# Overexpression of a glycine-rich protein gene in *Lablab purpureus* improves abiotic stress tolerance

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**ABSTRACT.** Glycine-rich protein (GRP) is involved in the response to abiotic and biotic stresses in plants. A novel GRP gene in *Lablab purpureus* has been identified. The cDNA of *LpGRP* was obtained from an SSH library constructed with root tissues of *L. purpureus* MEIDOU 2012 by waterholding for 10 days. The function of *LpGRP* was also evaluated in *Arabidopsis*. The cDNA of *LpGRP* has 555 bp and encodes a 184-amino acid protein. *LpGRP* was induced by drought and improved tolerance to abiotic stress. In *LpGRP* overexpressing *Arabidopsis*, the tolerance of transgenic seedlings to drought and salt was improved, and transgenic seeds showed insensitivity to both ABA and NaCl. The insensitivity to ABA indicated that there was crosstalk between *LpGRP* and ABA-responsive genes. These results indicated

that *LpGRP* is a drought-responsive gene that can increase the drought and salt tolerance of *Arabidopsis* seedlings overexpressing *LpGRP*.

**Key words:** Abiotic stress; *Arabidopsis thaliana*; Glycine-rich protein; *Lablab purpureus*

## INTRODUCTION

*Lablab purpureus* is a multipurpose leguminous crop that is used for food and fodder and also for weed control due to its strong capacity for nitrogen fixation and large biomass (Maass et al., 2005; Yuan et al., 2009). *L. purpureus* possesses characteristics that are important for tolerance to abiotic stresses, including dehydration and salinity (Murphy and Colucci 1999; D'Souza and Devaraj, 2010).

Glycine-rich proteins (GRPs) contain more than 60% glycine residues in $(Gly\text{-}X)_n$ motifs (Mousavi and Hotta, 2005). A number of GRP-encoding genes have been identified and can be divided into the following three main classes: 1) cell wall components that make up plant repair system during the protoxylem-stretching phase (Ringli et al., 2001), 2) RNA-binding proteins, or RNA-GRPs that are involved in post-transcriptional gene regulation (Kim et al., 2007), and 3) cytokeratin-like proteins (CL-GRPs) that are thought to protect plasma membranes from increasing osmotic pressure (Long et al., 2013).

Over the past several years, the functional roles of GRPs in the stress response have been investigated, and some members of this family in *Arabidopsis* have been shown to enhance seed germination and seedling tolerance to cold stress (Kim et al., 2010a,b). GRPs are also involved in responses to water stress, including drought and waterlogging. The grain yield of rice (*Oryza sativa*) during drought stress was improved following the incorporation of *AtGRP2* and *AtGRP7* into the rice genome (Yang et al., 2014). In contrast, NtGRP1 is a negative modulator in tobacco (*Nicotiana tabacum*), in which it increases stress tolerance to flooding.

Despite gaining increasing knowledge on the functions of GRPs in *Arabidopsis*, rice, and other plants, our understanding of GRPs in leguminous plants is limited. A study of MsGRP in alfalfa (*Medicago sativa*) showed that this RNA-binding protein may be involved in salinity and ABA stress responses (Long et al., 2013), and proteomic analysis of *Pisum sativum* indicated the existence of a glycine-rich RNA binding protein that is induced by abiotic and biotic stresses (Amey et al., 2008); however, the function of GRP in *L. purpureus* has not been determined.

In this study, we identified a new GRP-encoding gene named *LpGRP* in *L. purpureus*. *LpGRP* overexpression in *Arabidopsis* improves tolerance to drought and salinity and increases seed germination during ABA stress.

## MATERIAL AND METHODS

### Plants materials and drought treatment

*L. purpureus* germplasm MEIDOU 2012, which is drought-tolerant, was collected from Hebei Province, China (Yao et al., 2013). Seeds were germinated in PVC pots, 10 cm in diameter and 9 cm in height, under a 16-h/8-h light-dark cycle at 28°C. Drought stress was applied by waterholding for 10 days after 10 days of germination, while the control group

was well-watered every day. Samples were collected from the leaves, roots, shoots, and stems every other day during stress treatment.

## RNA extraction and cDNA synthesis

RNA was extracted from 100 mg tissue from each sample using the UNlQ-10 Total RNA Isolation Kit (Sangon, China), and the PrimeScript RT Reagent Kit (Takara, Japan) was used for cDNA synthesis.

## Isolation of the GRP transcript from *L. purpureus*

EST clones were obtained from an SSH library constructed with root tissues from MEIDOU 2012 that had not been watered for 10 days (Yao et al., 2013). Next, 5'- and 3'-*LpGRP* cDNA fragments were generated with the SMARTer RACE cDNA Amplification Kit and the Advantage 2 PCR Kit (Clontech, USA) using an EST sequence (GenBank accession No. JZ150166.1) and the following primers: 1) 5'-GGAGCGGCTGAAGGAGGTGAAGC-3' and 2) 5'-ATGGAGGTCCGAATGGAGGGTCC-3'. The full-length LpGRP sequence was amplified with the following primers: 1) 5'-TATACTTAATAATACACTGTCTTT-3' and 2) 5'-ACATGGGATCACACACATATCCTACA-3'.

## LpGRP sequence analysis

Sequence analysis was performed with BLAST in NCBI and the phylogenetic analysis was completed using MAGA 4.0 with the neighbor-joining method (Tamura et al., 2007). The protein sequence was scanned using the Prosite (http://prosite.expasy.org/) and Pfam databases (http://pfam.xfam.org/) and the motifs were predicted.

## *Arabidopsis* transformation

The full-length *LpGRP* cDNA was cloned into the pRI201-AN vector (Takara), which carries an NPT II marker, and then was transformed into *Arabidopsis* plants by the floral dip method (Clough and Bent, 1998) with *Agrobacterium tumefaciens* strain GV3101.

## Drought and salt tolerance assays in transgenic *Arabidopsis* seedlings

For drought stress treatment, four seedlings were placed in a pot (10 cm in diameter and 9 cm in height) and 10 pots were used in the experiment. Seedlings were watered constantly for 14 days before being subjected to dehydration. After 2 weeks without water, plants in all pots were watered, and plant regrowth was evaluated after 7 days.

The salt tolerance of transgenic plants was evaluated by the root length of seedlings. Seeds were germinated on MS plates. Wild-type (WT) and transgenic *Arabidopsis* seedlings were transferred to MS plates containing 0, 50, 100, or 150 mM NaCl. After 7 days of treatment, the root lengths of the seedlings were determined. Three biology replicates were analyzed and each replicate consisted of 15 seedlings for each independent line.

## Germination assay

The sensitivity of transgenic *Arabidopsis* seeds to ABA and salt was tested on MS plates containing varying concentrations of ABA (0.0 or 0.5 μM) or NaCl (0, 5, 100, 150, or 200 mM) (Kim et al., 2012). Seeds were incubated on MS plates at 4°C for 48 h and then moved to a growth chamber under 16-h/8-h light-dark cycles at 24°C. Seeds were considered to have germinated when radicles completely penetrated the seed coat. Germination was scored daily for up to 10 days after the seeds were placed at room temperature. Three biology replicates for each sample were used and 25 seeds of each independent line were analyzed in each replicate.

## Statistical analysis

Statistical analysis of data was performed with SPSS 16.0 (SPSS Software, USA) and the significance of the results was evaluated by one-way ANOVA and the Dunnett test.

# RESULTS

## *LpGRP* encodes a glycine-rich protein

*LpGRP* is 555 bp long and encodes a 184-aa protein containing a glycine-rich domain (PS50315) and a domain of unknown function (DUF3720). The DUF3720 domain is located at the N-terminus, which consists of 32 aa, and the glycine-rich domain is found next to the DUF3720 domain (67-171 aa) and includes 15-aa residues (Figure 1A). Alignment analysis with related GRPs revealed that the glycine-rich domain in LpGRP is highly conserved (Figure 1B). Phylogenetic tree analysis predicted LpGRP to be an ortholog of other GRPs, specifically those from Leguminosae, such as *Phaseolus vulgaris*, *Glycine max*, and *Medicago sativa* (Figure 1C).

## *LpGRP* expression patterns vary in different *L. purpureus* tissues

*LpGRP* expression was analyzed in different tissues under drought stress. *LpGRP* expression in root, leaf, and stem tissues increased after 2 days of dehydration and gradually accumulated up to 10 days of drought treatment. In shoot tissues, expression peaked after 8 days and began to decrease following 10 days of treatment (Figure 2)

## *LpGRP* overexpression improves stress tolerance in transgenic *Arabidopsis*

The role of LpGRP in the response to abiotic stress was investigated in *Arabidopsis* overexpressing *LpGRP*. Three independent *LpGRP* expression lines were confirmed by RT-PCR (Figure 3) and subsequently used to examine tolerance to drought and salt stresses.

The tolerance of transgenic *Arabidopsis* to drought stress was examined by determining the seedling survival rate in soil. The 14-day-old seedlings grown in soil without water for 2 weeks were watered to determine the function of *LpGRP* in the soil drought response. The seedlings wilted and were yellowish when water was withheld for 14 days; however, the transgenic lines showed a much stronger recovery rate than did the WT plants (Figure 4). Only 28.33% of the WT seedlings survived compared with more than 70% of the transgenic seedlings (Table 1).

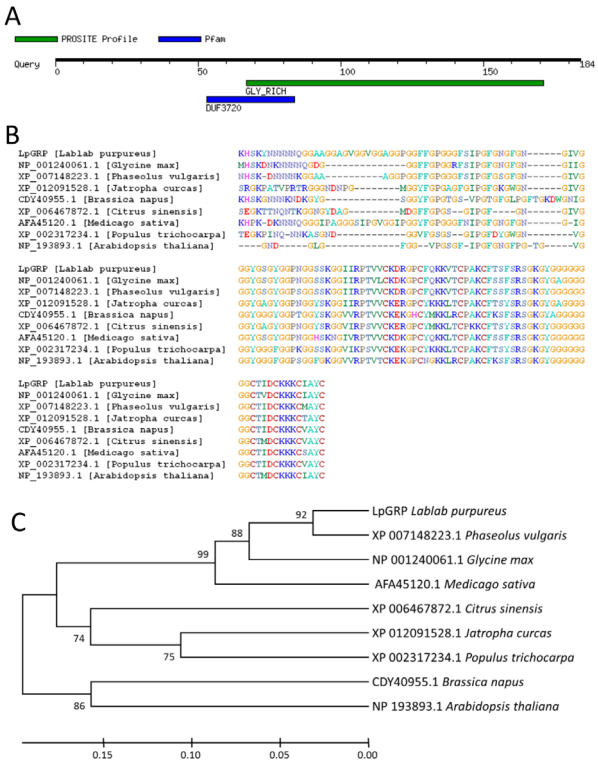


**Figure 1.** LpGRP protein motif analysis. **A.** A glycine-rich domain (PS50315) and an unknown functional domain (DUF3720) are predicted. **B.** Clustal W alignment comparing the amino acid sequence of LpGRP with other plant glycine-rich proteins (GRPs). The glycine-rich domain is highly conserved. **C.** Phylogenetic tree of LpGRP and other GRPs. The phylogenetic tree was constructed with the neighbor-joining method using the protein and amino acid sequences. Accession numbers are followed by the species name for each sequence. Bootstrap analysis was performed with 1000 repetitions to verify the tree reliability.

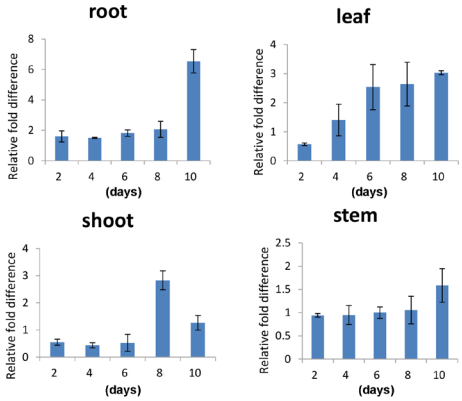


**Figure 2.** *LpGRP* expression analysis in *Lablab purpureus* under drought stress. Relative *LpGRP* expression levels (fold difference) under drought stress for 2, 4, 6, 8, and 10 days were evaluated using qRT-PCR analysis. Error bars represent the standard error of the mean (N = 3).

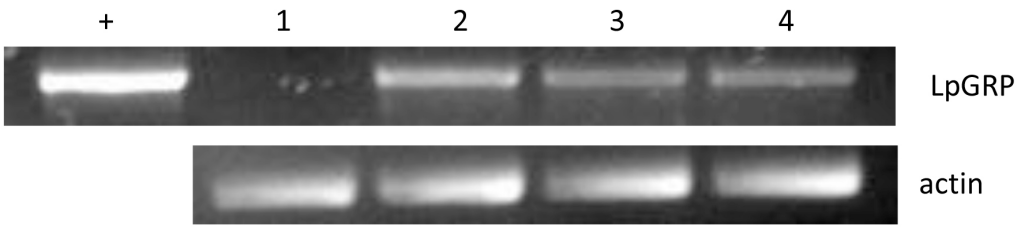


**Figure 3.** RT-PCR of *LpGRP* in wild-type (WT) and three transgenic lines. *Lane* + = pRI201-GRP plasmid; *lane 1* = WT; *lane 2* = transgenic *line 2*; *lane 3* = transgenic *line 5*; and *lane 4* = transgenic *line 7*. Actin represents RT-PCR amplification of *Actin* in transgenic *lines 2*, *5* and *7*.


**Figure 4.** Effect of soil drought on wild-type (WT) and three independent transgenic lines. From left to right, the samples are WT, *line 2*, *line 5*, and *line 7*. **A.** Fourteen-day-old plants grown in soil with watering for 2 weeks. **B.** Watering for 2 weeks after 14 days of watering. **C.** Fourteen-day-old plants grown in soil with waterholding for 2 weeks. **D.** Rewatering for 2 weeks after 14 days of drought.

**Table 1.** Survival rate of *LpGRP*-overexpressing *Arabidopsis* plants subjected to drought stress.

| Lines | Total seedlings | Surviving seedlings | Survival rate |
|---|---|---|---|
| WT | 60 | 17 | 28.33% |
| L3 | 60 | 51 | 85.00% |
| L5 | 60 | 44 | 73.33% |
| L7 | 60 | 49 | 81.67% |

*Arabidopsis* plants overexpressing *LpGRP* were also tolerant to salinity. Three independent lines were transplanted onto MS plates containing 0, 50, 100, or 150 mM NaCl and root length was investigated after 7 days. There were no significant differences between the transgenic lines and the WT plants in the absence of NaCl. In contrast, roots of the transgenic lines were significantly longer than those of the WT plants as the NaCl concentration increased (Figure 5).

## LpGRP-overexpressing *Arabidopsis* seeds are insensitive to ABA and NaCl

Germination of *LpGRP*-overexpressing *Arabidopsis* seeds was evaluated in the presence of NaCl and ABA stress. There were no significant differences in the germination rate between the transgenic lines and WT plants in the absence of ABA. In contrast, the germination rate of both the WT and transgenic plants was decreased in the presence of exogenous ABA (0.5 μM), although the rate was significantly higher in the *LpGRP*-overexpressing lines;

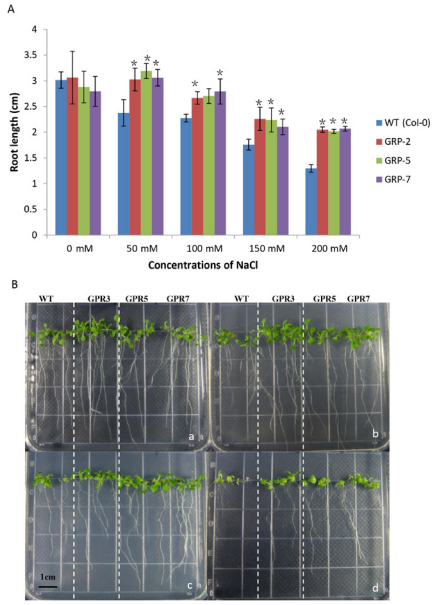


**Figure 5.** Root lengths of three independent transgenic lines and wild-type (WT) plants under NaCl treatment. **A.** Statistical analysis of the seedlings' root lengths. Data are reported as means ± SD (N = 12). *Indicates a significant difference (P ≤ 0.05) compared with WT. **B.** Root length of seedlings after 7-days growth on MS plates with different NaCl concentrations. (**a**) Seedlings under 0 mM NaCl stress for 7 days; (**b**) seedlings under 50 mM NaCl stress for 7 days; (**c**) seedlings under 100 mM NaCl stress for 7 days; and (**d**) seedlings under 150 mM NaCl stress for 7 days.

approximately 20% of the transgenic seeds germinated compared with 0% of the WT seeds (Figure 6). At ABA concentrations higher than 1.0 μM, the germination of both transgenic and WT seeds was completely inhibited.

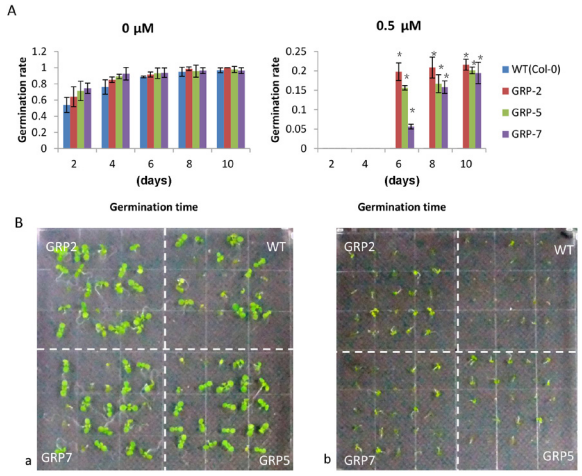


**Figure 6.** Germination of three independent transgenic lines and wild-type (WT) plants under ABA stress. **A.** Statistical analysis of germination. Data are reported as means ± SD (N = 12). *Indicates a significant difference (P ≤ 0.05) compared with WT. **B.** Germination of WT and three independent lines after (a) 0 μM and (b) 0.5 μM ABA treatment for 10 days.

Differences in germination between the transgenic and WT lines were also observed when NaCl was introduced. Germination was inhibited at 2 days in the presence of 100 and 150 mM NaCl, and at 4 days in the presence of 200 mM NaCl; however, after 6 days, all of the seeds germinated at all NaCl concentrations. The germination rate of the transgenic seeds was significantly higher than that of the WT seeds when NaCl was present (Figure 7).

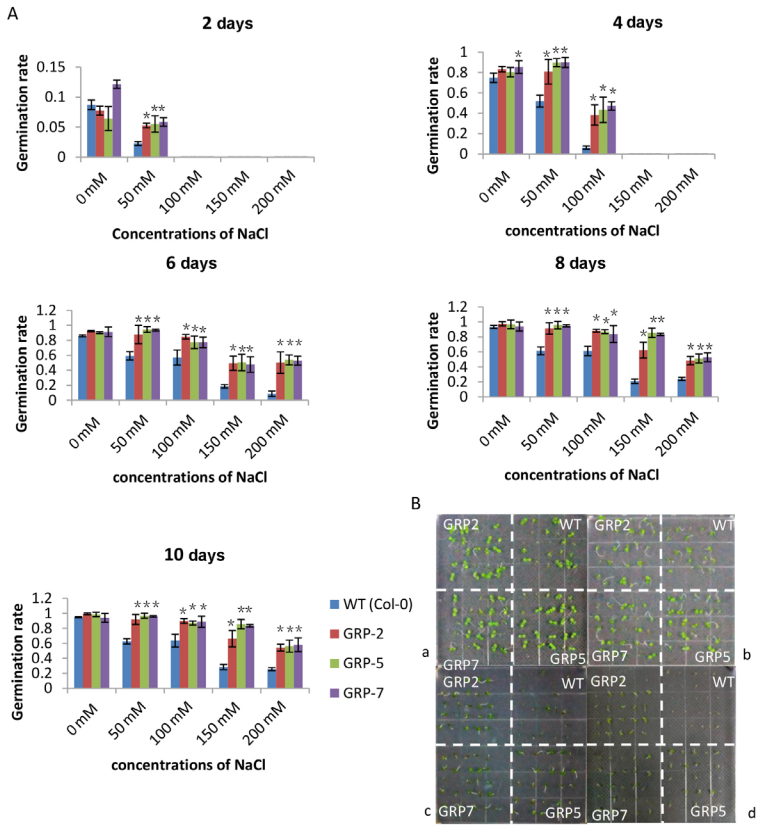


**Figure 7.** Germination of three independent transgenic lines and wild-type (WT) plants under NaCl stress. **A.** Statistical analysis of germination. Data are reported as means ± SD (N = 12). *Indicates a significant difference (P ≤ 0.05) compared with WT. **B.** Germination of WT and three independent lines after (a) 0 mM, (b) 50 mM, (c) 100 mM, and (d) 150 mM NaCl treatment for 10 days.

## DISCUSSION

In this study, we describe *LpGRP*, a novel GRP family gene in *L. purpureus*. *LpGRP* encodes a GRP of unknown function. This gene shares 90.48, 86.99, 80.38, and 56.49% identity with *P. vulgaris* (XP_007148223.1), *G. max* (NP_001240061.1), *M. sativa* (AFA45120.1), and *A. thaliana* (NP_193893.1), respectively, and the function of these genes remains uncharacterized. In *Arabidopsis*, the GRP gene *AT4G21620* is involved in phosphate-starvation tolerance (Hammond et al., 2003), while the functions of the other proteins are unknown.

GRPs are thought to have multifunctional roles in plants, and are involved in plant

growth, development, and abiotic/biotic stress responses. Overexpression of the glycine-rich gene in transgenic plants increases abiotic stress tolerance, leading to a higher survival rate under dehydration, longer root under salinity, and insensitivity to cold and ABA. For example, *AtGRDP2*, which is a non-canonical GRP-encoding gene, accelerates plant growth and development in *Arabidopsis* and in lettuce lines overexpressing *AtGRDP2* (Ortega-Amaro et al., 2015), while *AtGRP5* and *AtGRP7* overexpression has been shown to generate plants with longer roots and to promote floral transitions (Streitner et al., 2008; Mangeon et al., 2009). *MsGRP*, a salt stress-induced GRP gene, retards salt and ABA sensitivity in *MsGRP*-overexpressing lines (Long et al., 2013) and *BnGRP* affects seed germination and freezing tolerance of transgenic *Arabidopsis* under cold stress (Kim et al., 2012).

We examined the function of *LpGRP* in *L. purpureus* by overexpressing this gene in *Arabidopsis*. Increased tolerance to dehydration and salinity was observed in *Arabidopsis* during germination and at the seedling stage, which indicated that *LpGRP* is involved in the abiotic stress response. In addition, the *LpGRP*-overexpressing seeds were insensitive to ABA, indicating a relationship between abiotic stress responses and plant hormones.

Previous studies suggest that there is crosstalk between auxin and ABA during abiotic stress. It has been proposed that ABA-responsive gene expression is regulated by auxin, which might contribute to the positive regulation of abiotic stress (Shi et al., 2014). With an extraneous ABA inhibitor treatment, levels of both ABA and IAA decreased in rice while drought and cold tolerance increased (Du et al., 2013). OsGH3-2, an enzyme that catalyzes IAA conjugation to amino acids, is also involved in the modulation of endogenous free IAA and ABA homeostasis and affects drought and cold tolerance in rice (Du et al., 2012). Overexpression of *AtGRDP2* in *Arabidopsis* and lettuce downregulates *ARF2* expression and increases tolerance to salinity (Ortega-Amaro et al., 2015). In our study, *LpGRP*-overexpression lines showed insensitivity to ABA and salt during seed germination, indicating that there is crosstalk between *LpGRP* and ABA-related genes under abiotic stress.

Taken together, these results indicate that LpGRP is a novel glycine-rich gene in *L. purpureus* that is important for the drought response, salinity with different mechanisms of LpGRP and plant hormones.

## Conflicts of interest

The authors declare no conflict of interest.

## ACKNOWLEDGMENTS

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