

The stay-green or delayed senescence trait is accountable for the preservation of green coloration in the stem and leaf, during physiological maturity. It has also been known to play a significant role in the increase of grain size. The mechanism of senescence may get affected by number of candidate genes which are expected to have differential expression in stay green and non-stay green genotypes/RILs. Stay-green trait not only negatively associated with spot blotch disease but play a crucial role under drought and heat stress conditions. The trait is well known in sorghum and contributes about 15-20% additional yields in comparison to the non-stay green plants. Stay-green has been studied at molecular level in sorghum, maize and rice but little information is available in wheat. The stay-green trait has been reported to influence grain yield of different crops especially under drought stress conditions. Positive correlation of stay green trait with high grain yield has been found in sorghum (Rosenow et al, 1983; Victor et al, 1989; Evangelista and Tangonan, 1990), soybean (Phillips et al, 1984) and maize (Duvick, 1984; Russell, 1991). Stay green trait in wheat has been associated with increase in leaf area, rate and duration of grain filling and photosynthetic competence (Spano et al, 2003). Stay green duration of flag leaves and harvest index showed positive correlation with water use efficiency during grain formation of wheat (Gorny and Garczynski, 2002). In sorghum, stay-green genotypes are reported to not only remain green but also contain significantly more carbohydrate in the stem at all maturity stages than go-brown types and have a higher grain weight (Tuinstra et al, 1997; Mc Bee et al, 1983). Under water limited conditions, stay green genotypes of sorghum retain more green leaf are than do genotypes not possessing this trait, and they also continue to fill grain normally under drought conditions (Rosenow et al., 1983). Moreover, there is a positive association between stay-green and grain yield under water-limited environments (Borrell and Hammer, 2000). The beneficial effect of stay green trait towards grain yield is also reported in soybean (Philips et al., 1984), maize (Duvick, 1984; Russel, 1991; Ceppi et al., 1987) and sunflower (Cuckadar-Olmedo and Miller, 1997). Stay green trait is also known to reduce lodging (Woodfin et al., 1981) and there is good association with resistance to stem rots as well (Rosenow et al, 1983; Evangelista and Tangonan, 1990) suggesting that

stay green leaves remain photosynthetically active. The genotypes showing stay green trait must be having such genes, which prevents degradation of chlorophyll molecules.

2. MATERIAL AND METHODS

This experiment was carried out at the experimental farm of TERI Gram (The Energy and Resources Institute) at Gurgaon, Haryana in Rabi seasons of 2012 and 2013. Off-season facility at Wellington, Tamilnadu (India) was also utilized to advance the generations of the crosses during May-October to save time. A set of 100 released varieties and lines and an RIL derived (Fig 1) from the cross (Sonalika x Chirya3) was evaluated for the stay green trait (delayed senescence in comparison of the standard reference genotype) in the field. For this purpose, descriptor for stay green trait at different growth stages as developed by BBCH-scale was followed. Stay green trait was measured using two approaches (i) difference of leaf and spike greenness scores on a 0–9 scale (ii) a new parameter ‘‘leaf area under greenness’’ (LAUG). In the first approach, SG trait was recorded on the basis of visual scores (0–9 scale) for both flag leaf and spike at the late dough (GS 87) stage. The difference between flag leaf and spike scores was considered to group genotypes as SG (>3–6), moderately stay green (MSG) ([2–\3), moderately non stay green (MNSG) ([>1–<2) and non-stay green (NSG) (0–<1). In the second approach, LAUG was determined using a modified formula earlier used for leaf area under decline (LAUD). This approach was based on the method employed for estimating AUDPC. In the present approach (LAUG), a 0–9 scale was used and Yi was taken as the difference of green area under spike and flag leaf.

Estimation of Chlorophyll content

The chlorophyll content of the flag leaf at different growth stages i.e. GS69, GS73, GS77 and GS83 was estimated following Hiscox and Israelton (1979). In this method, 50 mg of leaf samples were incubated in 7.0 ml dimethyl sulfoxide (DMSO) at 65°C for 2 hours. At the end of the incubation period, the supernatant was decanted and the volume was made up to 10 ml with DMSO. The absorbance of the extract was read at 645 and 663 nm in spectrophotometer (Shimadzu-UV2450) keeping DMSO as a blank. The amount of chlorophyll was calculated by using the following formula;

$$Chlorophyll\ a = (12.7\ x\ A\ 663) - \left(2.69\ x\ A\ 645\ x \frac{V}{1000\ x\ W}\right)\left({}^{mg}/_{g\ fr.wt.}\right)$$

$$Chlorophyll\ b = (22.9\ x\ A\ 645) - \left(4.68\ x\ A\ 663\ x \frac{V}{1000\ x\ W}\right)\left(\frac{mg}{g} fr.wt.\right)$$

$$Total\ Chlorophyll = (20.2\ x\ A\ 645) - \left(8.02\ x\ A\ 663\ x \frac{V}{1000\ x\ W}\right)\left({}^{mg}/_{g\ fr.wt.}\right)$$

Designing of primers using stay green genes from other crops

The NCBI sequence data base was searched to find out known stay green genes from rice, sorghum, barley, pearl millet durum wheat and other cereals. The RNA/DNA or hypothetical protein sequences were downloaded. Using the these sequence, BLAST was done against wheat EST and gDNA database of IWGSC (International Wheat Genome Sequence Consortium) using BLAST tool (http://urgi.versailles.inra.fr/srs83/displayTool.do?toolName= BlastN) and NCBI. The BLAST rights on pre-released genomic sequence of wheat chromosomes were kindly provided by Unité de Recherche Génomique Info (URGI), Clermont, France, upon request. Primers were designed from the homologous and orthologous DNA sequences matches with stay green genes or DNA regions.

Extraction of total RNA and DNase I treatment

RNA concentration was measured and integrity was checked on a denaturing gel. cDNA synthesis with the help of degraded anchored oligo (dT) primer set - T12MA,T12MC, T12MG, T12MT (where M is A,C or G) PCR reaction (Master mix will contain H2O, 10 x PCR reaction buffer, 4dNTP mix (25 µM), T12MN primer, cDNA, 0.2 µl Taq DNA polymerase (5 U/µl), 1 µl [a-33P] dCTP aliquot and arbitrary primer (2 pmol/µl).

Run a denaturing Polyacrylamide gel and autoradiography

We had identified the candidate genes and checked their expression at different growth stages viz., 63, 69, 73, 77 and 83 growth stages (Zadoks et al., 1974). PCR fragment was isolated (cut out band of interest with a clean razor blade) and reamplification of band of interest and used it directly for RT-PCR.

RT-PCR

Further confirmation of expression of different candidate genes was done at RT-PCR level. Gene specific primers were constructed (Table 1) based on clones sequence corresponding to stay green genes. Extraction of total RNA following standard protocol Measure RNA concentration and try to equalize the amount on a denaturing gel.

Table 1: Informative markers selected for QRT-PCR analysis.

Primers Name	Sequence (5`->3`)	Sequence (5`->3`)
4540 F1R2	CACTCTTGCTTGGC ATTTCA	ACCTTGTAATCACGGCC AAG
5563-F	TGGGAAACTCTCTT GGAGGT	GCCCATGGAAAAATCTT TGA

4540-F	CACTCTTGCTTGGCATTTCA	GCGGCTGACATTAAGCTTTC
2693-F	ACAAAGATCCGAGCAACGTG	TTGGCGTAGAACTCGTGGAT
SGR3	TCCAAGAAATCAGTTAGTGT	TGAACTTGTCTCTATTTCGT

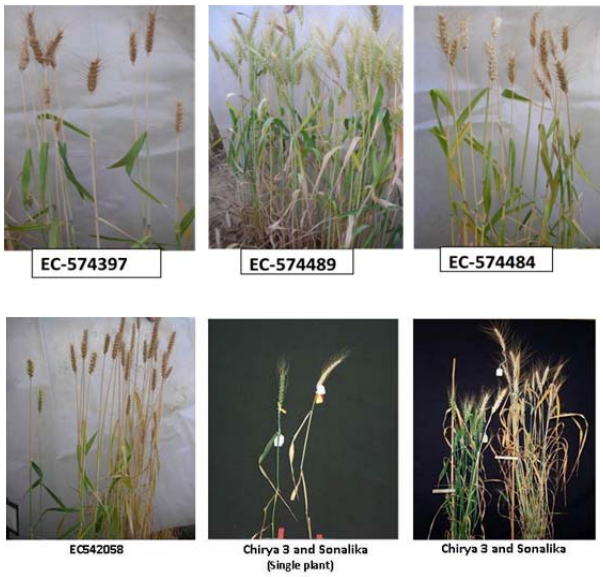


Fig. 1: Selected stay green and non- stay green wheat genotypes

Synthesis of cDNA using reverse transcriptase

Barley Ubiquitin gene transcript was Used as loading control of the templates to equalize the cDNA concentration. RT-PCR was done using cDNA as templates synthesized from total RNA extracted from leaf samples collected at different time points stages viz., 63, 69, 73, 77 and 83 growth stages (Zadoks et al., 1974) and study the expression pattern of different candidate genes. Best candidate gene was recommended for the development of transgenic plants

3. RESULTS AND DISCUSSION

The amount of chlorophyll 'a' was higher than chlorophyll 'b' in most of the genotypes (Table 2). This study depicted that the chlorophyll content in both stay green and non-stay green genotypes was almost similar during the initial stages, i.e. GS69 and GS73. However, during later growth stages i.e. GS77 and GS83, Sonalika (non-stay green) showed a significant decrease in the amount of chlorophyll content whereas in stay green genotypes, the chlorophyll content remained significantly high. Chlorophyll degradation is responsible for the maturity of the plant (Fig.2). This study provides the clear cut indication that the phenotypically selected lines have some kind of mechanism to slow down the degradation of chlorophyll at the later plant growth stages.

Table 2: Chlorophyll a and b content of the selected genotypes at different growth stages

	GS69 Flowering complete		GS73 Early milk		GS77 Late milk		GS83 Early dough	
	Chl *'a'*	Chl *"b'*	Chl *'a'*	Chl *"b'*	Chl *'a'*	Chl *"b'*	Chl *'a'*	Chl *"b'*
EC-574397	1.477	1.168	1.411	1.087	1.371	0.966	1.252	0.789
EC-574489	1.376	1.268	1.326	1.187	1.26	1.127	1.156	0.826
EC-574484	1.354	1.166	1.227	1.114	1.127	0.928	1.036	0.897
EC-542058	1.472	0.977	1.326	0.945	1.288	0.917	1.224	0.688
Chirya 3	1.652	1.342	1.528	1.289	1.532	1.244	1.375	1.121
Sonalika	1.457	1.126	1.324	0.961	1.124	0.808	0.378	0.269

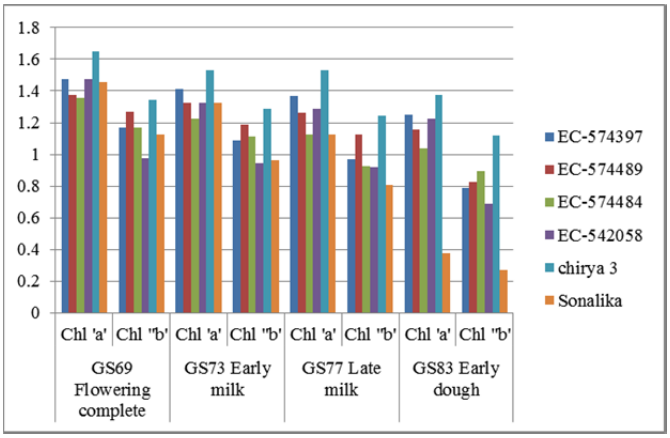
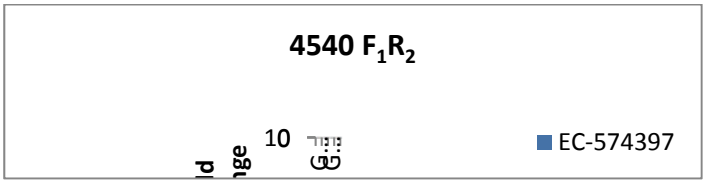
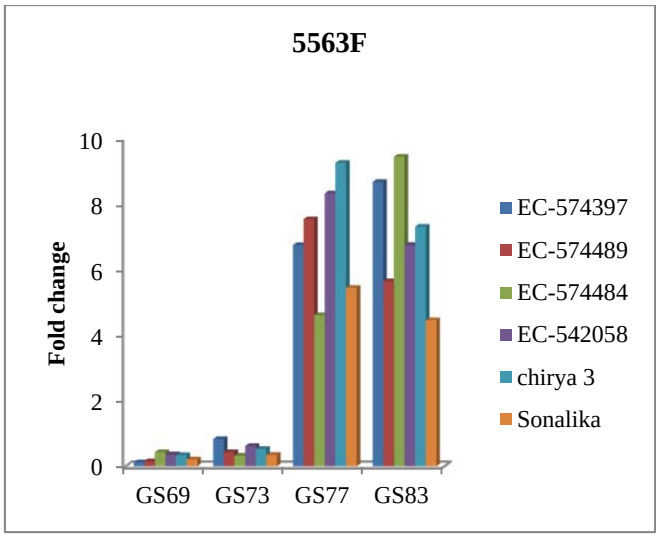
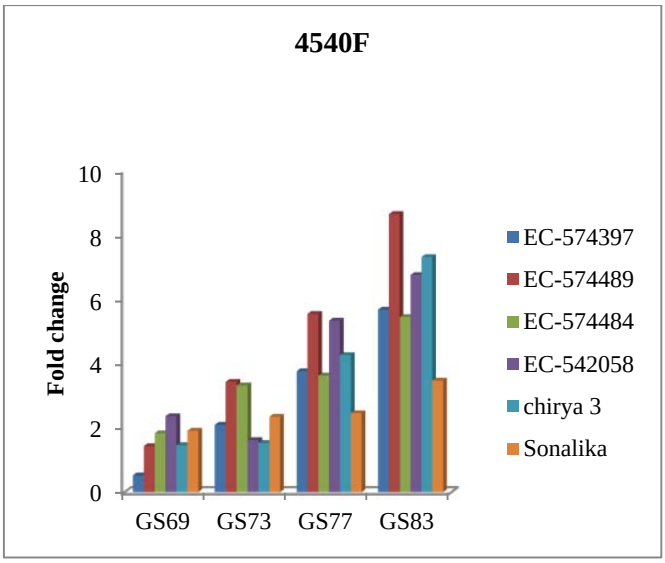


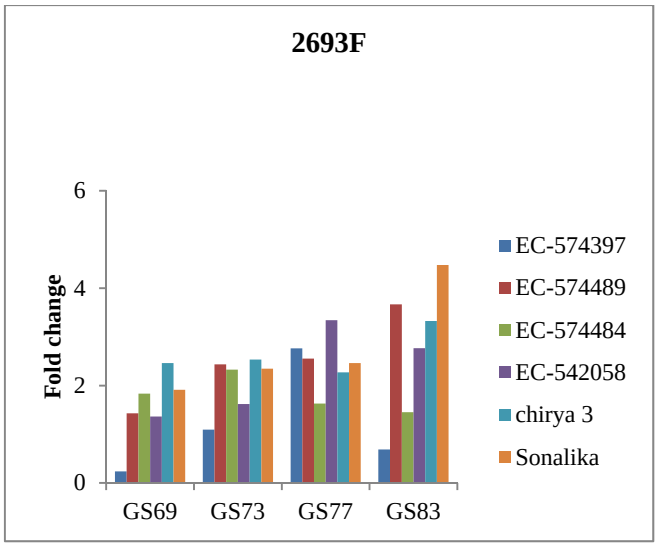
Fig. 2: Graphical presentation of Chlorophyll content of different genotypes in different growth stages

Quantitative RT-PCR was done for all the five primers related to stay green gene. The expression profile depicted (Fig 7)that 3 primers out of 5 showed a differential expression profile within stay green and non-stay green genotypes. Primer 4540 F1R2 and 5563 F showed very low expression in all the genotypes of GS69 and GS73 while, in GS77 and GS83 expression was very high in all selected stay green genotypes in comparison with Sonalika (non-stay green line). Primer 4540 F also found to be made similar kind of expression however its expression in GS69 and GS73 was significantly higher than above mentioned primers.









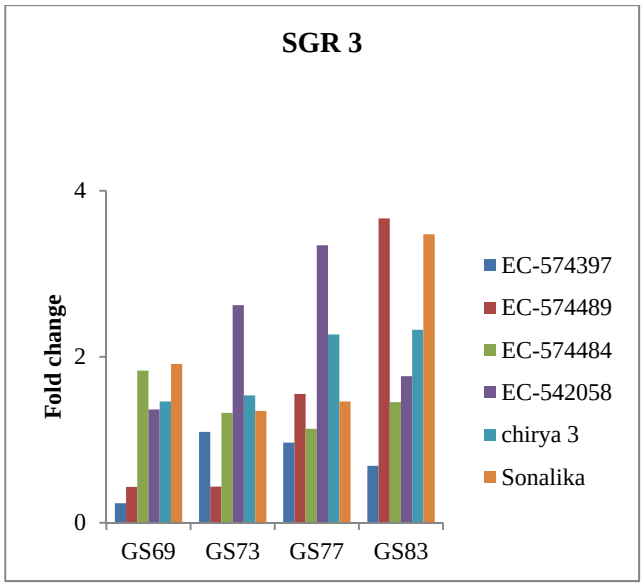


Fig. 7: Expression of the 4540F1R2, 5563 F, 4540 F, 2693 F, SGR 3 gene in flag leaves of six genotypes

Interestingly these three primer were designed from same cds from Oryza Sativa Japonica group NYC3 mRNA. These results indicating that this region (sequence) of the genome may be highly correlated with stay green trait in wheat. While other two primers could not show differential expression within the contrasting genotypes. There expression was low and found almost consistent in all growth stages. The results are quit expected because insertion/deletion within the gene is very rare most of the distinct phenotypes arise due to singe base substitution of SNPs so the quantitate estimation of gene expression is prerequisite. Further investigation on expression and regulation of this gene may provide information on the mechanism of this trait and its potential relationship with drought tolerance in wheat.

It appeared to be due to the influence of the environment on the expression of stay green. Genes for physiological traits like stay green and control of translocation of non-structural carbohydrates from stems to grain may have implications on yield potential in cereals including wheat and may play a crucial role in grain development, particularly when assimilates are limited. It has been suggested that stay green enhances grain yield, but it has yet to be tested for economic viability. Improved cultivars with stay green attributes provide a better option for drought and high temperature environments. The detected genes in the present study provide preliminary information for further investigation and to initiate a marker assisted selection strategy. Newly identified wheat varieties can also be used in the future breeding program against terminal heat stress and for the development of high yielding stay green cultivars.

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