BIOLOGIA PLANTARUM 55 (4): 721-724, 2011

BRIEF COMMUNICATION

Heat tolerance in rice mutants is associated with reduced accumulation of reactive oxygen species

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Abstract

Four mutants induced by ethylmethane sulphonate (N22-H-*dgl56,* N22-H-*dgl101,* N22-H-*dgl162* and N22-H-*dgl219*) with conspicuous dark green leaves were identified in the drought and heat-tolerant rice cultivar Nagina22 (N22), when screened under prolonged drought and heat conditions in field. During dark-induced senescence, these mutants maintained higher chlorophyll and carotenoid contents, and photochemical efficiency of photosystem 2 in comparison with N22. Following heat treatment, these mutants accumulated less reactive oxygen species (assayed by histochemical staining for H_2O_2 and superoxide radicals) and maintained higher chlorophyll content than N22.

Additional key words: chlorophyll, H_2O_2, *Oryza sativa*, photochemical efficiency, superoxide radicals.

Leaf de-greening is considered as a good indicator of progress of senescence (Matile 2000). Retention of greenness of the leaf till the grain filling period and even later is known as stay-green phenotype (Hörtensteiner 2006, 2009). Genetic variation exists for symptoms of leaf senescence and stay-green mutants are classified into different categories according to their chlorophyll retention during senescence (Thomas and Howarth 2000). In rice, several stay-green mutants have been characterized (Cha *et al.* 2002, Jiang *et al.* 2007, Eckardt 2009, Morita *et al.* 2009, Sato *et al.* 2009, Schelbert *et al.* 2009). Chloroplasts are the first organelles targeted during stress (Smart 1994). Photoprotective mechanisms, accumulation of reactive oxygen species (ROS) and antioxidant systems are used as a measure of cellular responses to abiotic stresses and senescence (Niyogi *et al.* 1997, Srivalli *et al.* 2003, Moussa and Abdel-Aziz 2008, Zentgraf and Hemleben 2008, Ahmad *et al.* 2009). In transgenic tobacco plants, pigment contents, photosystem 2 activity and antioxidant protection during senescence was mediated by cytokinin content (Procházková and Wilhelmová 2009). We screened mutants of upland rice cultivar N22 generated by ethylmethane sulphonate (EMS) under prolonged drought and high temperature stress in field conditions. Four mutants, which showed enhanced greenness and better drought tolerance than N22 plants, were selected. This study reports detailed characterization of these four mutants (N22-H-*dgl56,* N22-H-*dgl101,* N22-H-*dgl162* and N22-H-*dgl219*) showing significant alterations in the chlorophyll degradation during dark induced senescence or under heat stress. Recently, a gene from tomato has been shown to be responsible for stay green phenotype by inhibiting chlorophyll degradation (Hu *et al.* 2011).

The mutants showing dark green phenotype in vegetative and flowering stages for two seasons were

Received 6 January 2010, *accepted* 29 June 2010.
Abbreviations: *dgl* - dark green leaf mutant; DAB - diaminobenzidine; EMS - ethylmethane sulphonate; NBT - nitroblue tetrazolium, ROS - reactive oxygen species; SOD - superoxide dismutase.
Acknowledgements: We thank Department of Biotechnology, Govt. of India (BT/PR9264/AGR/02/406(04)/2007) for financial support and Dr. R..P. Sharma, NRCPB, Delhi, Coordinator, DBT Network Project on Nagina22 mutants and Dr. S.R. Voleti for useful discussions. We thank the other Principal Investigators in the network project: Dr. A.K. Singh, IARI, New Delhi; Dr. T. Mohapatra, NRCPB, New Delhi for generating mutant population and Dr. K. Singh, PAU, Ludhiana; Dr. S. Robin, TNAU, Coimbatore and Dr. M. Sheshshayee, UAS, Bangalore. The mutant lines have been deposited in the mutant bank of the network project. We thank G. Ashok Reddy for field assistance. We thank the instrumentation unit and the central facility of CCMB, Hyderabad for their help.
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selected from M3 generation of EMS-mutants of rice (*Oryza sativa* L.) cv. Nagina 22 (N22) and were named asN22-H-*dgl56,* N22-H-*dgl101,* N22-H-*dgl162* and N22-H-*dgl219*. They were grown in M4 generation to confirm that they are breeding true and genetically fixed through advancement to M5. For all experiments, plants were grown in a greenhouse till four-leaf stage in pots containing soil under natural irradiance, temperature of 28 °C and air humidity of 60 %. For dark treatment, 15 plants were covered with cardboard boxes for 5 d in greenhouse. For heat treatment, equal sized leaf samples were maintained at 40 °C in water bath for 72 h.

For all experiments, middle portions of the 3rd leaf from the apex were taken. Pigments were extracted in 80 % acetone. Chlorophylls and carotenoids were determined by UV-VIS spectrophotometer *Lambda 35* (*Perkin Elmer*, MA, USA) according to Lichtenthaler and Wellbum (1983). The photochemical efficiency of photosystem 2 was measured as variable to maximum chlorophyll fluorescence ratio (F_v/F_m) using chlorophyll fluorescence meter (*FIM1500*, *ADC*, Hoddesdon, UK). For native polyacrylamide gel electrophoresis (PAGE), chlorophyll-protein complexes were isolated according to Jiang *et al.* (2007). Detection of H_2O_2 and superoxide radicals was done according to Fitzgerald *et al.* (2004). Each experiment was repeated 3 - 5 times and the results were pooled for statistical analysis.

Under normal growth conditions, N22-H-*dgl101* and N22-H-*dgl162* leaves had more than double amount of total chlorophyll compared to N22, N22-H-*dgl56* and N22-H-*dgl219* (Fig. 1*A*). After 5 d in dark, total chlorophyll in N22, N22-H-*dgl101* and N22-H-*dgl162* leaves was reduced considerably but remained similar in N22-H-*dgl219* and showed a slight increase in N22-H-*dgl56*. Under normal growth conditions, total carotenoid content in N22-H-*dgl56,* N22-H-*dgl219* and N22 was lower than in N22-H-*dgl101* and N22-H-*dgl162* (Fig. 1*B*). After 5d in dark, the carotenoid content was reduced to 28 % of the initial amounts in N22, whereas all the mutants had higher total carotenoid content compared to N22. F_v/F_m was between 0.76 - 0.82 in N22 and all the mutants under normal growth conditions (Fig. 1*C*). After 5 d in dark, F_v/F_m in N22 fell to 0.15. In all the mutants, it was higher ranging from 0.21 to 0.54.

Integrity of chlorophyll-protein complexes in the thylakoid membrane was studied by native PAGE. The gel profiles showed two bands for the reaction center proteins, two bands for the light harvesting protein complex and one band for the free proteins (data not shown). After 5 d in dark, reduced amount of reaction center proteins was observed and light harvesting complex proteins and free proteins were absent in N22 and N22-H-*dgl219* samples. In N22-H-*dgl56*, N22-H-*dgl101* and N22-H-*dgl162*, the reaction center proteins were more stable and one of the bands of light harvesting complex proteins was retained. The stable chlorophyll complexes in the mutants were in agreement with their higher F_v/F_m after dark treatment. These results showed that the stay-green phenotype of all the mutants was associated with higher chlorophyll and carotenoid

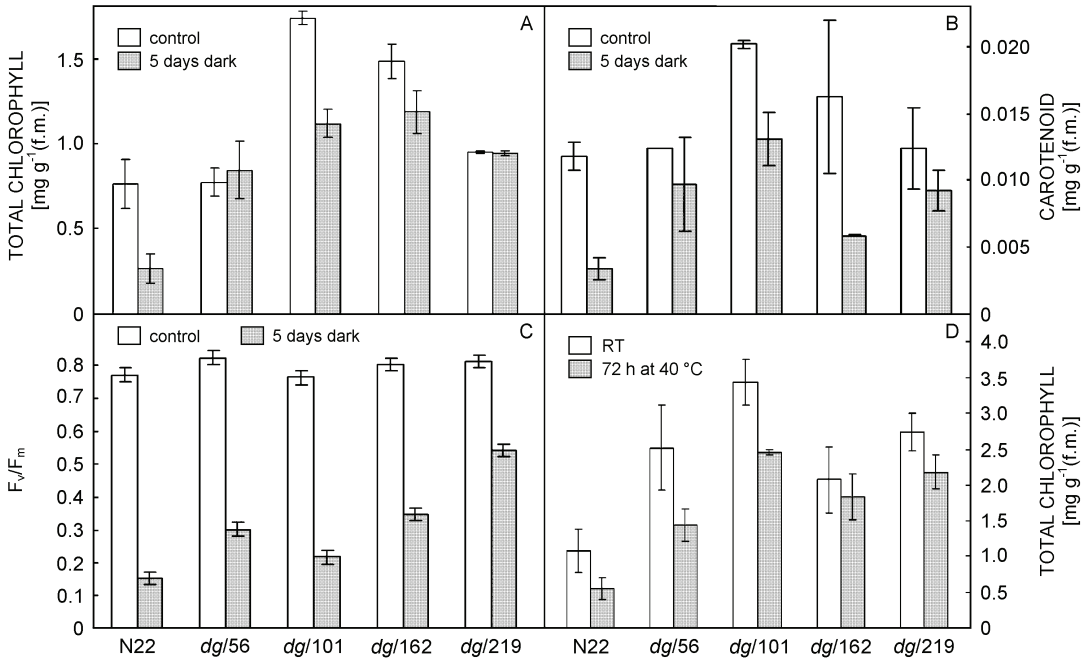

Fig. 1. The leaf chlorophylls (*A*) and carotenoid (*B*) contents and photochemical efficiency (F_v/F_m; *C*) in 4-week-old N22 and mutant plants grown under normal conditions or after 5 d in dark. *D* - chlorophyll content in cut leaves of 4-week-old N22 and mutants after incubation at 40 °C for 72 h. Means ± SE of five independent experiments.

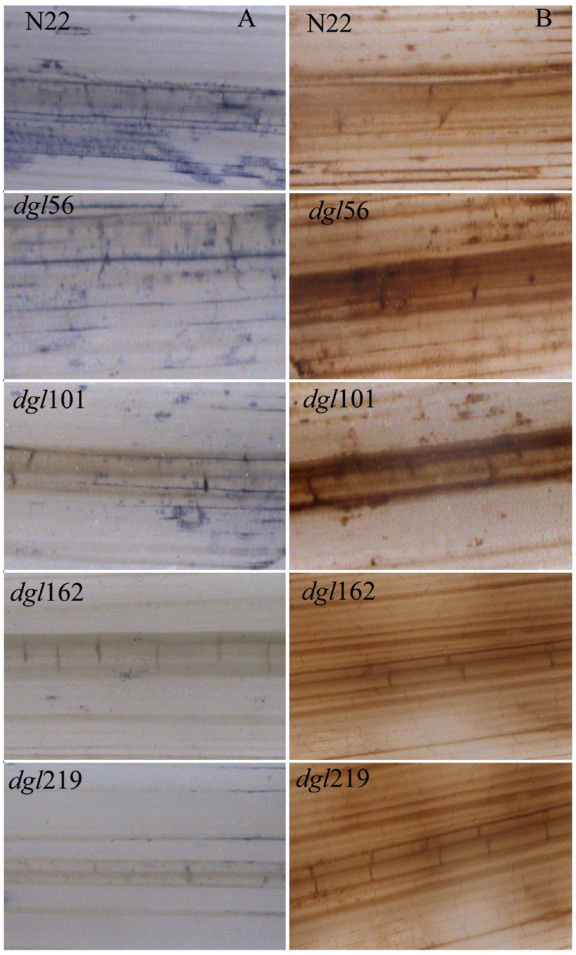

Fig. 2. Accumulation of superoxide radical (*A*) and H_2O_2 (*B*) in leaves of 4-week-old N22 and mutant plants after incubation at 40 °C for 72 h. Leaves were infiltrated with nitroblue tetrazolium (*A*) or diaminobenzidine (*B*) and precipitates with O_2^- and H_2O_2 were detected using light microscopy.

contents, higher photochemical efficiency and more stable pigment complexes as compared to N22 under conditions of dark-induced senescence.

N22 is known to be a drought and heat tolerant cultivar and has been used as a donor parent in breeding for drought tolerance (Markandeya *et al.* 2007, Jagadish *et al.* 2008). Further, the mutant and N22 leaves were evaluated for heat tolerance. After heat treatment (40 °C for 72 h), N22 leaves turned yellow retaining only 51 % of total chlorophyll present in samples incubated at room temperature (Fig. 1*D*). The mutant leaves were more heat tolerant compared to N22 leaves as they retained more chlorophyll (Fig. 1*D*). Drought or heat can induce production of hydrogen peroxide and superoxide radicals (Asada 2006). Accumulation of O_2^- and H_2O_2 was analyzed by staining with NBT and DAB, respectively, following 72 h of heat treatment at 40 °C. The accumulation of O_2^- and H_2O_2 in leaf samples of N22 and N22-H-*dgl56* was high and N22-H-*dgl101* also showed detectable amounts (Fig. 2*A*,*B*). On the other hand, N22-H-*dgl162* and N22-H-*dgl219* showed negligible accumulation of ROS in response to heat treatment (Fig. 2*A*,*B*). These results indicate that the reduced level of ROS leads to heat tolerance in leaves of N22-H-*dgl162* and N22-H-*dgl219* mutants and retaining of high chlorophyll contents. Maintenance of chlorophyll content under dark-induced senescence in gibberellic acid pre-treated *Pelargonium* leaves was also due to lower ROS accumulation (Rosenwasser *et al.* 2010). Tolerance to elevated temperatures was reported in lily plants by increased activity of antioxidants and decrease in ROS production (Yin *et al.* 2008). A superhybrid of rice showed lower decrease in F_v/F_m and higher activities of antioxidative enzymes under chilling stress as compared to the parental cultivars (Zhang *et al.* 2010). The role of antioxidative systems in reducing the levels of ROS in N22-H-*dgl162* and N22-H-*dgl219* under heat stress remains to be elucidated in future experiments.

It can be concluded that four stay-green mutants have higher contents of chlorophylls and carotenoids, and photochemical efficiency than N22. Cut leaves of mutants retained higher chlorophyll content and accumulated less ROS upon heat treatment compared to N22. These rice mutants may have agronomic importance in coping with expected increase in temperature in the context of climate change.

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