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THE OVER-EXPRESSION OF A GLYCINE-RICH RNA-BINDING PROTEIN IN *NICOTIANA TABACUM* AND ITS ROLES IN PLANT STRESS TOLERANCE

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Abstract

Glycine-rich RNA-binding proteins (GR-RBPs) have been explored in different plant species for their role in development and environmental stress responses. *Nt*GR-RBP1, a tobacco glycine-rich RNA-binding protein has recently been suggested as RNA chaperone with reference to protective role in bacteria upon exposure to various stresses. In the present study, we aimed to test the roles of *Nt*GR-RBP1 in plants. The cDNA sequence of *Nt*GR-RBP1 was amplified and cloned into entry vector using pENTR/D-TOPO cloning kit followed by sub-cloning into a plant GATEWAY® expression vector resulting in construction of pH7WG2-*Nt*GR-RBP1. *Nt*GR-RBP1 recombinants were confirmed through colony PCR and sequencing. Further, pH7WG2-*Nt*GR-RBP1 was cloned into *Agrobacterium tumefaciens* AGL1 competent cells and confirmed by PCR after screening on streptomycin and hygromycin. The culture was subsequently used for the transformation of *Nicotiana tabacum* cv samsun by *Agrobacterium*-mediated plant transformation using the leaf disc method. Explants were screened using hygromycin as selection marker and later confirmed through PCR. Seed germination assay showed that *Nt*GR-RBP1 had significant role in response to cold, heat and drought stress, while salt stress has negative effect on germination. Transformed plants will be used further for in depth functional characterization of *Nt*GR-RBP1.

Key words: Glycine-rich RNA-binding Protein, *Nicotiana tabacum*, RNA chaperone.

Introduction

Plant Glycine Rich RNA-Binding proteins (GR-RBPs) were isolated owing to their differential expression and modulations towards stimuli such as biotic and abiotic stresses about two decades ago. Belonging to the Class IVA of RBPs, these bipartite proteins contain an RNA recognition motif (RRM) at N-terminus and a glycine rich region at the C-terminus (Mangeon *et al*., 2010). GR-RBPs play crucial roles towards growth and development of different plant species (Staiger *et al.*, 2003). Their multifunctional roles include transport and localization of RNA, mRNA splicing, post-transcriptional metabolism of RNA, polyadenylation, translation and degradation (Glisovic *et al.*, 2008). GR-RBPs ubiquitously exist in plants and the expression of GR-RBP encoding genes is regulated in response to a variety of abiotic stresses, i.e. cold, heat, drought, salinity, wounding and flooding, biotic stress, such as pathogen attack (Gomez *et al*., 1998; Maruyama *et al*., 1999; Glisovic *et al*., 2008; Califice *et al*., 2012; Khan *et al*., 2013). Other stress conditions that also regulate the expression of these proteins include phytohormones, viral infection, UV irradiation, and heavy metals (Naqvi *et al*., 1998; Sachetto-Martins *et al*., 2000; Aneeta *et al*., 2002; Mangeon *et al*., 2010). During cold stress canonical cold shock domain proteins (CSPs) along with GR-RBPs play protective role in plant adaptation (Nakaminami *et al.*, 2006). In various studies, heterologous expression of GR-RBPs rescued reduced growth rates of plants during cold adaptation that had their GR-RBP genes deleted. (Kim *et al*., 2010). *Arabidopsis thaliana* and *Oryza sativa* have been investigated deeply for illuminating the response of GR-RBPs in encounter to different stress conditions, for example, a CCHC-type zinc finger motif containing protein at its C-terminus conferred freezing tolerance in *Arabidopsis*, while when expressed, it has a negative impact on seed germination and seedling growth upon dehydration and salt stress (Kim *et al*., 2007a). For instance, over-expression of GR-RBP2 during salt stress had no effect on seedling growth while it improved seed germination (Kim *et al*., 2007b). The RNA chaperone activity of *At*GR-RBP2 was confirmed in *E. coli* in response to cold acclimation (Kim *et al*., 2007c). Over-expression of another GR-RBP i.e. *At*GR-RBP7, during cold stress caused transport of mRNAs to the cytoplasm from the nucleus and promoted freezing tolerance by regulation of stomatal apertures (Kim *et al*., 2008). A rice *Os*GR-RBP4 was reported to be involved in heat stress (Sahi *et al*., 2007) while, over-expression of *Sa*GR-RBP1 from *Suaeda asparagoides* conferred salt tolerance in *Arabidopsis* (Ayarpadikannan *et al*., 2014). Furthermore, seven *Bn*GR-GRPs were found in *Brassica napus* with the almost same sequence of N-terminal RRM domain while there were significant differences in the sequence of C-terminal glycine rich region. Transcript expression of all *Bn*GRPs was remarkably elevated in response to cold, but was seen down-regulated in response to dehydration and salt stress. The functional roles of *Bn*GRP1 as RNA chaperone were determined by over-expression of *Bn*GRP1 in cold sensitive mutant BX04 *Escherichia coli, which* resulted in improved cold tolerance, while the heterologous expression of the same gene in Arabidopsis showed the elevated seed germination rate and plants showed freezing tolerance (Kim *et al*., 2012).

The stress related role of *Nt*GR-RBP1 was previously explored by checking the transcript expression of *Nt*GR-RBP1in response to cold, salt and wounding stress, the level of mRNA was upregulated in response to cold stress and it was modulated in salt stress, while wounding caused no effect, so it was concluded that *Nt*GR-RBP1 has a

definite role in response to cold stress (Khan *et al*., 2013). By sequence analysis, *Nt*GR-RBP1 is a homologue of *At*GR-RBP7, in Nuclear Magnetic Resonance spectroscopy experiments, CTG comprising nucleic acids were more efficiently melted by *Nt*GR-RBP1 than the isolated *Nt*RRM domain. Upon cold treatment RNA adopts secondary structures while *Nt*GR-RBP1unfolds and helps in efficient translation of mRNA hence providing its suggestive role as RNA chaperone (Khan *et al*., 2014). Expression of *Nt*GR-RBP1in *E. coli* under stress conditions such as salinity, drought, cold and heat was observed by spot assay and liquid culture assays that exhibited the recombinant *E. coli* had better growth and were more tolerant (Jabeen *et al*., 2017). In the present study, we tried to further elucidate the role of *Nt*GR-RBP1 as plant RNA chaperone by over-expression of *Nt*GR-RBP1 in *N. tabacum* as a model plant utilizing GATEWAY®® technology. Stress tolerance of seeds from T^2 generation of transformed plants was checked through seed germination assay against different stress conditions i.e. cold, heat, salt and drought stress. Seeds of transgenic plant exhibited improved stress tolerance as compared to the wild type seeds, suggesting some role of *Nt*GR-RBP1during exposure to adverse environmental conditions.

Materials and Methods

Plant material: Seeds of *Nicotiana tabacum* cv samsun were acquired from National Agriculture and Research Council (NARC), Islamabad. Surface sterilization was done with 30% Clorox followed 3-5 washes with distilled autoclaved water, followed by culturing on half strength MS media supplemented with vitamins (Murashige & Skoog, 1962) containing 30 g/L of sucrose and 6 g/L agar. Plants were kept in a plant culture room under 16-h light/8-h dark photoperiod and 25ºC.

RNA extraction, RT-PCR and cloning: A pair of primers was designed for directional cloning, adding an overhang of CACC at 5' end of forward primer i.e., GRP-TOPO-F: 5'-CACCATGGCTGAAGTTGAATACAGGT-3' and GRP-TOPO-R: 5'-TTAACTCCTCCAGCTTCCTTC-3'. RNA was extracted using Gene JET RNA Purification Kit by (Thermo Scientific) that was used immediately in cDNA synthesis (Thermo Scientific). Thermal profile with an initial denaturation at 95°C for 3mins, followed by 30 cycles, i.e. denaturation at 95°C for 20 sec, annealing at 58°C for 30 sec and extension at 72°C for 40 sec respectively and a final extension for 10 min at 72°C. PCR products were visualized on 1% agarose. After confirmation, for directional TOPO cloning a blunt end PCR product was produced by the help of Pfu DNA polymerase and ligated into pENTR, an entry vector by using Topo cloning kit (Invitrogen) following manufacturer's instructions. Purified plasmids from few selected clones were further PCR amplified with gene specific primers. Confirmed recombinant entry vectors were further used for construction of plant Gateway® expression vector using Gateway®™ conversion technology, LR Clonase™ II Enzyme Mix (Invitrogen) was used to carry out the LR recombination reaction, in which 150 ng/µL purified recombinant entry clone was mixed with 150 ng/µL destination/expression vector in a 1.5 mL microcentrifuge tube and 2 µL of LR Clonase™ II Enzyme Mix was added followed by incubation at 25°C for 2 hrs. To terminate the reaction 1 µL of proteinase K solution was added with incubation at 37°C for 10 mins. This reaction mixture was transformed into *E. coli* competent cells through electroporation and further confirmed by colony PCR and PCR with purified plasmid as template DNA with gene specific primers. Commercial sequencing further confirmed the presence of the target gene.

***Agrobacterium*-mediated plant transformation of *N. tabacum*:** *Agrobacterium tumefaciens* AGL1 strain was transformed with PH7WG2-*Nt*GR-RBP1 expression vector through electroporation and confirmed by PCR amplification of purified plasmid using gene specific primers. *Agrobacterium*- mediated plant transformation was done by using the leaf disc method (Bottino *et al*., 1989). About 4-8 weeks old young plant leaves of *N. tabacum* were used to prepare leaf discs of approximately 4-6 cm in diameter/length that were surface sterilized with 10% Clorox. For co-cultivation, 25 µL of starter culture was added in 25 mL of LB liquid with rifampicin (50 mg/mL), streptomycin (50 mg/mL) and hygromycin (50 mg/mL) followed by incubation at 28°C till OD_{600} reached 0.4. The culture was pelleted down at 3000 g for 10 mins and the pellet was properly resuspended in 25 mL liquid ½ strength MS media. Subsequently, the transformation mixture was incubated at 28°C with continuous shaking till OD_{600} reached 0.6, prior to dipping of leaf discs 100 µM acetosyringone was added to prepared culture. Leaf discs were dipped in 0.6 OD_{600} maintained transformation mixture for 25-30 mins and blot dried onto filter paper to absorb excess culture. Then leaf discs were placed on co-cultivation media containing 200 µM acetosyringone and placed in the dark for 36 hrs. Later, leaf discs were washed with 500 mg/mL cefotaxime twice followed by a final wash with autoclaved distilled water. The blot dried leaf discs were placed on ½ strength MS selection media containing 250-500 mg/mL of cefotaxime and 50 mg/mL hygromycin. Cultures were maintained by changing selection media after every 3 weeks. Explants obtained via direct somatic embryogenesis were shifted to rooting media for root development. Later, developed explants were shifted to shooting media and kept on selection until confirmed through PCR by using isolated DNA from transformed plants as template and amplified with hygromycin primers.

Seed germination assay: To check the abiotic stress tolerance, seeds from both transgenic and wild type plants were surface sterilized with 30% Clorox followed by 4-5 washes with distilled autoclaved water. Four stress conditions were used i.e. cold, heat, salt and drought stresses. Following conditions were set for each stress condition i.e. for cold stress seeds from both wild type and transgenic plants were cultured on ½ MS medium and plates were incubated in growth chamber at 12°C. While for heat stress plates were incubated in growth chamber at 38°C. For salt stress seeds from both wild type and transgenic plants were cultured on ½ MS medium supplemented with 50 mM, 100 mM, 250 mM NaCl. Plates were incubated in growth chamber at 25°C. For

drought Stress, seeds from both wild type plants were cultured on ½ MS medium supplemented with 50 mM, 100 mM, 250 mM Mannitol and plates were incubated in growth chamber at 25°C. For experimental control, seeds from both wild type and transgenic plants were cultured on ½ MS medium and incubated in growth chamber at 25°C, all the growth chambers were set at photoperiod of 16/8 hours with 40% humidity.

Results and Discussion

*Nt*GR-RBP1 of tobacco, ~16kDa protein consists of *Nt*RRM domain and a glycine-rich region, both comprising of nearly 85 residues each. Sequence alignment shows that *Nt*GR-RBP1 is conserved in various organisms with orthologs in *zea mays* and *Arabidopsis thaliana* with 73% and 76% identity, respectively. Furthermore, homology with human, bacteria and mouse was also observed (Khan *et al*., 2014). Most recently, the role of *Nt*GR-RBP1 as RNA chaperone by ectopic expression of *Nt*GR-RBP1 in *E. coli* during various stress treatments resulted in better growth and survival than the non-recombinants (Jabeen *et al*., 2017). However, as a plant protein, the over-expression of *Nt*GR-RBP1 in plants may offer a better insight and detailed information with reference to its function as RNA chaperone, which undoubtedly requires an efficient plant expression system. According to various reports, over-expression of a gene may or may not alter the expression of other genes, for example, *At*GR-RBP7 was found to promote the floral transition in native plant over-expressing *At*GR-RBP7, which also acted as flowering time regulator and controlled expression of various other transcripts (Streitner *et al*., 2008). In plants, over-expressing *At*GR-RBP7, phytohormones involved in GA biosynthesis were reduced that suggested its regulatory role with respect to late-flowering (Lohr *et al*., 2014) along with consequent differential expression of one third of expressed genes (Streitner *et al*., 2010). Moreover, *Bn*GR-RBP1 over-expressing plants showed increased freezing tolerance that also support the role of GR-RBPs as RNA chaperone during cold (Kim *et al*., 2012). In a recent study, adaptation of iCLIP was combined with RIP-seq that identified 452 high-confidence targets regulated by *At*GR-RBP7 in mutants as well as plants over-expressing this protein, among these targets stress related genes occupy the huge number (Meyer *et al*., 2017). Therefore, we aimed at generating plants over-expressing *Nt*GR-RBP1 by devising an efficient plant expression system so that the role of this protein can be studied. In order to evaluate the stress tolerance in plants over-expressing *Nt*GR-RBP1, seeds from these plants were subjected to different stress conditions and their germination rate was checked.

TOPO cloning for recombinant entry vector: After RNA isolation and cDNA synthesis, purified PCR product (Fig. 1A) was cloned into entry vector. Recombinant entry vector was propagated into TOP10 *E. coli* competent cells followed by colony PCR and another PCR taking plasmid as template (Fig. 1C).

Recombinant plant expression vector: Gateway® technology (Invitrogen) is based on a phage λ mediated recombination reaction being also site-specific. Gateway® technology is fast, precise in results and enzyme free cloning, i.e. one does not require ligase enzyme unlike conventional cloning methods. Eliminating the need of restriction enzymes and sub-cloning steps, this also diminishes the chance of unwanted chunks of sequences away. Furthermore, the use of multi-site Gateway® technology can be used for cloning multiple genes once in all through same recombination technique (Marsischky & Labaer, 2004). Recombinant plant Gateway® expression vector pH7WG2 -*Nt*GR-RBP1 was transformed into *E. coli* DH5α competent cells through electroporation, followed by confirmation through colony PCR and PCR with plasmid DNA taken as template (Fig. 1B, C, D).

Plant transformation: Confirmed recombinant expression vector pH7WG2 -*Nt*GR-RBP1 was transformed into *Agrobacterium tumefaciens* AGL1 strain followed by confirmation through colony PCR prior to proceeding towards plant transformation. Explants were obtained through direct somatic embryogenesis (Fig. 2). Hygromycin (50 mg/mL) helped with selection of transformed explants while cefotaxime was increased up to 500 mg/mL to avoid the excess growth of *Agrobacterium* that contaminated few cultures. Prior use of rooting hormone IAA at a concentration of 0.2 mg/L was performed because it was observed that as the roots emerged, the selection became faster due the selection pressure of hygromycin selection marker and it became easier to distinguish between a transformed and an untransformed explant. Rooted explants were shifted to shooting media containing 1.5mg/mL of BAP, a shooting hormone, along with hygromycin and cefotaxime. As soon as the plants gained some height and sufficient mass they were scrutinized for being transformants or escapes. PCR with hygromycin primers confirmed the transformants (Fig. 1E) After confirmation they were shifted to ½ MS medium without hygromycin but cefotaxime was not removed till the cultures were shifted to soil for acclimatization. Plants remained stable upon shifting to a green house, interestingly, they started flowering earlier than the season so we can also hypothesize that *Nt*GR-RBP1 may also act as flowering time regulator and play its role in floral transition as seen in the case of *At*GR-RBP7 (Streitner *et al*., 2008), an ortholog of *Nt*GR-RBP1 with 76% sequence identity (Khan *et al*., 2014) and despite of the weather fluctuations, they remained unharmed. Therefore, studying the role of these transformants under stress conditions will not only be interesting but will also increase the importance of *Nt*GR-RBP1 in different environmental stresses which we aim to study later.

Seed germination assay: GR-RBPs help in rescuing plants from different stress conditions (Mangeon *et al*., 2010). Seed germination assay was set to evaluate the response of plants over-expressing *Nt*GR-RBP1 as compared to wild type plants. Seeds from wild type and transgenic plants were used in the experiment. Over all, it was observed that transgenic seeds showed faster germination as compared to wild type plants. The seeds were considered germinated when a small outgrowth protruded bursting the seed coat; it later formed hypocotyls and epicotyls. In control conditions, when comparison was made between transgenic and wild type seed germination rate, by 4th day all the transgenic seeds were germinated. While all wild type seeds germinated by 7th day. This showed transgenic seeds grew faster in comparison to wild type.

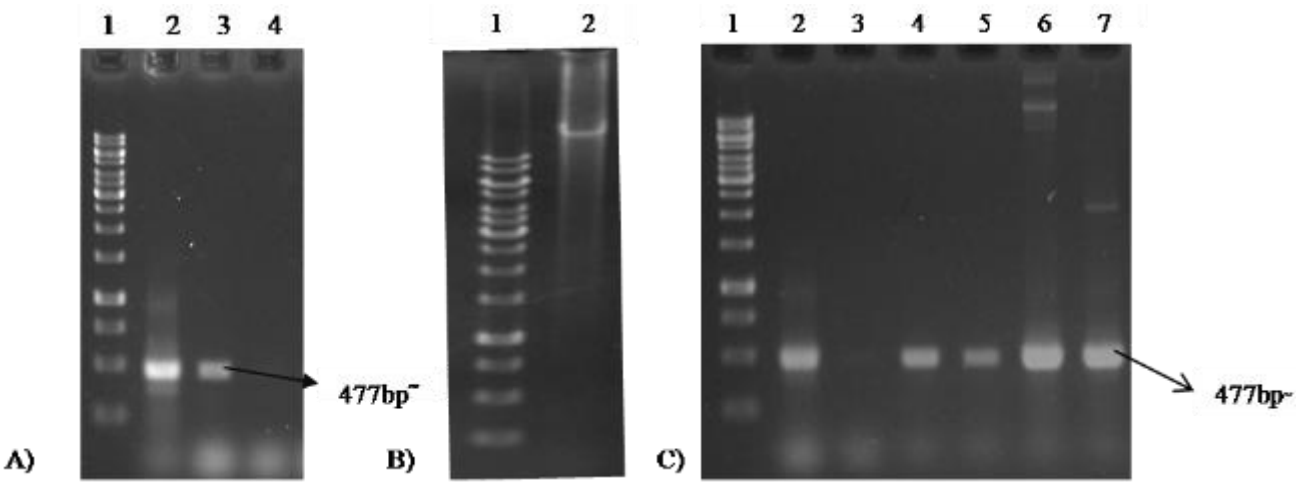

Fig. 1. A) PCR amplification of cDNA of *Nt*GR-RBP1 with gene specific primers, Lane 1: 1kb DNA ladder (Fermentas), Lane: 2-3 PCR products, Lane 4: Negative control B) isolated Recombinant plant Gateway® expression vector (pH7WG2 -*Nt*GR-RBP1), Lane 1: 1kb DNA ladder (Fermentas), Lane 2: isolated plasmid i.e. pH7WG2 -*Nt*GR-RBP1 C) PCR Confirmation of *Nt*GR-RBP1 gene on different dilutions of isolated recombinant entry clone and recombinant plant Gateway® expression vector (pH7WG2 -*Nt*GR-RBP1) with gene specific primers, Lane 1: 1kb DNA ladder (Fermentas), Lane 2: Positive control, Lane 3: Negative control, Lane 4 & 6: PCR amplification of isolated expression vector (pH7WG2 -*Nt*GR-RBP1), Lane 5 & 7: PCR amplification of isolated recombinant entry clone.

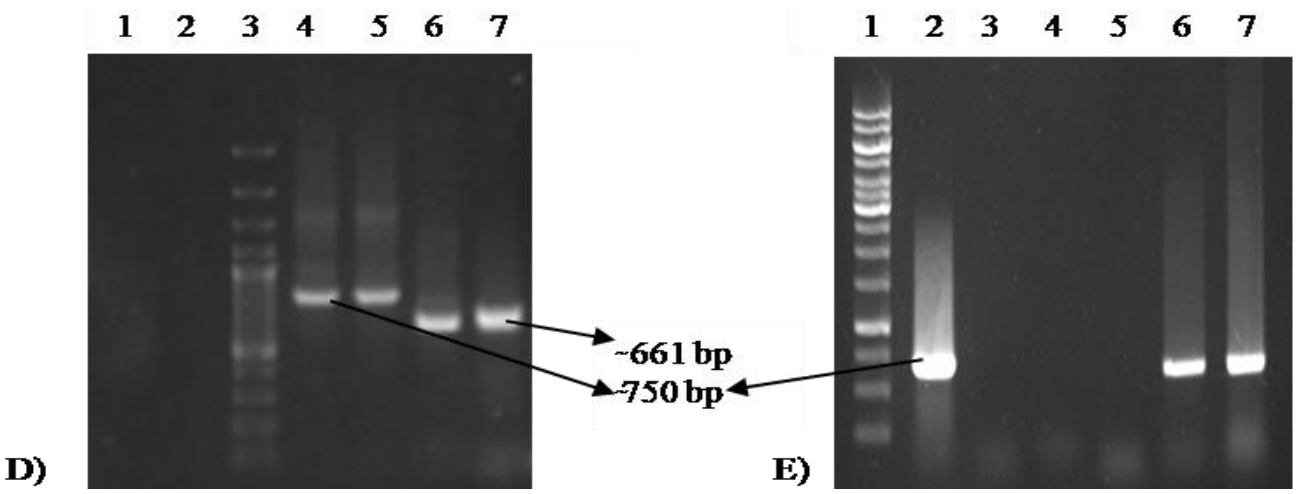

Fig. 1. D) PCR confirmation of pH7WG2 -*Nt*GR-RBP1 with Hygromycin F&R primer pair, Lane 1: Negative control Hygro F&R primers, Lane 2: Negative control using P35S forward and gene specific Reverse primer for *Nt*GR-RBP1 as primer, Lane 3: 100bp DNA ladder (Thermo Scientific), Lane 4 & 5: PCR amplification of pH7WG2 -*Nt*GR-RBP1 with Hygromycin F&R primer pair, 750 bp PCR product. Lane 6 & 7: PCR amplification of pH7WG2 -*Nt*GR-RBP1 with P35S forward and gene specific reverse as primer pair, expected PCR product of 661 bp. E) PCR Confirmation of transgenic plants using hygromycin primer pair, Lane 1: 1kb DNA ladder (Fermentas), Lane 2: Positive control, Lane 3 & 4: Negative control, Lane 6 & 7: PCR product of ~750 bp from extracted DNA.

Germination during cold stress: During cold stress condition at 12°C, wild type seeds started to germinate at 5th day but only 30% seeds germinated till 10th day, whereas, for transgenic over-expressing *Nt*GR-RBP1, seeds started to germinate on 2nd day and germination rate reached 53% by the end of 6th day, while on10th day germination rate reached 97% (Fig. 3A). During cold stress plants show certain degree of freezing tolerance by underlined molecular mechanisms (Chang et al., 2020). In a study *At*GR-RBP2 was induced upon cold treatment while, *At*GR-RBP4 and *At*GR-RBP7 were up-regulated upon cold treatment in *A. thaliana*. *At*GR-RBP2 over-expressing lines showed freezing tolerance and better seed germination rate as compared to wild type plants, while the knock out mutants were unable to survive in chilling temperatures (Kim *et al.,* 2007). It was concluded that seeds of transgenic plant over-expressing *Nt*GR-RBP1 showed better tolerance to cold stress and still managed to germinate as compared to wild type seeds, highlighting the role of *Nt*GR-RBP1 in cold stress tolerance.

Germination during heat stress: *At*GR-RBP7 is a close homologue of *Nt*GR-RBP1, Level of *At*GR-RBP7 was not as affected by heat stress as compared to cold stress but was increased (Wienkoop *et al*., 2008). So it was interesting to see the response of *Nt*GR-RBP1 in response to heat stress, it was observed that the seeds of both transgenic and wild type started to germinate after 2nd day. While, after 6th day, 100% transgenic seeds germinated, whereas, wild type seeds germination rate was 88% and didn't reach up to 100% (Fig. 3B). These results depicted the role of *Nt*GR-RBP1 in response to heat stress.

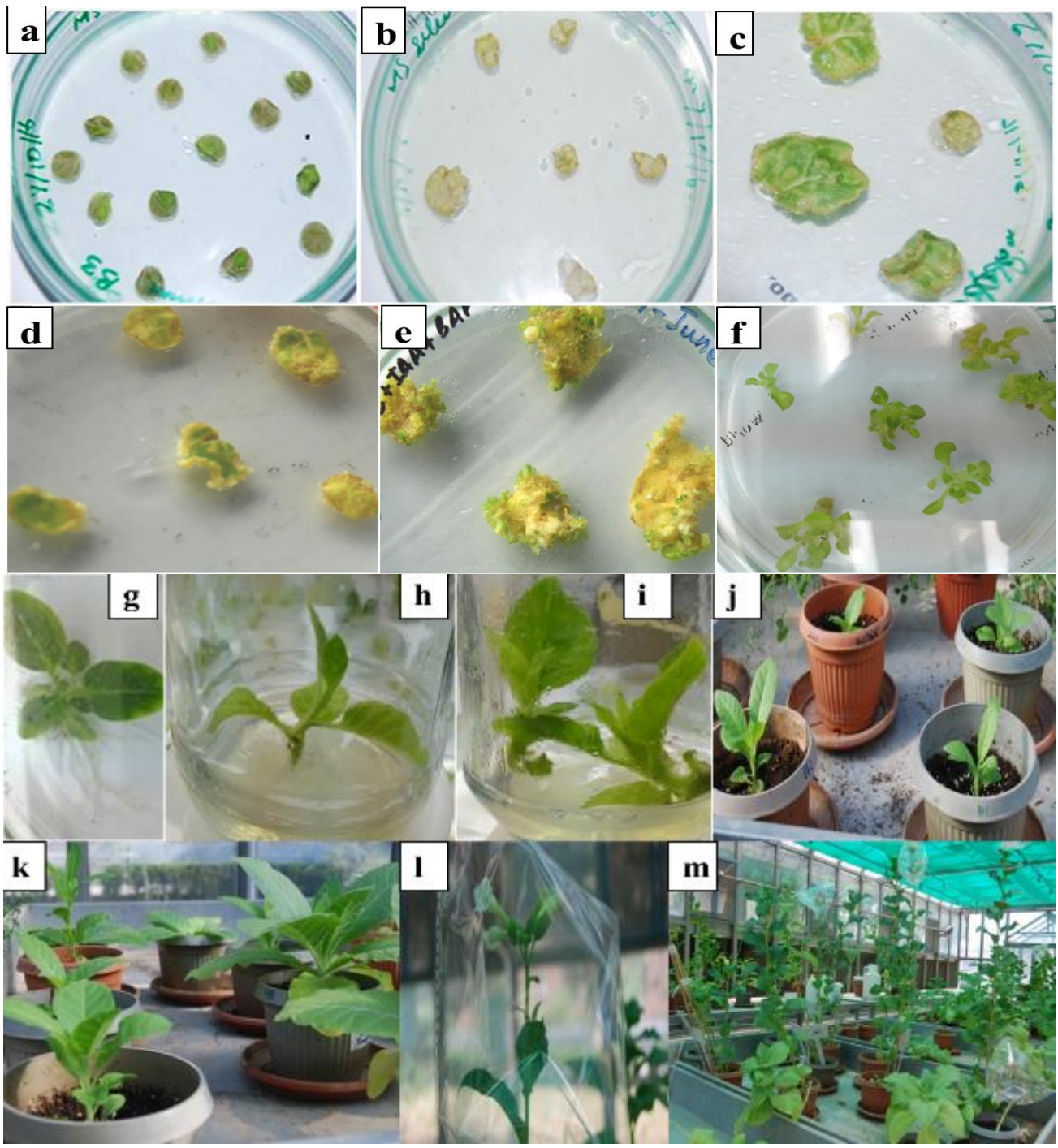

Fig. 2. Stages of *Agrobacterium* mediated plant transformation of *Nicotiana tabacum*, a: Explants placed on selection medium containing hygromycin and cefotaxime, after Co-cultivation. b: Negative control i.e. non-transformed explants on selection media (with antibiotic) after 15 days. c: Positive control i.e. non-transformed explants on simple ½ MS medium (without antibiotic) after 15 days. d: Treated explants with transformed *Agrobacterium* culture with pH7WG2-*Nt*GR-RBP1 after 15 Days on selection medium. e: Treated explants with transformed *Agrobacterium* culture with pH7WG2-*Nt*GR-RBP1after 35 days on selection medium. f: explants gained from somatic embryogenesis on selection medium after 50 Days. g: Explants shifted to Rooting medium for proper selection on selection medium. h & i: resistant explants that survived selection shifted to shooting medium PCR also confirmed the presence of hygromycin resistant gene at this stage. J & k: Shifting of confirmed transgenic plants to peat moss in green house for acclimation. l & m: transformed plants exhibiting flowering stage in green house.

Germination during salt stress: Salinity is one of the major reasons of yearly loss of crop yield in many parts of the world. All GR-RBPs may not respond towards salt stress e.g. *At*GR-RBP4 under high salt stress i.e., 250 mM NaCl didn't rescue from stress condition and seed germination was retarded (Kwak *et al*., 2005). It was observed that during higher salt concentration of 250 mM, wild type and transgenics over-expressing *Nt*GR-RBP1 behaved almost similar by 10th day of germination. Only, 25% seeds of transgenic and 27% seeds of wild type were germinated, suggesting that the higher salt concentration had negative effect on germination (Fig. 3C).

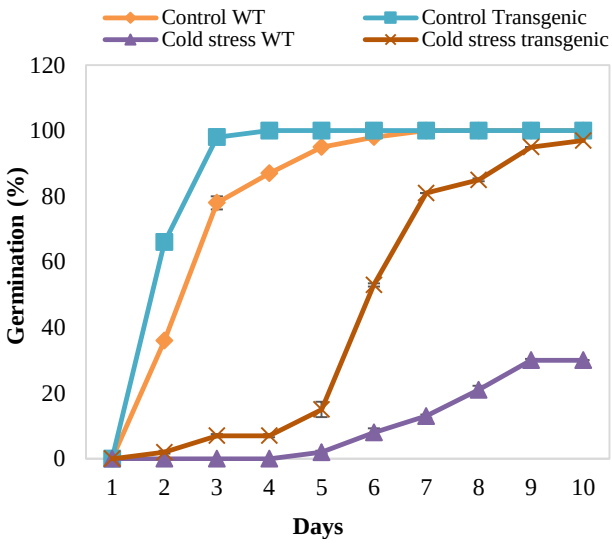

Fig. 3A. Seed Germination rate during cold stress at 12°C
Seed germination rate of wild type seeds differed to transgenic seeds. In Control conditions, 100% germination was achieved by wild type seeds at 7th day, while seeds from transgenic plants germinated 100% at 4th day, showing faster growth in comparison. Interestingly, 97% seeds from transgenic plants germinated as compared to 30% seeds from wild type plants germinated by 10th day during cold stress at 12°C. Values are means ± SE. Values are taken from three independent experiments (n=20).

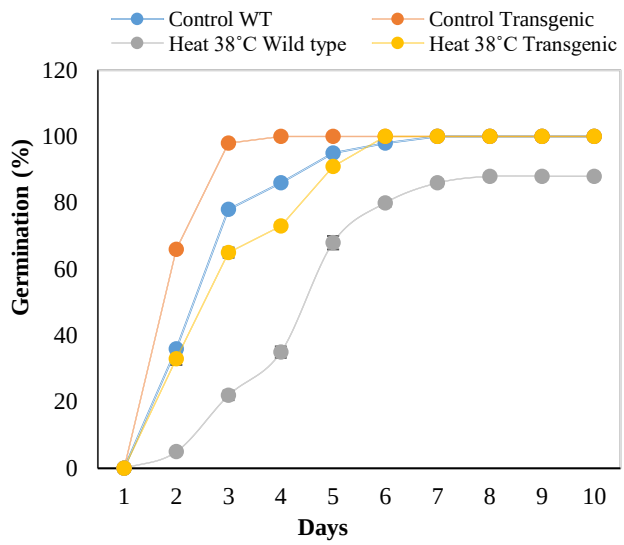

Fig. 3B. Seed Germination rate during Heat stress at 38°C
Germination rate reached 100% for transgenic seeds by 10th day as compared to 88% seed germination rate for wild type seeds. Values are calculated as means ± SE. Values are taken from three independent experiments (n=20). Seeds of transgenic line showed increased germination rate in comparison to wild type during heat stress.

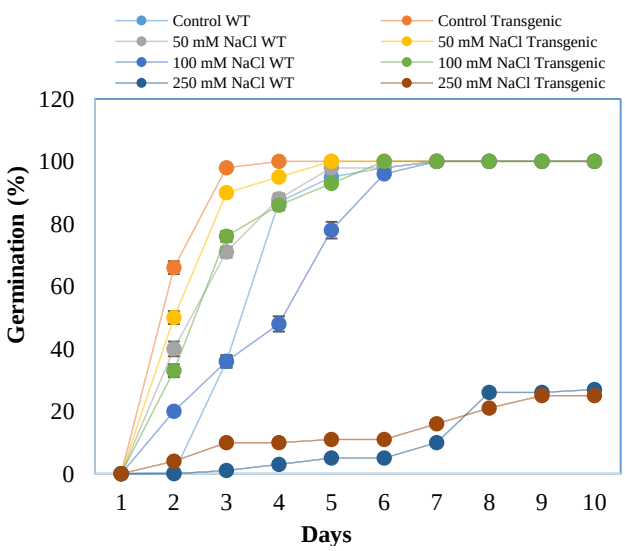

Fig. 3C. Seed germination during salt stress.
Higher salt concentration of 250 mM had negative effect on germination, whereas at low salt concentration germination rate was still higher in transgenic seeds as compared to seeds from wild type plants. Only, 25% seeds of transgenic and 27% of wild type germinated by 10th day, hence affected equally. Values are calculated as means ± SE. Values are taken from three independent experiments (n=20). Seeds of transgenic plants showed better growth in comparison to wild type in control conditions.

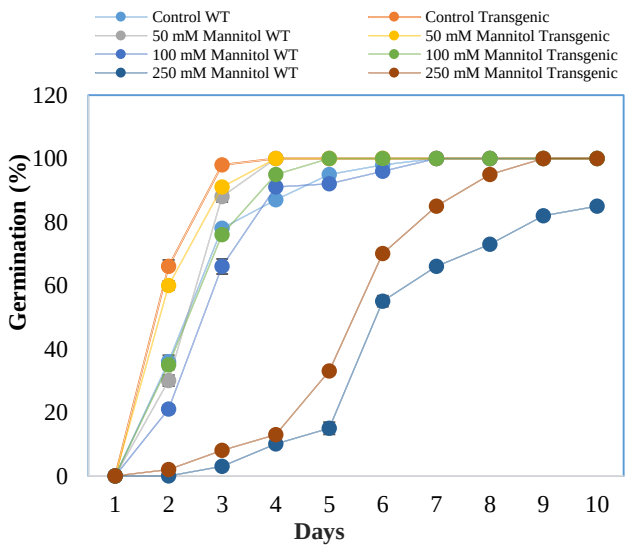

Fig. 3D. Seed germination during drought stress
At higher Mannitol concentration of 250 mM, seed germination rate of transgenic seeds was better as compared to wild type seeds. Transgenic seeds germinated 100% by 10th day in comparison to 85% seeds of wild type. Values are means ± SE. Values are taken from three independent experiments (n=20). Seeds of transgenic line showed better growth in comparison to wild type.

Germination during drought stress: GR-RBPs have not been explored deeply in response to drought stress but have been found to play significant role in response to drought stress. In a study by Ciuzan *et al.*, (2015), *At*GR-RBP2 knockouts behaved well in drought conditions contrary to the study conducted by Yang *et al.*, (2014), who demonstrated that *At*GR-RBP2 and *At*GR-RBP7 were over-expressed in rice. The transformed plants were more tolerant to drought stress caused by water deficit in comparison to wild type plants. This difference in result may suggest that GR-RBPs behave independently in different plant species. Therefore, the rate of seed germination in response to drought stress was monitored in plants over-expressing *Nt*GR-RBP1. It was observed

that higher mannitol concentration of 250 mM affected seed germination in both transgenic and wild type, where seeds from transgenic plants showed tolerance to drought stress better than wild type. After 5th day, only 15% seeds of wild type, whereas, 33% seeds of transgenic germinated and after 10th day 100% seeds of transgenic germinated as compared to 85% seeds of wild type (Fig. 3D), hence it was concluded that seeds from transgenic plant showed better tolerance as compared to wild type.

Conclusion

Transformed plants over-expressing *Nt*GR-RBP1 under the control of p35S promoter have been engineered for further studies. Additionally, p35S promoter from cauliflower mosaic virus is highly active in many plants that are transformed, thus the response of transformed plants was initially evaluated against different stress treatments through seed germination assay. In comparison to seeds from plants over-expressing *Nt*GR-RBP1 and seeds from wild type, transgenic seeds showed tolerance in response to cold, heat and drought stress, while salt stress had negative effect on germination in both. Transgenic plants over-expressing *Nt*GR-RBP1 will be used for in depth characterization of *Nt*GR-RBP1 and its role in plant stress physiology.

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