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Stay-green rice has greater drought resistance: one unique, functional SG Rice increases grain production in dry conditions

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ABSTRACT

As the relationship between stay-greenness and drought tolerance in rice is not well known, three stay-green (SG) Rice-induced heir wildtype, Nagina 22 (N22), were examined. The dark-induced senescence experiment was used to confirm the mutant phenotype. Agronomic metrics, oxidative stress (OS) related enzyme activity, and transcript profiling of 15 candidate genes involved in chlorophyll catabolism and senescence were used to assess mutants' performance under well-irrigated and drought stress conditions. The mutants' whole genome sequencing data was used to derive the sequence information for the potential genes. While SGM-1 and SGM-2 completely lacked senescence, SGM-3 exhibited delayed senescence. Compared to the WT, mutants had steady levels of expression across time. However, ATG6a transcript abundance was significantly rising with time in SGM-3. Even while all the rice performed better under drought conditions across the board, only SGM3 had a higher grain yield. The mutants have higher ascorbate peroxidase activity than N22. Under drought, all 15 genes showed overexpression, with N22 and SGR-30 showing the greatest up regulation.

Keywords: Drought tolerance, Rice, and Stay-green trait

INTRODUCTION

Drought stress has become a serious concern to global food security due to the constant loss in water resource availability on the one hand and rising utilisation of those same resources on the other [1-2]. In order to adapt to drought stress, plants that are rooted in the ground change their morphological, physiological,and molecular processes [3-5]. Depending on the stage of growth and length of the stress, a plant's response to drought will vary in intensity. The magnitude of drought stress experienced by a plant depends on the growth stage of the plant and the duration of the stress. Rice (Oryzasativa L.), a monocot with shallow root architecture but huge water requirement for cultivation, is more prone to water deficit stress than other major food crops [6]. Although rice is susceptible to drought stress at all development stages, reproductive stage stress has the most negative consequences because it reduces grain yield [7]. According to [8], drought stress destabilises the photosynthetic apparatus, which causes changes in the carbon and nitrogen metabolism and, eventually, a smaller sink size. Drought tolerance can be achieved in one of two ways: by completing the life cycle in a shorter amount of time (escape), or by developing morpho-physiological adaptations like root architecture, water use efficiency, osmoregulation, stomatal and non-stomatal limitations, and molecular changes to withstand the stress condition (true tolerance) [9-11]. Since chlorophyll, the most abundant pigment on earth is a phototoxic compound, it is degraded by a highly conserved autophagy mechanism in plants [12-13. Plant autophagy plays a crucial role in the onset of leaf senescence while certain autophagy genes are also involved in stress response [14-15].

Leaf senescence, the final stage of plant development, wherein the shift from the carbon capture phase to the nitrogen remobilization phase occurs as the final source of nutrient supply to the developing grains, is a highly regulated molecular mechanism [16]. Delayed senescence, i.e., stay-green (SG) phenotype, associated with elevated levels of cytokinins showed drought tolerance capacity without compromising yield [17]. SGtraitcan also be a result of better maintenance of water intake and supply equilibrium after anthesis[18]. The adverse impact of drought stress can be partially mitigated by enhanced photosynthetic activity during post- anthesis[19]. Thus mutants with delayed senescence that exhibit prolonged photosynthetic activity can have enhanced productivity and better drought tolerance as a result of better water maintenance. SG mutants with retention of photosynthetic

activity are termed functional while those without photosynthetic activity are termed as cosmetic mutants [20].
Even under well-irrigated conditions, functional stay-green mutants are of greater value as they possess yield-enhancing qualities. Several QTLs viz, rdgf2a, rdgf2b, rdgf3, rdgf8a, rdgf9, rdgf10, qCCAI-9, qCCAJ-9, qRCRJ-9, etc. responsible for SG phenotype have been mapped in rice, but they have been reported for their negative correlation with yield [21-23]. Further, a recessive stay green allele, sgr has been mapped on chromosome 9 which showed type-C SG phenotype with reduced photosynthetic activity [24-25]. Another cosmetic mutant was reported with a decrease in chlorophyll a and other chlorophyll protein complexes but retention of chlorophyll b and LHC II [26]. Both these mutants had impairment in chlorophyll catabolism. A type-B functional stay green mutant, SNU-SG1 was derived from a japonica cultivar by EMS-induced mutagenesis. This mutant showed a delayed decline in the rate of photosynthesis and chlorophyll content compared to some of the elite japonica cultivars. Though QTLs for the functional stay-greenness in this mutant have been mapped on chromosome 9, the molecular basis is not yet known [27-31]. The present study was undertaken with the objective of characterizing three rice for their stay-greenness, and drought tolerance ability and to understand the molecular aspects behind their association between stay-greenness and drought tolerance.

Materials and Methods

Plant materials and dark-induced senescence assay

Four individual flag leaves were labeled at the heading stage to measure the photosynthetic activity of SG mutants and WT. The net photosynthetic rate was measured using a portable photosynthesis system (Li-6400XT, LI-COR Biosciences, Lincoln, NE, USA) at 11:30–12:30 hrs from the 7th day after anthesis till physiological maturity under 1200 µmol photon m−2 s−1 light intensity. The light was provided by a red/blue LED light source system in both years. The gas exchange parameters including (µmol m−2 s−1), (mol m−2 s−1), (µmol mol−1), and (mmol m−2 s−1) were acquired. The atmospheric CO2 concentration, air temperature, and relative air humidity were 380–390 µmol mol−1, 26–28°C, and 60.21–65.10%, respectively, during the data acquisition period of both study years.

Sampling for candidate gene expression profiling under DIS

In order to study the expression pattern of the candidate genes under senescence, it was important to conduct the DIS assay without detaching the flag leaf from the plant. To provide a dark environment to the flag leaves in-vivo, a modified protocol by [28] was followed. A scaffold was made using aluminum foil around the flag leaf which was wrapped with butter paper to minimize the high temperature effect on the leaf. Sampling was done on Day-0, Day-3, Day-6, and Day-9 in three biological replicates.

Drought stress treatment and sampling

Three varieties of SG rice, designated SGR10, SGR20, and SGR30, were cultivated alongside N22 in order to test each variety's ability to withstand drought. At the booting stage, drought stress was produced by denying water for ten days. Physiological parameters such as relative water content (RWC), chlorophyll content, and agronomical characters namely, plant height, panicle length, flag leaf length, number of productive tillers (NPT), and yield per plot were recorded. Flag leaf samples were collected after the drought stress period for gene expression analysis and enzymatic studies. For comparison of gene expression and enzyme activity under drought, flag leaf samples from the well-irrigated control plants grown in the IARI farm, but not subjected to DIS assay were used. The harvest index (HI) under well-irrigated conditions was calculated for the SG mutants and N22 by weighing the shoot biomass and the grain weight. HI was expressed in terms of the percentage of grain weight to shoot biomass ratio.

RNA extraction and gene expression profiling

15 candidate genes were chosen for the expression analysis of senescence-associated genes, including genes involved in autophagy and senescence (ATG4a, ATG5a, ATG6a, ATG7, ATG8, Fd-GOGAT, SAG12), chlorophyll catabolism (NYC1, NYC3, NOL, SGR, PAO, RCCR1, CHL), and genes that regulate oxidative and drought stress (DREB2A).The details of the primers used are given in Supplementary Table 1.
Total RNA was isolated by the Trizol method from flag leaves which were collected from drought treatment plots (sampled at 10 days after withholding water) and well-watered plots subjected to DISassay on Day-0, Day-3, Day-6, and Day-9.DNase treatment was carried out using Turbo DNA DNase free (Ambion, USA) according to the manufacturer's instruction to get rid of genomic DNA contamination. The integrity and quality of the RNA were checked on 1.5% agarose gel. RNA concentration was determined using Nanodrop, ND-8000 spectrophotometer (Thermo Fisher Scientific, USA). RNA samples with good quality and integrity were used for cDNA synthesis. For single-stranded cDNA synthesis 1µg of total RNA along with dNTP, reverse transcriptase (RT) enzyme, buffer, oligo-dT, and hexamer primers fromSuperscript III first strand synthesis kit (Thermo Fisher Scientific, USA) were used. Semi-quantitative and quantitative RT-PCR was performed to analyze the expression profile of candidate genes. cDNA was diluted 5 fold and used as a template for Semi-quantitative and qRT-PCR. Actin was used as the housekeeping gene for normalization. For semi-quantitative PCR, the following reaction condition was used: initial denaturation at 94 °C for 5 min, 30 cycle of 94 °C for 30s, 60 °C for 30s, and 72 °C for 1 min with a final extension of 72 °C for 7 min. The qRT-PCR reactions had total volume of 10 µl with 10 mM of each gene-specific primer, diluted ROX, 1 µl cDNA (1/5 dilution), Brilliant-III ultra-fast SYBR Green qPCR master mix (Agilent Technologies), and RNase-free water. The experiment was performed on the AriaMX Real-time PCR system (Agilent Technologies). To check the specificity of PCR, amplificationmelt-curve analysis was carried out at the end of the PCR. Three biological and two technical replicates were used for each experiment and analysis was done by the Livak method. Results were expressed as fold change with reference to the control samples.

Enzyme assay

Leaf extracts for superoxide dismutase (SOD; EC 1.15.1.1), Glutathione reductase (GR; EC 1.6.4.2), Ascorbate peroxidase (APX; EC 1.11.1.1) and Catalase (EC 1.11.7.6) was prepared from the flag leaf of control and drought samples. The samples were pre-frozen in liquid nitrogen to prevent proteolytic activity, and ground in 3 ml extraction buffer containing 0.1 M

phosphate buffer (pH 7.5) and 0.5 mM EDTA. Extracts were centrifuged for 20 min at 15,000 g and the supernatant was collected and used for the enzyme assay. The concentration of the extracted protein was estimated by the Bradford method before performing enzyme assays. Each enzyme assay was scaled down to a miniprep of 200 µl volume and the spectrophotometric measurements were done in a 96-well plate reader (Varioskan™, Thermo Scientific, USA). Enzyme assays were performed using three biological and three technical replicates. SOD, APX, and GR activity were assayed and the activity of respective enzymes was calculated as explained in [29]. Catalase (CAT) was assayed by measuring the disintegration of H2O2. The reaction mixture (200 µl) consisted of 10 µl of dilute enzyme extract and 100 µl of 0.1 M phosphate buffer (pH 7) and 60 µl of water. The reaction was initiated by adding 30 µl of 75 mM H2O2. A decrease in absorbance at 240 nm was observed every 30 s for 3 min with UV- visible spectrophotometer. The molar extinction coefficient of 39.4 M−1 cm−1 was used for the calculation of enzyme activity.

Statistical analysis

The data in all figures were expressed as mean standard error (SE). Normality and homogeneity of variances were tested through Kolmogorov-Smirnov and Levene testing, respectively. One-way analysis of variance (ANOVA) was conducted to understand the differences among the genotypes under both well-irrigated and drought treatments. Tukey's test was conducted for mean comparisons. All these were carried out using SPSS package v.19.

Sequence analysis

The whole genome sequence resource of these three mutants and the WT are readily available to us [29]. Structural variations between the WT and the three mutants were identified for the 15 candidate genes selected for expression analysis in this study. High-quality reads from the mutants were mapped directly onto the N22 genic sequence assembly using bwa v0.7.12 [30]. The sequences of the candidate genes from the mutants and WT were extracted using bedtoolsgetfasta[31]. SNPs were called and amino acid changes were identified using an in-house Python script.

Results

DIS assay of the SG mutants

The initial chlorophyll concentration (day 0) was the highest in the mutant, SGM-2 (4.97 mg g-1) followed by SGM-1 and N22 with nearly equal chlorophyll content (~4.5mg g-1) while SGM-3 had the least chlorophyll content (Fig. 1A). Invariably, all the three mutants had higher chlorophyll content than the wild type (WT) on day 10. The rate of decline in chlorophyll content was gradual and the slowest in SGM-3 which showed signs of deep decline only on day 8. SGM-2 showed a sharp decline in chlorophyll content on day 2 and on day 8. SGM-3 was nearly similar to N22 in appearance on day 10 while the other two mutants remained green even on day 10 (Fig. 1B and 1C). Further, on physiological maturity, SGM-1 and SGM-2 panicles remained completely green while SGM-3 turned brown with some tinges of greenness (Fig. 1D). Thus all three mutants proved to be of stay-green type by the DIS assay (Figs. 1A). While SGM-1 and SGM-2 showed complete lack of senescence, suggesting impairment in chlorophyll metabolism, SGM-3 showed delayed senescence.

Performance of the thestay-green mutants under drought stress

Under drought, the RWC content of all four lines was significantly different from that of well-irrigated control. SGM20 had the highest RWC under both treatments (82.2 % under well-irrigated and 80% under drought stress) and it also showed the lowest reduction in RWC (2.5%) under drought stress. SGM-30 though had the lowest RWC under both the treatments the % reduction in RWC under stress was minimal (4%). Though N22 and SGM-1 had similar RWC under well-irrigated (~80%) and drought stress (75%) conditions, they showed a steep decline in RWC under stress (7.5 % and 6.5 % respectively). SGR-30 retained its chlorophyll content almost unchanged under drought stress, while the other two mutants showed minimal differences. Overall, under drought, the change in chlorophyll content was not significant in the mutants while WT showed a steep and significant decline. For the agronomic traits also, the WT showed a significant reduction in performance under drought treatment. However, the SG mutants behaved differently for different traits. For instance, the drought did not have any influence on the plant height of the SG mutants Moreover, SGM-2 and SGM-3 did not show any alterations in NPT under drought. However, the most important trait, yield/ plot (g m-2) indicated that only the WT and SGM-3 performed better under drought stress though there was a decline in their performance (~130 g m-2) compared to the irrigated control (~150 g m-2). The other two SG mutants, SGM-1 and SGM-2 showed very poor performance under both the treatments for yield (100-110 g m-2) though they did not show any reduction in performance under drought stress compared to the control conditions. Thus, the three SG mutants performed better than the WT under drought stress in terms of both the physiological and agronomic parameters other than yield; only SGM-3 had the best performance among the mutants for yield.

Oxidative stress-associated enzyme studies on the mutants under drought stress

All four oxidative stress management (OSM) related enzymes, APx, GR, SOD, and CAT showed enhanced activity under drought stress compared to well-watered control conditions in the three mutants and the WTshowing that all four lines had better OSM under drought (Fig. 5A to 5D). The only exception was the WT for APx activity. In terms of enzyme activity under drought stress, no definite hierarchy in performance emerged among the mutants and the WT could be found, as for different enzymes, different lines performed the best. For APx enzyme, SGM-1 and SGM-2 showed the highest activity while for GR the highest activity was seen in SGM-3. N22, a drought-tolerant genotype, performed better than the mutants with respect to catalase activity whereas for SOD, SGM-1 and N22 activity were on par (Fig. 5C and 5D). Comparison in terms of 'fold increase in enzyme activity under drought' clearly showed that SGM-3 was the best performer, as it had the highest fold increase for APx (1.65) and GR (2.89) and the second best fold increase for SOD activity (Supplementary Table 3).

Expression analysis of the chlorophyll catabolism and senescence-related genes under drought

Upregulation of DREB2A in all four genotypes under drought conditions indicated that drought stress was really experienced by the plants (Fig. 6). Further, all 15 genes showed upregulation

from less than one fold (0.3) to 27.4 fold across the SG mutants, and the WT indicating moderate to high response to drought (Fig. 6). Among the five autophagy associated genes, N22 showed drastic upregulation in all the five genes (8 to 27 fold) compared to the mutants, except for SGM-3 in case of ATG4a and ATG7. In both these genes, SGM3 showed more upregulation (13 fold) than N22 (7-8 fold). SGM-3 also had the highest upregulation for NOL, SGR, and PAO while SGM-1 showed the highest expression for NYC1 and SAG12. SGM-2 showed the highest upregulation only for CHL. Further, GR involved in OSM and Fd-GOGAT involved in nitrogen remobilization showed higher expression in N22 than in the mutants. Thus, overall SGM-3 and N22 differed from the other two mutants in their expression of genes associated with senescence and chlorophyll catabolism under drought.

Discussion

All three mutants showed enhanced chlorophyll retention both under dark incubation and drought. While SGM-10 and SGM-20 retained higher levels of chlorophyll throughout the DIS time period, SGM-3 maintained a consistent level followed by a decline at the end. This phenomenon elucidates the nature of the cosmetic and functional stay-green phenotype respectively [35]. Though the SGR-30 mutant was smaller in stature than N22, with a significant reduction in PH, FLL, and NPT, it did have grain yield equivalent to the WT, and N22 under irrigated conditions (Fig. 4). On the other hand, SGM-1 and SGM-2 with bigger stature than the WT, produced grain yield much lesser than the WT. Such an increase in biomass without accompanied increase in grain yield has been reported in rice transgenics with delayed leaf senescence [33] and this could be a typical feature of cosmetic SG mutants [34]. Thus, SGM-1 and SGM-2 were inferred as cosmetic SG mutants while SGM-3 was a functional SG mutant.

For biomass-related and physiological traits, the SG mutants showed the least reduction in their performance under drought stress compared to the control. Hence, the SG mutants were certainly better than the WT for the secondary traits under drought stress. As SGM-3 alone is a functional SG mutant it could perform equally well for grain yield too under drought. Among the wide range of mechanisms contributing to drought tolerance and leaf senescence, a drastic increase in the ROS levels due to alterations in the electron transport chain leading to cell death is one of them ((Palma et al., 2006; Khanna-Chopra., 2012). Nagina 22 is known for its better oxidative stress management mechanism under drought [36-37]. It was demonstrated by Prakash et al., (2016) that N22 had a better response for SOD and GR but not for APx through enzyme activity, gene sequence, and protein stability studies. In the current study, the same trend was found with additional information on catalase in which again N22 was found to give a better response under drought. The SG mutants though more or less had the same response akin to N22 under drought for these enzymes, they did show drastic enhancement in APx activity suggesting that rice SG mutants do have better drought tolerance, as found in sorghum and wheat [38]. SGM-3 mutant had the best expression for GR under dark incubation in the time-course analysis through expression and enzyme activity studies, compared to the other SG mutants and the WT, indicating that the oxidative damage during senescence was very limited in this mutant.

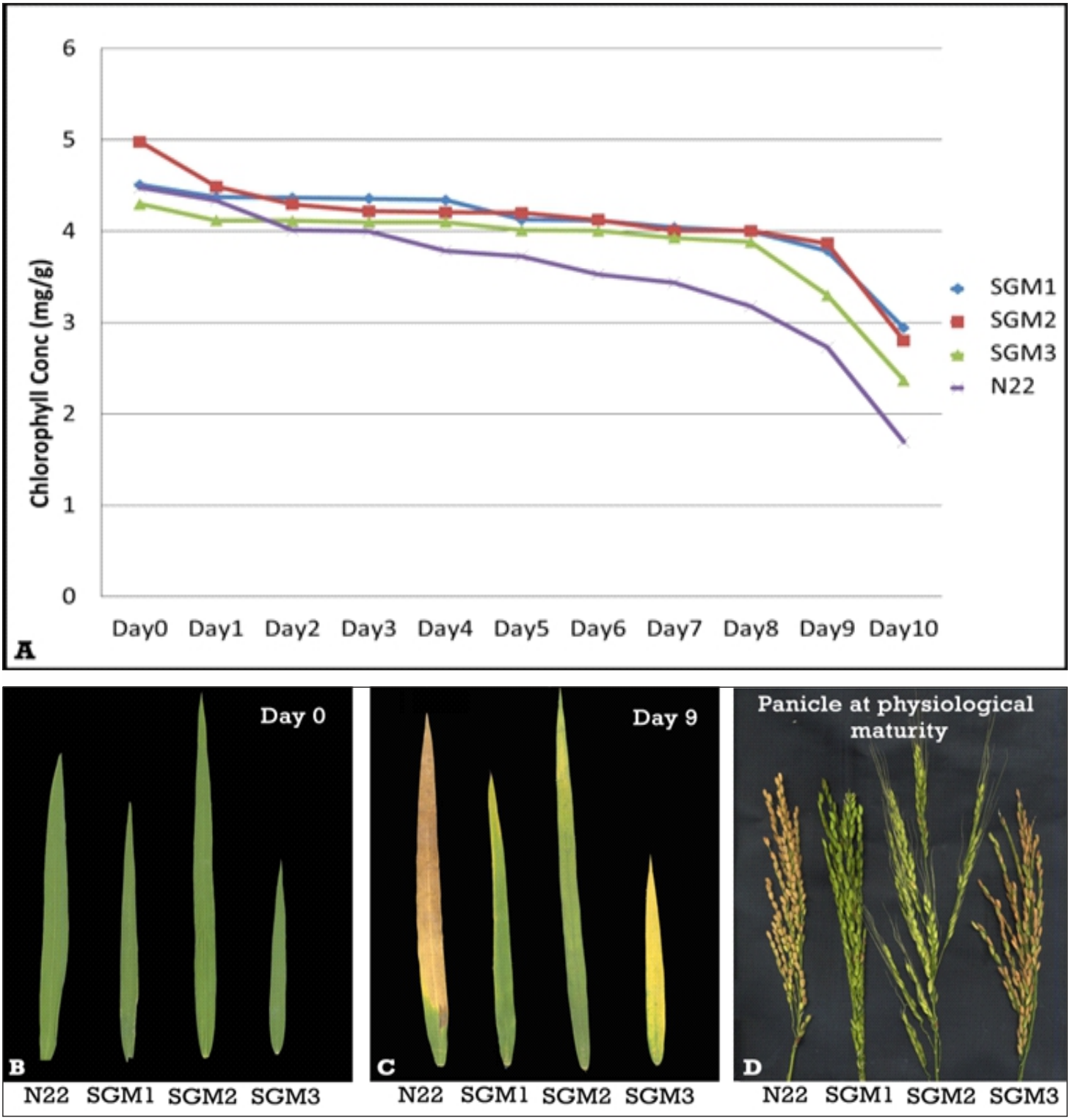
Fd-GOGAT is the primary enzyme for photorespiration and N assimilation of grains [39] for which SGM-2 performed the poorest under dark incubation while both SGM-1 and SGM-2 showed poor performance under drought. Only SGM-3 showed a moderate performance for this enzyme showing that the N assimilation in this mutant is functional. The contrasting transcript level for the autophagy-related genes in SGM-1 and SGM-2, compared to N22 clearly showed that senescence is severely impaired in these mutants. As leaf senescence is a major determinant of yield (Jordan et al., 2012), the impairment in senescence resulted in their low yield potential. In contrast, the transcript levels of SGM-3 matched that of N22 for the autophagy-related genes, especially, in the latter part of the time-course analysis showing that senescence is delayed in this mutant but not impaired which was also corroborated by its better performance in grain yield.

Table 1:

S. No.	Gene Name	Gene ID	SNP position	SNP Change	Amino acid position	Amino Acid Change
1	ATG5	Os02g0117800	365	T? C	122	L? P
2	Fd- GOGAT	Os07g0658400	2293	A? G	765	I? V
			3877	A? T	1293	I? L
			3905	C? T	1302	A? V
			4088	A? C	1363	Q? P
			4249	G? A	1417	V? I
3	NYC1	Os01g0227100	161	C? G	54	P? R
4	NYC3	Os06g0354700	1394	G? C	465	C? S
			44	G? C	15	C? S

Figure Legend

Fig. 1: Time-course relative gene expression analysis of 15 genes involved in chlorophyll catabolism, senescence and nitrogen assimilation in three stay-green mutants (SGM1 to SGM3) and the wild type, Nagina 22 (N22) under dark incubation.



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