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Inheritance and genetic analysis of EMS induced stay green mutants and their wild type Nagina22

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Abstract
The present study was conducted to analyze the inheritance of the stay green (SG) trait in EMS induced SG mutants (SGM-1 and SGM-3) with their wild type (WT) Nagina 22 (N22). A total of 504 and 540 F_2 progenies of SGM-1 X N22 and SGM-3 X N22 were developed and Dark Induced Senescence (DIS) assay of these two F_2 populations was performed. Under DIS condition, on day-7 the chlorophyll content of F_2 progenies ranged from 2.76 mg g^{-1} to 4.13 mg g^{-1} in SGM-1 X N22 and 2.73 to 3.94 mg g^{-1} in SGM-3 X N22. Out of 504 F_2 progenies (SGM-1 X N22), 366 were of mutant type and 138 were of N22 type, whereas in the SGM-3 X N22 population 387 F_2 progenies were of mutant type and 153 were of the WT. In both the populations, 3:1 (SGM: N22) segregation ratio was observed. Hence these two SG mutants showed monogenic dominance inheritance and the causal genes can be mapped using any of the Mutmap approaches or its varaiants.

Keywords: stay green, dark induced senescence, f_2 population

Introduction
Rice (*Oryza sativa* L.) forms the basic nutritional source for more than half the world population. Global climatic changes has led to detrimental effects on rice productivity worldwide. In addition, consistent increase in the population has furthered the demand for increased crop productivity [1]. Rice has a rich genetic diversity for several agronomic traits such as plant height, productive tillers, panicle length, grain length, grain width etc., which have been explored well for their inheritance and gene/ Quantitative Trait Loci (QTLs) mapping using appropriate mapping populations. So far around 1600 QTLs have been mapped for different morphological traits, 835 for abiotic stresses and 402 QTLs for different biotic stresses in different mapping populations [2]. As far as SG trait is concerned, only a few QTLs have been reported in rice and among those, the functional SG QTLs are yet to be studied.

Leaf senescence is the final stage of growth and development in plants. It is defined as a highly sophisticated genetically programmed sequence of structural, biochemical and physiological events [3]. Leaf yellowing due to chlorophyll degradation is a phenotypic marker of plant senescence and it is the salvage of nutrient supply to the plants. Chlorophyll is a potential phototoxin for the cells and therefore chlorophyll degradation is an essential metabolic process in plant cells [4]. For that reason, inhibition of chlorophyll degradation leads to accumulation of phototoxic intermediates and ROS production [5]. Delay in the senescence mechanism leads to stay green phenotype. Stay green mutants are classified into two groups namely functional (Type A and B) and cosmetic (Type C, D and E) based on the rate of chlorophyll degradation [6]. Functional stay greenness is of agronomical importance as the delay in senescence leads to prolonged photosynthesis thereby increasing crop yield. Besides the agronomic importance, stay green mutants also possess abiotic stress tolerance potential which has been extensively studied in a wide range of crops such as wheat, barley, maize etc. [7]. Mapping of genes associated with SG trait and introgressing the same into elite rice varieties could help in enhancing crop productivity. In the present study two SG mutants earlier reported for the SG trait [7] were selected for inheritance studies so that the strategy for mapping of these mutants can be devised. The mutants were crossed with their wild type (WT), Nagina 22 (N22) to develop F_2 mapping populations and the F_2 progenies were screened for their SG phenotype by dark induced senescence (DIS) assay.

Materials and Methods
Population development
We earlier reported identification of three EMS induced SG mutants, of which one, namely, SGM-2, was mapped to *RCCR1* gene with a nonsense mutation at amino acid position 330 [7]. In the present study the other two mutants, namely SGM-1 and SGM-3 were selected for crossing with their wild type (WT), Nagina 22 (N22) to generate first filial (F_1) generation. These F_1 seeds were grown as individual plants in the experimental field at Indian Agricultural Research Institute, New Delhi, India during *kharif* 2016. In the subsequent year, the F_2 mapping populations raised from the F_2 seeds were grown in double spacing (40 cm × 40 cm). Three flag leaves in each F_2 progeny were tagged during booting.

Dark Induced Senescence (DIS) assay
Flag leaf samples were collected from each F_2 individual post-anthesis in triplicates and DIS assay was carried out [7-9]. The flag leaf samples were incubated under dark conditions at room temperature. From the DIS assay performed in the parents [7], two time points *viz*, Day-0 and Day-7 were selected for chlorophyll estimation in the F2 progenies Day-0 to determine the initial chlorophyll content and Day-7, when the decline in chlorophyll content was prominent in the WT parent as against the SG mutants, to determine the SG nature of the progeny. In brief, 0.5 mg of fresh leaf samples were added in 1 ml of DMSO (Dimethyl

Sulfoxide). Subsequently, 24 h after incubation, 200 µl of the extracts were taken in a 96 well microtitre plate in three technical replicates for each biological replicate and absorbance of the samples was measured at two wavelengths *viz*, 645 nm and 663 nm using a 96 well plate reader (Varioskan™, Thermo Scientific, USA). Only 200 µl of DMSO, without extract, served as blank in each plate. Finally, using Arnon's equation, total chlorophyll content was calculated [10, 11]. After seven days, the same exercise was repeated to measure the chlorophyll content of the dark incubated samples.

Statistical analysis

From the chlorophyll data of two time points (Day-0 and Day-7), the percent reduction in the chlorophyll content was calculated for each progeny and the frequency distribution was plotted in a graph. The segregation ratio of the F_2 individuals was determined using Chi-square test.

Results and Discussion

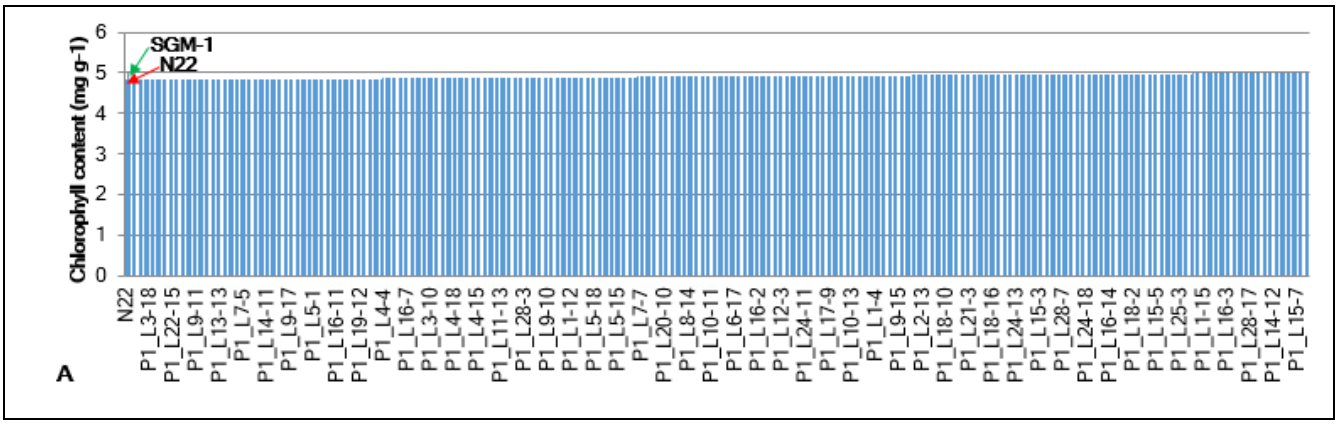
A total of 504 and 540 F2 progenies of SGM-1 X N22 and SGM-3 X N22 respectively, were used to determine the segregating pattern of the stay green trait. Total chlorophyll content of the F_2 progenies of SGM-1 X N22 cross ranged from 4.81 to 4.98 mg g^{-1} on day-0 which corresponded to their parents' N22 (4.8 mg g^{-1}) and SGM-1 (4.99 mg g^{-1}) post-anthesis. Under DIS, on day-7 the chlorophyll content of parents N22 and SGM-1 had reduced to 3.17 mg g^{-1} and 3.97 mg g^{-1} and their F_2 progenies had a minimum of 2.76 mg g^{-1} and 4.13 mg g^{-1} (Fig. 1B). The F_2 progenies of SGM-3 X N22 had total chlorophyll content ranging from 4.29 to 4.81 mg g^{-1} on day-0 and 2.73 to 3.94 mg g^{-1} on day 7 after DIS (Fig. 2A). These values were in line with the performance of the parents which had initial chlorophyll content of 4.81 mg g^{-1} (N22) and 4.31 mg g^{-1} (SGM-3) on day-0 and 3.17 mg g^{-1} (N22) and 3.88 mg g^{-1} (SGM-3) on day-7 after DIS (Fig. 2A and B). Based on the chlorophyll content on day-7, the progenies are categorized as mutant type and N22 type individuals. Out of the 504 SGM-1 X N22 progenies, 366 were mutant type (3.75 mg g^{-1} to 4.12 mg g^{-1}) and 138 were N22 type (2.73 mg g^{-1} to 3.12 mg g^{-1}), whereas in the SGM-3 X N22 population 387 F_2 progenies were mutant type (3.78 mg g^{-1} to 3.95 mg g^{-1}) and 153 were N22 type (2.75 mg g^{-1} to 3.10 mg g^{-1}).

Further, we calculated the percent reduction of chlorophyll content on Day-7 in the F_2 progenies which ranged from 17% to 43 % (366 SGM-1 type and 138 N22 type progenies) in case of SGM-1 X N22 and in case of SGM-3 X N22 population, the percent reduction in chlorophyll content on Day-7 ranged from 8% to 43% (387 SGM-3 type and 153 N22 type). Both the data, chlorophyll content on Day 7 as well as that of percent reduction, showed a clear cut bimodal frequency distribution wherein the class intervals were distinctly different for the two classes. Further we calculated segregation analysis for 3:1 ratio for both the populations (Table 1). In both the populations, a clear 3:1 segregation ratio was observed depicting that the inheritance of SG trait is monogenic and dominant.

QTLs for SG trait have been mapped in crops such as wheat, maize, barley, rice and sorghum [12]. In maize, QTL mapping of F_2 mapping population derived from A150-3-2 (stay green inbred line) and Mo17 (normal inbred line) revealed 14 QTLs associated with different stay-green associated traits such as green leaf area per plant and green leaf number per plant at the grain-ripening stage [13]. 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, yet have negative correlation with yield [14-16]. In sorghum, four major effect QTLs (Stg1, Stg2, Stg3 and Stg4), responsible for stay-greenness were reported to retain larger green leaf area under drought environment during grain filling stage [17]. Through EMS induced mutagenesis, a functional stay-green mutant (SNU-SG1) was derived from a japonica cultivar which showed delayed decline in the rate of photosynthesis and chlorophyll content compared to some of the elite japonica cultivars. The QTLs for the functional stay-greenness in this mutant have been mapped on chromosome 9, but the molecular basis is unexplored [18].

Table 1: Chi square analysis of the F_2 mapping populations (SGM-1 X N22 and SGM-3 X N22) for SG trait

Cross	Phenotype class	Observed frequency (O)	Expected frequency (E)	O-E	(O-E)^2/E	Cal χ2 value	Table χ2 value (P:0.05; df=1)
SGM1 X N22	Mutant type	366	378	12	0.381	1.524	3.84
	N22 type	138	126	-12	1.143		
	Total	504	126				
SGM 3 X N22	Mutant type	387	405	18	0.8	3.2	3.84
	N22 type	153	135	-18	2.4		
	Total	540	135				



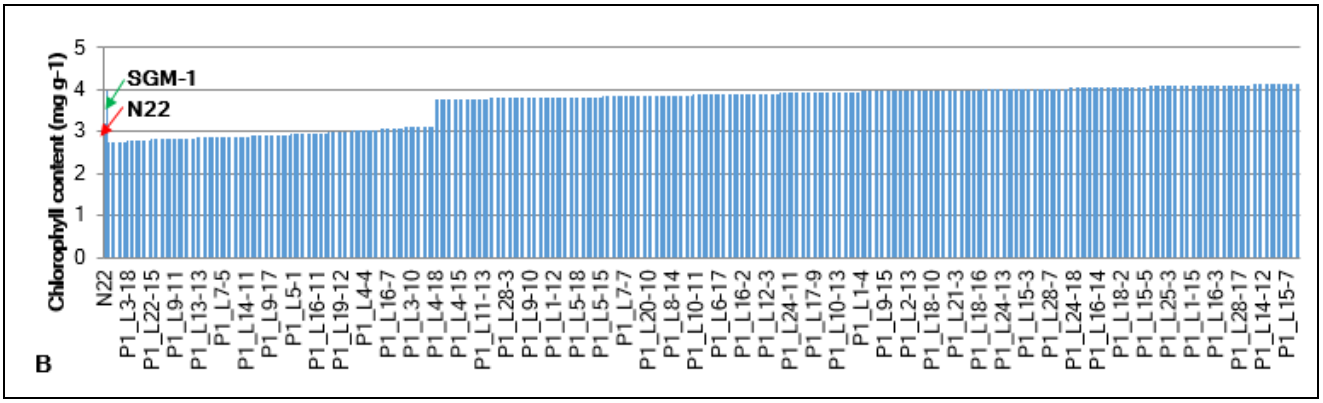


Fig 1: Total chlorophyll content observed in the F2 mapping population SGM-1 X N22 under DIS (A: Day-0; B: Day-7)

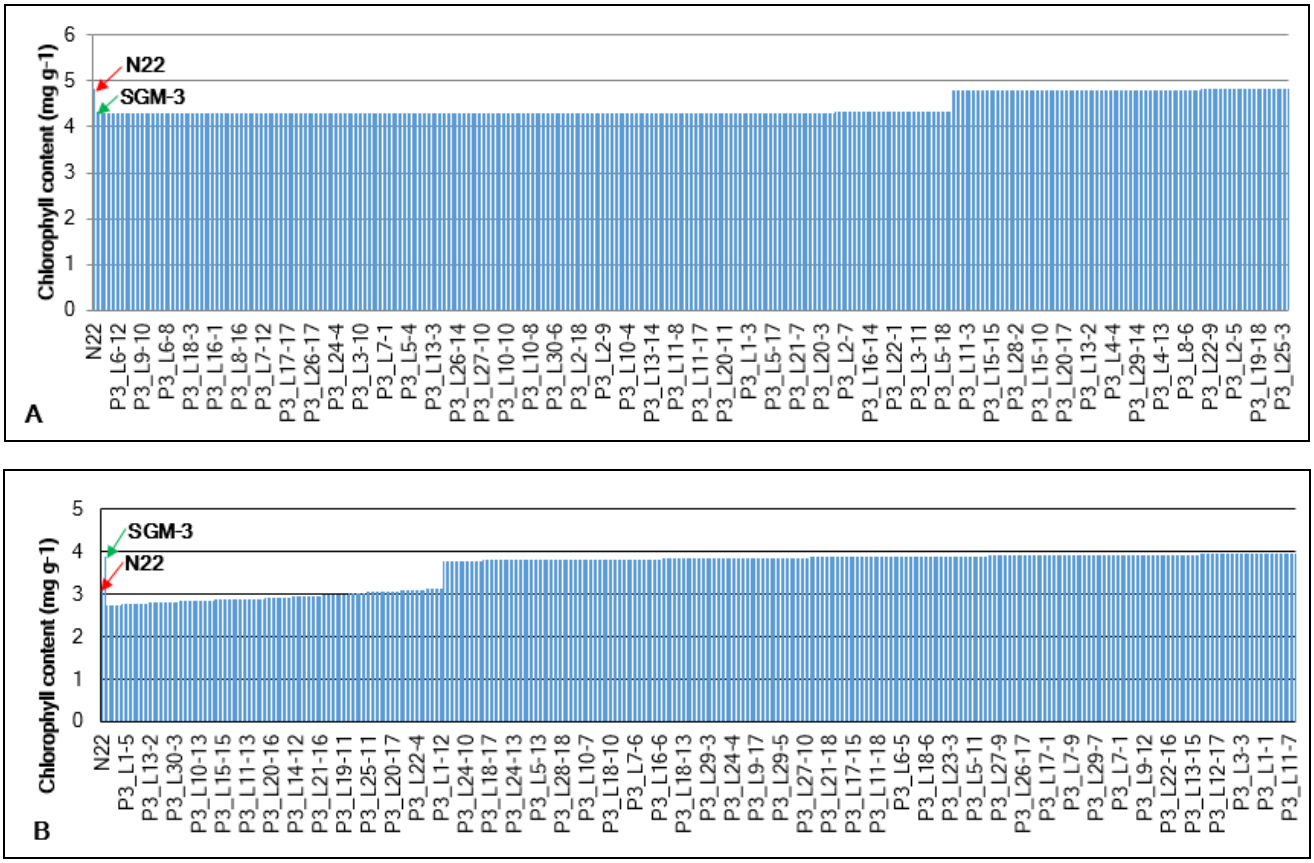


Fig 2: Total chlorophyll content observed in the F2 mapping population SGM-3 X N22 under DIS (A: Day-0; B: Day-7)

Conclusion

From this study we could demonstrate that the inheritance stay-green trait is controlled by a single nuclear gene by estimating the chlorophyll content on Day-7 of dark incubation as well the percent reduction of chlorophyll content on Day-7 compared to Day-0. Further, mapping of the causal gene mutations using Mutmap approach or any of its modifications could be attempted and the candidate gene can be utilized in breeding programs for enhanced yield especially from the SGM-3 which showed functional stay-green nature in earlier studies.

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