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Molecules and Cells

Flooding Stress-Induced Glycine-Rich RNA-Binding Protein from *Nicotiana tabacum*

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A cDNA clone for a transcript preferentially expressed during an early phase of flooding was isolated from *Nicotiana tabacum*. Nucleotide sequencing of the cDNA clone identified an open reading frame that has high homology to the previously reported glycine-rich RNA-binding proteins. The open reading frame consists of 157 amino acids with an N-terminal RNA-recognition motif and a C-terminal glycine-rich domain, and thus the cDNA clone was designated as *Nicotiana tabaccum* glycine-rich RNA-binding protein-1 (*NtGRP1*). Expression of *NtGRP1* was upregulated under flooding stress and also increased, but at much lower levels, under conditions of cold, drought, heat, high salt content, and abscisic acid treatment. RNA homopolymer-binding assay showed that NtGRP1 binds to all the RNA homopolymers tested with a higher affinity to poly r(G) and poly r(A) than to poly r(U) and poly r(C). Nucleic acid-binding assays showed that NtGRP1 binds to ssDNA, dsDNA, and mRNA. NtGRP1 suppressed expression of the fire luciferase gene *in vitro*, and the suppression of luciferase gene expression could be rescued by addition of oligonucleotides. Collectively, the data suggest NtGRP1 as a negative modulator of gene expression by binding to DNA or RNA in bulk that could be advantageous for plants in a stress condition like flooding.

INTRODUCTION

Flooding often causes severe damage to crops and this is especially important in about 16% of the production area worldwide (Boyer, 1962). Although this problem occurs mainly in tropical rainforests, plants can also undergo such stress in cooler regions during much of the year when soil water remains saturated due to bad drainage and slow evaporation. Occasional heavy rainfalls also affect crops in temperate zones. Although there are plants that can cope with prolonged flooding conditions, most plants are tolerant to flooding in a very restricted level (Drew et al., 2000). When plants susceptible to flooding stress are subjected to submergence, most genes are silenced while some genes are newly induced. The proteins that are specifically up-regulated when this type of stress occurs are referred to as anaerobic proteins, and the proteins most studied so far mainly comprised metabolic pathway enzymes (Dat et al., 2004; Dennis et al., 2000). However, much more diverse genes are induced when plants become submerged. These include putative transcription factor genes, signal transduction pathway component genes, and some without predicted functions (Agarwal and Grover, 2005; Klok et al., 2002). Among the flooding stress-induced proteins, we were interested in studying the GRPs that have been reported to function in posttranscriptional gene regulation.

Regulation of gene expression at the posttranscriptional level is one major regulatory domain that influences and controls growth, development, and differentiation of organisms that often relate to stress response. Posttranscriptional gene regulation includes pre-mRNA splicing, capping and poly adenylation, mRNA transport, mRNA stability, and translation of the functional mRNA (Higgins, 1991; Simpson and Filipowicz, 1996). In these processes, regulation is mainly achieved either directly by the RNA-binding proteins (RBPs), or indirectly by the RBPs modulating function of other regulatory factors. RBPs that contain one or more RNA recognition motifs (RRMs) at the N-terminus and a variety of auxiliary motifs at the C-terminus, such as glycine-rich, arginine-rich, SR-repeat, RD-repeat, and acidic domain, have been identified (Alb and Pages, 1998; Bove et al., 2008; Fukami-Kobayashi et al., 1993; Fusaro et al., 2007; Kenan et al., 1991; Mousavi and Hotta, 2005; Nakaminami et al., 2006; Palusa et al., 2007). The RRM contains two essential motifs, designated ribonucleoprotein (RNP)-1, positioned centrally, and RNP-2, positioned toward the N-terminus (Query et al., 1989).

Posttranscriptional regulatory functions of RBPs in plants have not been studied as much as those in other eukaryotes (Fedoroff, 2002). Although it has been shown that *Arabidopsis* genome encodes more than 200 putative RNA-binding proteins, only a few RBPs in plants have been studied for their function (Lorkovic and Barta, 2002; Rochaix, 2001). A gene encoding a protein containing RRMs at the N-terminus and glycine-rich region at the C-terminus (glycine-rich RNA-binding protein, GRP) was first isolated from maize (Gomez et al., 1988), following which, cDNAs encoding homologous proteins have been identified from various plant species (Carpenter et al.,

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1994; Condit et al., 1990; Gendra et al., 2004; Hirose et al., 1993; Horvath and Olson, 1998; Masaki et al., 2008; Moriguchi et al., 1997; Shinozuka et al., 2006; van Nocker and Vierstra, 1993). Given their possible role in RNA recognition and processing, plant GRPs with RNA-binding capacity may have important roles in plant cell physiology, and their involvement in the stress responses of plants has been indicated by several expression analyses. The GRP mRNA accumulation levels were shown to be modified following exposure to stresses such as wounding, salt, and dehydration (Kim et al., 2005; Sachetto-Martins et al., 2000). The involvement of plant GRPs during cold acclimation has also been implicated by the fact that GRP transcription was significantly induced by low temperatures (Dunn et al., 1996; Stephen et al., 2003). Although the functions of the plant GRPs remain elusive, affinity of the GRPs for various types of nucleic acids has been reported. In ribohomopolymer-binding assays, GRP proteins from maize, barley, and *Arabidopsis* show higher affinity to poly r(U) and poly r(G) than to poly r(C) and poly r(A) (Dunn et al., 1996; Karlson et al., 2002; Kim et al., 2005; Ludevid et al., 1992). GRPs bind not only to double stranded DNA but also to single stranded DNA (Hirose et al., 1993; Karlson et al., 2002).

As a step toward understanding the biological role of GRPs in plants under flooding stress condition, a cDNA clone for a GRP was isolated from tobacco (*Nicotiana tabacum*) under a flooding stress condition, and designated *NtGRP1*. Here we report experimental evidences for NtGRP1 that support its function as a comprehensive negative modulator of gene expression. It probably suggests that down regulation of expression of a wide range of genes in plants under stress conditions would be one mechanism to adapt to unfavorable conditions like flooding.

MATERIALS AND METHODS

Plant material and stress treatments

Tobacco (*Nicotiana tabacum* cv W38) plants were grown in soil in a growth room (16-h photoperiod, 23°C, 60% relative humidity, and 200 μE m^{-2} s^{-1} from fluorescent lamps), and 6-weeks old plants with 4 to 6 leaves were used as the source of plant tissue for all experiments, unless otherwise mentioned. Flooding stress was applied by fully immersing the pots in tap water for 2 or 4 h. For low-temperature treatment, the plants were placed at 4°C for 12, 24, or 48 h. For drought treatment, plants were placed on a filter paper and maintained for 12, 24, or 48 h. For salinity treatment, the root was immersed in 0.5 or 1 M NaCl solution for 12 h, and for ABA treatment, the root was immersed in 10 μM ABA solution for 12 or 24 h.

cDNA cloning for a GRP

A tobacco flooding-stressed cDNA library (Lee et al., 2007) was screened using ^{32}P-labeled single-stranded cDNA probes that were synthesized from poly(A)$^+$ RNA isolated from either flooding-stressed or unstressed tobacco plants. The cDNA clones showing induction under flooding stress were isolated, one-path nucleotide sequenced, and the sequences were analyzed based on the existing annotation for non-redundant databases at the NCBI (http://www.ncbi.nlm.nih.gov) using BLASTX (Altschul et al., 1997; Lee et al., 2008). Among the cDNA clones analyzed, a clone encoding GRP was isolated. Since the clone did not carry the 5′-terminus, PCR was carried out with a degenerative forward primer, GRP-dF (5′-ATGGC(A/T)GAAGT(T/A)GAATAC(A/C)G(G/T)TGC-3′), based on the highly conserved sequence among the most closely related GRP sequences and a reverse primer based on the nucleotide sequence in the partial cDNA clone, GRP-R (5′-TGAAGGAAG-CTGGAGGAGTTAA-3′). The PCR products were cloned into the pCR-Blunt II-TOPO vector (Invitrogen, USA), and the nucleotide sequencing of the insert confirmed that the PCR product covers the full ORF. The clone was named *NtGRP1* (*Nicotiana tabacum* glycine-rich RNA-binding protein 1).

RNA blot hybridization

Total RNA was isolated from tobacco according to the method of Sambrook et al. (1989) with minor modifications (Lee et al., 2007). Briefly, tissues were ground in liquid nitrogen with a mortar, and extracted with homogenization buffer (50 mM LiCl, 25 mM Tris-Cl pH 7.5, 35 mM EDTA, 35 mM EGTA, and 0.5% SDS) and then with phenol:chloroform:isoamylalcohol (25:24:1) mixture. The aqueous phase was separated by centrifugation, and extracted with chloroform:isoamylalcohol (1:1) mixture. RNA was precipitated by adding 4 M LiCl, and collected by centrifugation. Twenty μg of total RNA was loaded in each lane and subjected to electrophoresis on a 1.2% formaldehyde gel. RNA was then transferred onto a membrane (Hybond N; Amersham Bioscience, USA), and the membrane was prehybridized and hybridized with ^{32}P-labeled *NtGRP1* cDNA clone at 65°C in a solution of 0.5 M sodium phosphate (pH 7.2), 7% (w/v) SDS, and 1 mM EDTA (pH 7.0). The membrane was washed and exposed to X-ray film.

Production and purification of recombinant NtGRP1 proteins from *E. coli*

Three recombinant constructs of *NtGRP1* were made with the N-terminal His_6-fusion in the pBAD NH vector (Cho et al., 2005). The full ORF of NtGRP1 was prepared by PCR using primers of GRP F27 (5′-GGGGAGCTCATGGCTGAAGTTGAATACAG-3′) and GRP R28 (5′-GGGCATGCTTAACTCCTCCAGCTTCCT-3′). The cDNA containing the N-terminal region (from 1 to 85 amino acid of NtGRP1, designated as $NtGRP1^{1\text{-}85}$) was prepared using GRP F27 and GRP R39 (5′-GGGCATGCTTA-GACAGACTGGGCTTCGT-3′), and the cDNA containing the C-terminal region (from 47 to 156 amino acid of NtGRP1, designated as $NtGRP1^{47\text{-}156}$) was produced by primers of GRP F40 (5′- GGGAGCTCAGAGGATTTGGATTTGTTACC-3′) and GRP R28. The six underlined nucleotides are *Sac*I and *Sph*I restriction sites. Amplified PCR products were digested with *Sac*I and *Sph*I and ligated into the pBADNH vector. All DNA manipulations were performed according to the methods of Sambrook et al. (1989), and all constructs were confirmed by DNA sequencing. The resulting constructs were transformed into *E. coli* strain MC1061. Expression of H_6NtGRP1 and its truncated forms was induced by adding 0.1% L(+)-arabinose and incubating for 6 h at 37°C; the proteins were subsequently purified using an Ni-NTA agarose column (Qiagen, USA). Eluates were concentrated in Centricon spin columns (Milllipore, USA). Concentrated samples were spectrophotometrically quantified with the RC DC protein assay system (Bio-Rad Laboratories, USA).

Antibody production and protein blot analysis

The purified H_6NtGRP1 of 100 μg was intravenously injected into a rabbit to raise a polyclonal antibody. After the first injection, three additional injections of H_6NtGRP1 followed every 2 weeks. A week after the last injection, sera were collected and stored at 4°C in 50 mM phosphate buffered saline containing 30% glycerol at a concentration of 1 mg of IgG per ml (Sambrook et al., 1989). Protein blot analyses basically followed Sambrook et al. (1989). Briefly, proteins were separated on SDS-PAGE gel and blotted onto nitrocellulose membrane (Amersham Bioscience). The membrane was incubated with either

polyclonal anti-H_6NtGRP1 antibody or anti-luciferase antibody (Promega, USA). After three washes, the membrane was hybridized to the horseradish peroxidase-conjugated goat anti-rabbit secondary antibody and visualized using the enhanced chemiluminescence kit (Amersham Bioscience).

Binding assay on DNA

Gel-retardation assay for the binding activity of NtGRP1 onto ssDNA and dsDNA substrates was carried out as described previously (Nakaminami et al., 2006) with minor modifications. Briefly, 150 ng of either ssDNA (M13mp8) or dsDNA (pSPT18) were incubated with purified H_6NtGRP1 in a binding buffer (10 mM Tris-Cl pH 7.5). The protein was added in the following amounts: 0, 50, 100, 200, 300, and 500 ng. To test the binding affinity of NtGRP1 to ssDNA and dsDNA, 400 ng of H_6NtGRP1 was added to 150 ng of DNA in the binding buffer with variable concentrations of KCl (0, 100, 200, 400, and 800 mM). The mixtures were maintained on ice for 10 min before being subjected to agarose gel electrophoresis and visualization by ethidium bromide staining. The reaction volume was kept at 15 μl.

Binding assay on RNA

H_6NtGRP1, H_6NtGRP1$^{1-85}$, and H_6NtGRP1$^{47-156}$ proteins (300 μM each) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was washed with tris-buffered saline solution (20 mM Tris-Cl pH 7.5, 150 mM NaCl) for 20 min and prehybridized overnight at 4°C in a renaturation buffer (50 mM Tris-Cl pH 7.6, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1% BSA). It was then incubated for 2 h at 23°C in Northwestern binding buffer (10 mM Tris-Cl pH 7.6, 50 mM NaCl, 1 mM EDTA, and 1 mM DTT) with ^{32}P-labeled ribohomopolymers of 25 nucleotides. After washing four times with the northwestern binding buffer containing NaCl up to 500 mM at 23°C, the membranes were exposed to X-ray film. For the binding assay to luciferase mRNA (Promega), it was processed as the case of binding to DNA except additional RNase inhibitor RNasin (Promega) used and the incubation time kept at 15 min. Multiple bands observed in Fig. 3E for luciferase mRNA were due to the shorter and longer than expected transcripts of luciferase mRNA from the *in vitro* transcription process of luciferase gene (Promega). The mRNA and protein complexes were subjected to agarose gel electrophoresis and visualized by ethidium bromide staining.

Coupled *in vitro* transcription/translation

T_NT Coupled Reticulocyte Lysate System (Promega) was used for coupled *in vitro* transcription/translation reaction. Briefly, plasmid T7-luciferase DNA was mixed with T7 RNA polymerase, 0.02 mM amino acid mixture, RNase inhibitor, T_NT Coupled Reticulocyte Lysate, and H_6NtGRP1 (0-300 μM), and incubated at 30°C for 90 min. The amount of synthesized luciferase protein was quantified either by protein blot analysis as described above or by chemiluminescence assay. For the chemilumiscence assay, 5 μl of the 1/10 diluted *in vitro* translation product was mixed with 20 μl of luciferase assay reagent (Promega), and chemiluminescence was measured for 2 s using a luminometer (Turner design, USA).

Oligonucleotide competition assay

DNA oligomer (10-500 μM; TTTTTTTTTTTTTTTTTTTTGGATCC) or RNA oligomer (100-500 μM; 25 mer of rU) was added to the coupled *in vitro* transcription/translation reaction mixture with 100 μM H_6NtGRP1. The result was quantified by measuring chemiluminiscence from the luciferase activity as described above.

RESULTS

Isolation and structural characterization of NtGRP1 cDNA clone

We constructed a cDNA library representing mRNAs from tobacco plants exposed to a short-term flooding (Lee et al., 2007). From a differential screening of the cDNA library, we isolated cDNA clones with apparently increased expression under flooding. Nucleotide sequencing of the cDNA clones and homology search on the public database isolated a cDNA clone that showed high homology to the previously reported clones encoding GRPs. The cDNA clone was named *Nicotiana tabacum* glycine-rich RNA-binding protein-1 (*NtGRP1*). This clone contained an incomplete open reading frame (ORF) of 418 nucleotides and a 3′-untranslated region of 189 nucleotides that lacked a poly(A)-tail. The missing 5′-terminus of the ORF was obtained by degenerative polymerase chain reaction (PCR); the resulting ORF was initiated with ATG and was extended for 474 nucleotides, encoding a protein of 157 amino acids with a calculated molecular mass of 17.27 kDa. RNA-binding proteins commonly feature RRMs. RRMs, in turn, usually contain two highly conserved RNP motifs: RNP-1, which consists of eight amino acid residues (K/R)G(F/Y)(G/A)RVX(F/Y), and RNP-2, which contains six amino acid residues (L/I)(F/Y)(V/I)(G/K)(G/N)L (Hanano et al., 1996). NtGRP1 has a single RRM at the N-terminus that contains RNP-1 (RGFGFVTF) and RNP-2 (CFVGGL) sequences and a glycine-rich domain at the C-terminus. About 60% of the glycine residues in the glycine-rich domain are contiguous, and tyrosine residues and charged amino acid stretches (such as RRE or RRD) are inserted between the glycine stretches (Fig. 1A). Multiple alignment of NtGRP1 with other plant GRPs showed more than 70% identities toward the N-terminal region in the RRM (Fig. 1B). DNA blot hybridization for *NtGRP1* on *N. tabacum* genomic DNA showed a single band from two restriction digests that indicates *NtGRP1* as a single copy gene in the tobacco genome (Fig. 1C).

Effect of various stresses and ABA treatment on the expression of *NtGRP1*

Effect of various stresses such as flooding, cold, drought, heat and salinity, and abscisic acid (ABA) treatment on the transcript level of *NtGRP1* in tobacco plants was examined by RNA blot analyses. As shown in Fig. 2, only minor level of *NtGRP1* transcript was detected in the unstressed-tobacco plants. Expression of *NtGRP1* was markedly induced within an hour of flooding, increased till the 24-h time point, and then decreased. Expression of *NtGRP1* was also induced by low temperature, drought, salinity, and heat, but at much lower levels. Slight increase in the transcript level was also observed under the ABA treatment (Fig. 2).

Nucleic acid-binding activity of NtGRP1

For the analysis of NtGRP1 activity, NtGRP1 was expressed in *Escherichia coli* as a His_6-fused form (H_6NtGRP1) and purified using Ni-NTA column chromatography (Fig. 3A). H_6NtGRP1 showed strong binding activity to all four types of homoribonucleotide polymers at a lower salt concentration. At a higher salt concentration, H_6NtGRP1 bound more preferentially to poly r(A) and poly r(G) than to poly r(U) and poly r(C) (Fig. 3B). When the truncated NtGRP1 proteins were subjected to the homoriboguanine polymer binding at an NaCl concentration of 250 mM, both truncated forms, i.e. H_6NtGRP1$^{1-85}$ and H_6NtGRP1$^{47-156}$, showed drastically reduced binding activity to poly r(G) (Figs. 3C and 3D). Gel-retardation assay on the incubation mixture of

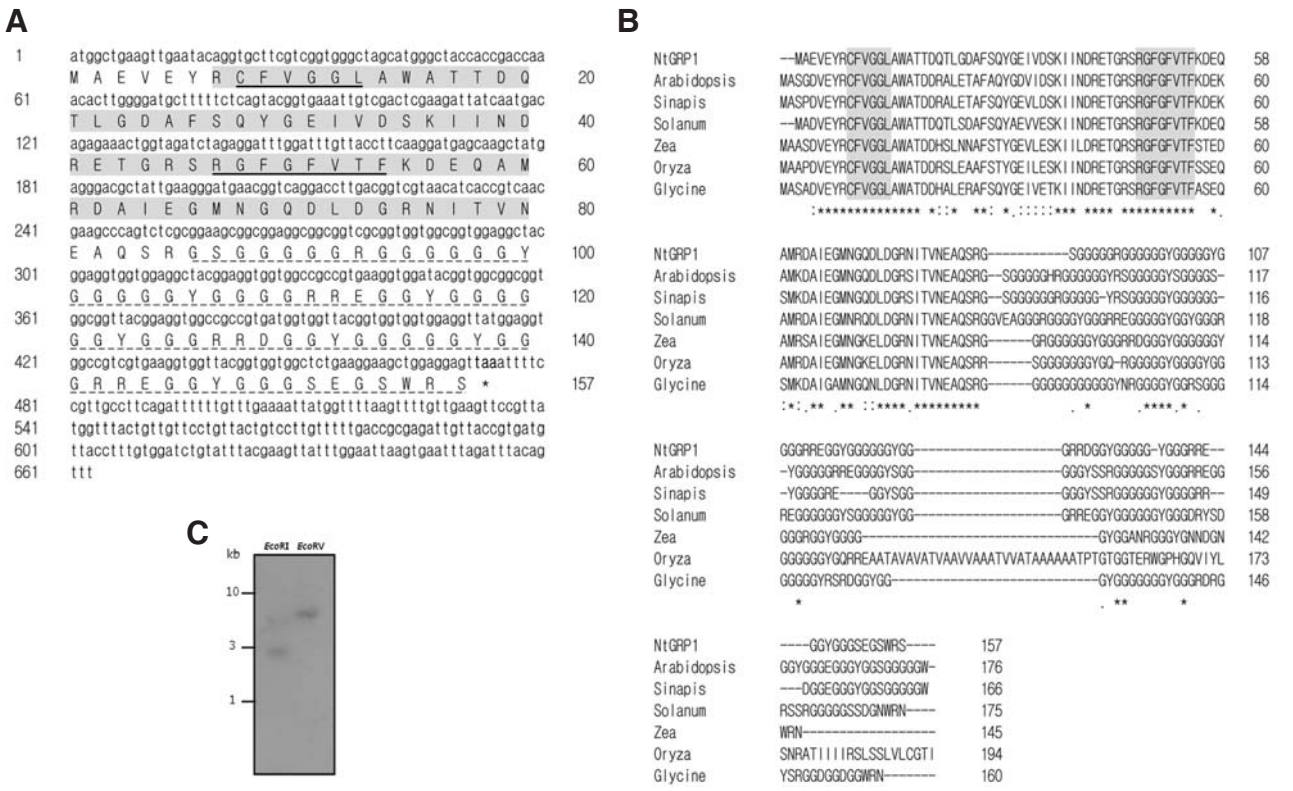

Fig. 1. cDNA clone for a flooding stress-induced GRP from *N. tabacum*. (A) Nucleotide and deduced amino acid sequences of *NtGRP1*. The RRM motif is in a grey-box, and the RNP-1 and RNP-2 sequences are underlined (RNP-2 is placed at the N-terminus). The glycine-rich region is indicated by a dashed underline. Numbers on the left indicate the position of nucleotide, and numbers on the right indicate the position of amino acid. (B) Multiple alignment of amino acid sequences of NtGRP1 and other plant GRPs in plants. The RNP-1 and RNP-2 motifs are indicated in grey. Amino acids common to all the aligned sequences are indicated by asterisks. The aligned sequences belong to the GRPs from *Arabidopsis thaliana* (Z14987), *Sinapsis alba* (L31374), *Solanum tuberosum* (Z49197), *Zea mays* (AF496726), *Oryza sativa* (AJ302060), and *Glycine max* (AF169205). (C) DNA blot hybridization analysis of tobacco genome for *NtGRP1*. Genomic DNA of tobacco was digested with *Eco*RI or *Eco*RV and subjected to DNA blot hybridization to the ^{32}P-labeled *NtGRP1* cDNA. The nucleotide sequence reported in this article has been submitted to NCBI under the accession number EU569289.

H_6NtGRP1 and luciferase mRNA showed shifting of mRNA band positions in case of the luciferase mRNA and H_6NtGRP1 mixture but not from the luciferase mRNA and BSA mixture (Fig. 3E). Binding activity of NtGRP1 was again tested on M13mp8 phage ssDNA and pSPT18 dsDNA. Gel mobility shift assays showed that NtGRP1 bound both ssDNA and dsDNA, but with different affinities. With the increasing amount of H_6NtGRP1, shifting of the DNA band was much more evident from the ssDNA than from the dsDNA. On the other hand, BSA did not affect the mobility of both forms of DNA (Fig. 3F). Binding affinity of NtGRP1 onto the ssDNA was not affected by salt concentration up to 800 mM, and in the case of the dsDNA, a slight increase in the binding was apparent as the salt concentration increased (Fig. 3G).

Effect of NtGRP1 on the coupled transcription/translation

To test NtGRP1 activity on the expression of genes, H_6NtGRP1 in different concentrations was added to the coupled *in vitro* transcription/translation system using the firefly luciferase gene as the reporter to monitor the concentration-dependent effect of NtGRP1 on gene expression. Protein blot analysis of the luciferase protein showed that addition of H_6NtGRP1 to the coupled *in vitro* transcription/translation mixture lowered the amount of luciferase protein produced from the luciferase gene in the mixture in a concentration-dependent manner (Fig. 4A). Suppression activity of NtGRP1 on the expression of luciferase gene was again confirmed by quantifying the activity of luciferase in the reaction mixture. The luminescence of luciferase decreased with the increase in the amount of NtGRP1 added (Fig. 4B).

Oligomer DNA and RNA recover expression of the reporter gene that was suppressed by NtGRP1

We hypothesized that the suppression effect of NtGRP1 on the expression of luciferase gene is due to the binding of NtGRP1 to DNA and RNA that interferes with transcription and translation of the luciferase gene. However, other possible mechanisms, such as interaction of NtGRP1 with other proteins functioning in transcription and/or translation, are possible that can bring about the inhibition of transcription and/or translation. To further define the suppression activity of NtGRP1, we added small deoxyoligonucleotide to the coupled *in vitro* transcription/translation mixture. If addition of this deoxyoligonucleotide to the mixture can lower the suppression activity of NtGRP1 on the expression of luciferase gene in the coupled *in vitro* transcription/translation mixture, it is likely to be due to competition between the deoxyoligonucleotide and the luciferase DNA for binding to NtGRP1. When the deoxyoligonucleotide was added to the coupled *in vitro* transcription/translation mixture with the luciferase gene and NtGRP1,

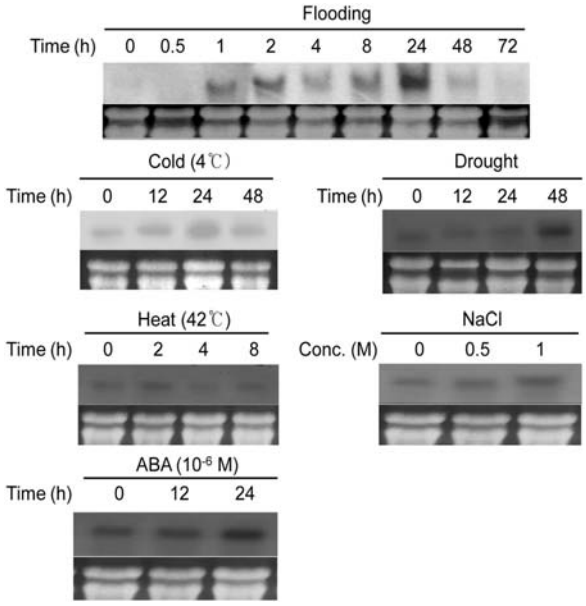

Fig. 2. Effects of ABA and various stresses on the level of *NtGRP1* transcript. Total RNA was isolated from tobacco plants that had been exposed to flooding, low temperature, drought, heat, salinity, and ABA. Each lane was loaded with 20 μg of total RNA. The number above each lane indicates the number of hours that the plant had been treated for, except salinity where the number indicates molarity of NaCl.

luminescence by luciferase was recovered from the suppressed level in a concentration-dependent manner up to 200 μM of the deoxyoligonucleotide in the mixture with 100 μM NtGRP1. Above this concentration, the luminescence was again suppressed (Fig. 5A). The suppression activity of NtGRP1 by binding to the transcript of luciferase was similarly evaluated. When an RNA oligomer instead of the deoxyoligonucleotide was used as a competitor, recovery from the suppressed level of luminescence by the luciferase gene in the coupled mixture was apparent for up to 200 μM of the RNA oligomer (Fig. 5B).

DISCUSSION

A cDNA clone for a glycine-rich RNA-binding protein (*NtGRP1*) was isolated from a cDNA library of flooding-stressed tobacco (Lee et al., 2007). Biological roles of GRPs in response to environmental stresses have been implicated based on the expression analysis of GRPs in plants exposed to various biotic and abiotic stresses including low temperature, drought stress, and viral infection (Kwak et al., 2005; Palusa et al., 2007; Raab et al., 2006; Stephen et al., 2003). In this study, we showed that *NtGRP1* expression is strongly induced by flooding stress (Fig. 2). This is the first report of a GRP being strongly induced under a flooding stress condition and expands the role of GRP related to flooding stress.

The N-terminal RRM domain of GRPs is highly conserved but the C-terminal glycine-rich region shows substantial diversity (Lorkovic and Barta, 2002). NtGRP1, which is comprised of 157 amino acids, is one of the smallest among the GRPs. The size difference among the GRPs is mainly from the difference in the glycine-rich region, and NtGRP1 has a shorter glycine-rich region compared to other GRPs. NtGRP1 has a significant number of arginine residues in the glycine-rich region (Fig. 1B) that are probably responsible for the interaction with nucleic acids as shown in Fig. 3. The N-terminal RRM domain interacts with nucleic acids (Fusaro et al., 2007; Maris et al., 2005), and the C-terminal glycine-rich region is known to interact with other proteins and distinguish substrate nucleic acids (Mousavi and Hotta, 2005). Previously, GRPs from other plant species showed preferential affinity to poly r(U) and poly r(G) (Dunn et al., 1996; Kim et al., 2005; Ludevid et al., 1992; Nomata et al., 2004). On the other hand, NtGRP1 showed higher affinity to poly r(A) and poly r(G) than to poly r(U) and poly r(C). At present, no experimental evidence is available to link the structural difference observed in the glycine-rich regions of NtGRP1 and other reported GRPs: however, higher proportion of arginine residues in the region compared with other GRPs attracted our attention associated with the differences in substrate affinity.

Genomic DNA blot analysis for *NtGRP1* suggests the presence of a single copy of the gene in the tobacco genome (Fig. 1C). RNA blot analyses for *NtGRP1* showed strong induction of *NtGRP1* transcript under the flooding condition, and under other abiotic stress conditions, the induction was marginal (Fig. 2). Signal transduction pathways involved in abiotic stresses are well known to be crosslinked, and thus a gene whose expression is induced under one abiotic stress condition is often induced by other abiotic stress conditions (Knight and Knight, 2001; Mahajan and Tuteja, 2005). However, the RNA blot analysis results shown in Fig. 2 demonstrate that *NtGRP1* is a directly flooding-stress-inducible GRP gene that is drastically induced under the flooding stress condition but not under other abiotic stress conditions. Signal transduction pathways induced under abiotic stress conditions are often described as ABA-dependent or ABA-independent (Knight and Knight, 2001; Mahajan and Tuteja, 2005). *NtGRP1* transcript level was slightly increased upon treatment with ABA (Fig. 2); this probably does not imply strong involvement of ABA related to NtGRP1 under flooding condition.

As mentioned earlier, NtGRP1 binds more preferentially to r(G) and r(A) than to r(U) and r(C) (Fig. 3B) that differentiates NtGRP1 from the previously reported binding affinity of GRPs to homoribonucleotide polymers (Dunn et al., 1996; Kim et al., 2005; Ludevid et al., 1992; Nomata et al., 2004). The histine tag attached at the N-terminus of NtGRP1 probably does not interfere with the binding activity of NtGRP1 to the nucleic acids at least at a high salt concentration such as 250 mM used in most buffers for the binding assays in this study. The truncated forms of NtGRP1 lost most of the binding activity (Figs. 3C and 3D). It probably indicates that NtGRP1 cannot accommodate significant deletion to carry out binding to nucleic acids. NtGRP1 binds not only to the homoribonucleotide polymers but also to mRNA (Fig. 3E); this proves its binding capacity to various types of RNAs probably in *in vivo*. The extent of NtGRP1 binding to single stranded form of DNA is strong, while its binding to double stranded form of DNA is significantly weaker (Figs. 3F and 3G). This differential affinity of NtGRP1 between ssDNA and dsDNA probably indicates that NtGRP1 is able to bind DNA much more preferentially either in the replication or transcription processes.

If NtGRP1 binds to RNA and ssDNA, what would be the consequence?

To test the NtGRP1 function as a gene expression regulator, we used an *in vitro* gene expression system with luciferase as a quantifiable reporter. We applied different concentrations of NtGRP1 on each experiment and found a dramatic decrease in expression of the luciferase gene as the amount of NtGRP1 increased (Fig. 4). Competition experiments using oligomer DNA and RNA supported the notion that NtGRP1 can bind to

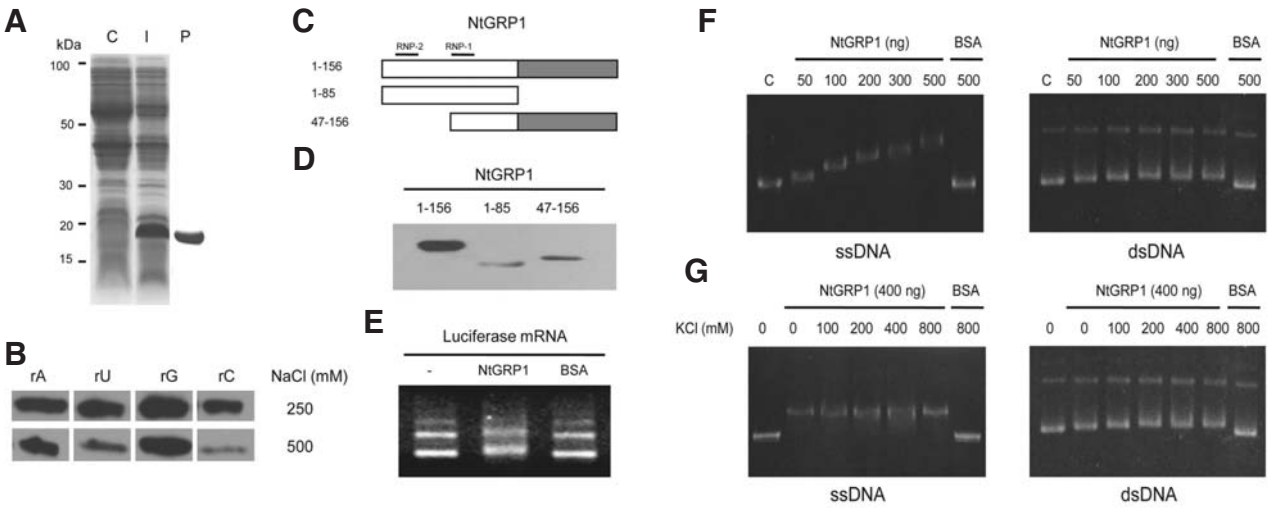

Fig. 3. Nucleic acid-binding activities of NtGRP1. (A) H_6NtGRP1 overexpressed in *E. coli* was purified, and separated by SDS-PAGE. C, *E. coli* cellular proteins without H_6NtGRP1; I, H_6NtGRP1 highly expressed in *E. coli* after arabinose addition; P, purified H_6NtGRP1. (B) North-Western blot analysis of NtGRP1. Purified H_6NtGRP1 protein was probed with ^{32}P-labeled ribohomopolymers. rA, rU, rG, and rC represent ribohomopolymers of A, U, G, and C, respectively. (C) Schematic structural representation of NtGRP1 and its truncated forms. Numerals represent amino acid positions from the N-terminus. (D) Northwestern blot analysis for H_6NtGRP1, H_6NtGRP1$^{1\text{-}85}$, and H_6NtGRP1$^{47\text{-}156}$. ^{32}P-labeled ribohomopolymer of guanine was used for the hybridization probe. (E) Analysis of mRNA-binding activity of NtGRP1 by gel mobility shift assay. Luciferase mRNA was incubated with H_6NtGRP1 for 15 min on ice, and the mixture was subjected to agarose gel electrophoresis. '-' stands for no addition of protein. Multiple bands in each lane are due to the combination of shorter and longer than expected transcripts of luciferase from the *in vitro* transcription. The band second from the bottom corresponds to the expected size of the luciferase transcript at about 1800 nucleotides (Promega). (F) Analysis of DNA-binding activity of NtGRP1 by gel mobility-shift assay. Purified H_6NtGR1 protein, up to 500 ng, was incubated with either ssDNA (M13mp8) or dsDNA (pSPT18) before agarose gel electrophoresis. (G) Analysis of NtGRP1 binding affinity to ssDNA and dsDNA. H_6NtGRP1 was mixed with either ssDNA or dsDNA of 150 ng at varying salt concentrations, i.e. up to 800 mM KCl, and incubated on ice for 15 min before agarose gel electrophoresis.

DNA and RNA, and thus can regulate gene expression (Fig. 5). The maximum level of recovery at 200 μM and then downshifting of the effect in the competition assay is likely due to the excessive amount of competitors that probably interferes with overall transcription and translation processes in the system (Hofweber et al., 2005). The negative effect in gene expression diverts NtGRP1 from the previously reported plant GRPs function known as RNA chaperones, i.e., GRPs can prevent the formation of secondary structures in mRNAs, and thus promote translation of the transcript under a stress condition like low temperature. The possibility of GRPs as a positive regulator in gene expression under abiotic stresses has been suggested for *Arabidopsis* atRZ1a and *E. coli* CspA (Bae et al., 2000; Fusaro et al., 2007; Jiang et al., 1997; Kim et al., 2005; 2007; Theiringer et al., 1998). Although we cannot provide experimental evidence at present to explain this difference, the structural difference between the aforementioned GRPs that carry cold-shock domains and NtGRP1 will probably be where the functional difference resides. Here, we provided experimental evidences that support NtGRP1 function as a negative regulator on gene expression by binding to DNA or RNA probably to a large extent. When plants encounter stresses, the synthesis of bulk proteins is temporally reduced. For an example, in the roots of maize seedlings under flooding stress, there is a rapid and dramatic shift in gene expression patterns and activities including about 70% reduction in total protein synthesis (Saab and Sachs, 1996). Although most gene expression is repressed in response to flooding, an important subset of genes is upregulated. *NtGRP1* was isolated from a cDNA library of tobacco at an early phase of flooding-stress (Lee et al., 2007) and showed highly increased expression under flooding. Linked

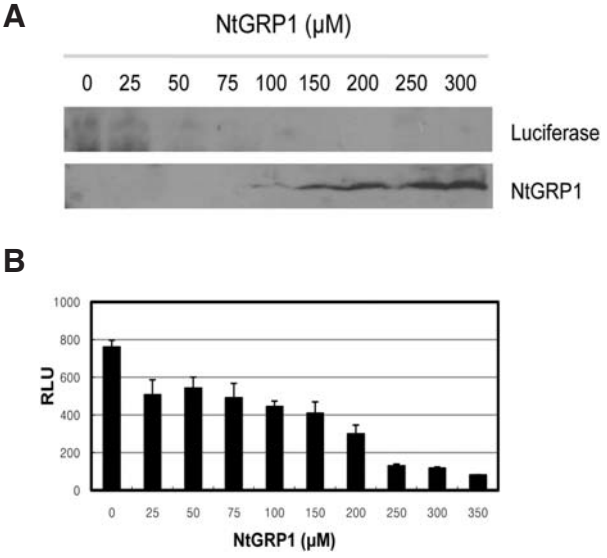

Fig. 4. Inhibitory activity of NtGRP1 on the expression of luciferase gene. H_6NtGRP1 (25-300 μM) was added to the *in vitro* coupled transcription/translation system of pT7-luciferase, and incubated for 90 min at 30°C. (A) The reaction product was separated by SDS-PAGE and subjected to protein blot analyses with either anti-luciferase antibody or anti-NtGRP1 antibody. (B) The reaction product was again analyzed by measuring chemiluminescence from the activity of luciferase. Chemiluminescence is represented as the relative light unit (RLU). Mean and standard error of the mean from five measurements for each datum point are presented.

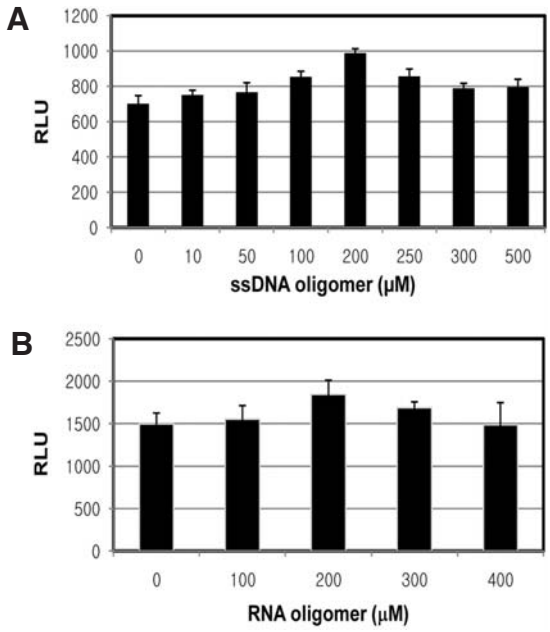

Fig. 5. Deoxyoligonucleotide and oligonucleotide release the suppression of luciferase expression by NtGRP1. *In vitro* translation of luciferase mRNA was carried out in the presence of H_6NtGRP1 and oligonucleotides. DNA (A) and RNA (B) oligomer of different concentrations (10-500 μM) were added to the reaction and incubated for 90 min at 30°C. Chemiluminescence from the reaction product was measured and presented as the relative light unit (RLU). Mean and standard error of the mean from five measurements for each datum point are presented.

to its binding activities to ssDNA and RNA and negative function on gene expression *in vitro*, we are tempted to hypothesize that the flooding-inducible NtGRP1 may function as a negative modulator of gene expression in bulk under flooding stress that could be beneficial for the plants to cope with the abiotic stress.

Analyses of NtGRP1 function *in vivo* including knockdown and overexpressing transgenic plants of *NtGRP1* would be the next approach to understanding the functional roles of NtGRP1 in gene regulation under stress condition.

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