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Characterization and Expression Analysis of Four Glycine-Rich RNA-Binding Proteins Involved in Osmotic Response in Tobacco (*Nicotiana tabacum cv.* Xanthi)

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Abstract

Plants have developed many signals and specific genes' regulations at both transcriptional and post-transcriptional levels in order to tolerate and adapt to various environmental stresses. RNA-binding proteins (RBPs) play crucial roles in the post-transcriptional regulation *via* mRNA splicing, polyadenylation, sequence editing, transport, mRNA stability, mRNA localization, and translation. In this paper, four cDNAs of glycine-rich RNA-binding proteins (GR-RBPs), named *NtRGP-1a*, *-1b*, *-2*, and *-3*, were isolated from *Nicotiana tabacum* by RT-PCR analysis, and special emphases were given to the sequences alignment, phylogenetic analysis and gene expression. Sequences alignment revealed minor difference of cDNA sequences, but no difference of deduced proteins between *N. sylvestris* and *N. tabacum*. Phylogenetic alignment revealed that four cDNAs in tobacco were clustered into two different groups. *NtRGP-2* and *-3* were evolutionarily closest to *Arabidopsis* GR-RBPs genes and related to animal GR-RBPs genes, while *NtRGP-1a* and *-1b* were closest to Gramineae GR-RBPs genes. The expression analyses of these four *NtRGPs* in response to different abiotic stressess showed the similar expression pattern. Moreover, the four *NtRGPs*, especially *NtRGP-1a* and *NtRGP-3*, were strongly induced by stresses including water, wound, cold, and high temperature, weakly induced by PEG, drought and SA, while reduced by NaCl and unaffected by ABA treatment. The fact that all of these abiotic stresses included in our experiments affected the water balance and resulted in osmotic stress on cellular level, suggests that NtRGPs in tobacco should be a family of crucial osmosis-related proteins, and may play a key role in signal transduction with ABA-independent pathway under abiotic stresses.

Key words: glycine-rich RNA-binding proteins, abiotic stresses, phylogenesis, expression pattern, osmotic stress

INTRODUCTION

Plants are often exposed to various environmental stresses which seriously affect plant growth and development during their life cycles. To tolerate and adapt to various stresses, plants have developed many signals and regulate the expression of specific stress-related genes.

The regulation of gene expression in living organisms occurs at the transcriptional and post-transcriptional level. RNA-binding proteins (RBPs) play crucial roles in the post-transcriptional regulation, including the splicing, polyadenylation, sequence editing, transport, mRNA stability, mRNA localization, and translation (Fedoroff 2002). Glycine-rich RNA-binding proteins (GR-RBPs) are characterized by containing one or more RNA-recognition motifs (RRMs) on the N-terminus and

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a glycine-rich domain on the C-terminus (Sachetto-Martins *et al*. 2000). Structurally, RRMs are composed of about 80-90 amino acids and each RRM has highly homologous RNA binding domains RNP-1 and RNP-2. Alignment of amino acid shows that RRMs are highly conserved and almost 60-80% identity in different RBPs, but the C-terminal glycine-rich domains (GDs) have substantial diversity (Stephen *et al*. 2003).

In contrast to the well-defined function of the RRM, the functional role of glycine-rich domain is still obscure. Some evidences showed that the GD has a positive role in RNA binding activity of the full-length protein, although it is not sufficient for RNA binding of its own. GD domain has been proposed to be involved in protein-protein interactions at least in the three distinct families of proteins: keratins and other intermediary filament proteins, loricrins, and the single-stranded RNA-binding proteins (Steinert *et al*. 1991). Glycine is the major component in total protein nitrogen in certain plant tissues, such as the seed-coat of soybean with 21% of glycine, the cell wall of milkweed stem with 31% of glycine, and coat of coleoptile cells with 27% of glycine (Varner and Cassab 1986). Glycine-rich proteins with 47 and 45.6% glycine content have been isolated from pumpkin seed-coat and cell wall of maize silk tissue, respectively (Tao *et al*. 2006). The glycine-rich protein (GRPs), PvGRP1.8 in bean was reported to be located in cell wall (Ringli *et al*. 2001), and the structural function was always attributed to a lot of new GRPs. However, an increasing number of studies have indicated that different GRPs may have different localizations and many other possible biological functions (Sachetto-Martins *et al*. 2000). RG or RGG motif characterized by the presence of multiple RG (Arginine-Glycine) and RGG (Arginine-Glycine-Glycine) peptides are commonly existed in GD domain. RG domains contribute specifically to protein function. For example, Saccharomyces Npl3 can support growth of cells with its own RG domain, whereas the RG domains of nucleolar proteins Nop1 and Gar1 only support partial Npl3 function (McBride *et al*. 2009). The RG-rich domain of Fragile X mental retardation protein FMRP binds to specific G-quartet-containing mRNAs (Stetler *et al*. 2006). RG-rich proteins are also frequent targets for arginine methylation, which can impact protein function through altering molecular interactions (Boisvert *et al*. 2005).

A protein containing RRMs at the N-terminus and glycine-rich region at the C-terminus (GR-RBPs) was initially isolated from maize (Gomez *et al*. 1988). Subsequently, GR-RBPs have been identified in various organisms ranging from prokaryotes to mammals, including cyanobacteria (Maruyama *et al*. 1999), moss (Nomata *et al*. 2004), *Arabidopsis thaliana* (Nocker and Vierstra 1993), tobacco (Hirose *et al*. 1993, 1994; Moriguchi *et al*. 1997), barley (Molina *et al*. 1997), Brassica (Bergeron *et al*. 1993), *Prunus avium* (Stephen *et al*. 2003), and mammals (Nishiyama *et al*. 1997a). Many studies have shown that mRNA levels of GR-RBPs were changed after exposure to various abiotic stresses such as cold, wound, water stress, salt, ABA, SA, and metal ion (Sachetto-Martins *et al*. 2000; Sanan-Misha *et al*. 2002). Based on these expression patterns, many transgenic plants were obtained to understand and testify their biological functions in response to abiotic or biotic stresses. For example, *LpGRP1* in perennial ryegrass plays a role in cold adaptation (Shinozuka *et al*. 2006). *AtGRP2* has a positive impact on seed germination and seedling growth of *Arabidopsis* plants under cold stress conditions (Kim *et al*. 2007b). *AtGRP4* negatively affects seed germination and seedling growth of *Arabidopsis* plants under salt or dehydration stress conditions (Kwak *et al*. 2005). *AtGRP7* has an RNA chaperone activity during the cold adaptation process in *Escherichia coli* (Kim *et al*. 2007a). *AtRZ-1a* which contains a CCHC-type zinc fingers at the C-terminus has a negative impact on seed germination and seedling growth of *Arabidopsis* under salt or dehydration stress conditions (Kim *et al*. 2007c).

In order to understand the function of GR-RBPs in tobacco plants experiencing abiotic stresses, we isolated four GR-RBPs genes from *Nicotiana tabacum* and analyzed the structures of their predicted proteins, phylogenesis, expression patterns as well as their possible physiological functions.

MATERIALS AND METHODS

Plant materials and growth condition

Seeds of *N. tabacum cv*. Xanthi (stored in our lab) were germinated on Murashige and Skoog medium (MS) and seedlings were transferred to soil after 10 d. Plants were grown in a green house with 16-h-light period

and 28°C day/20°C night temperature. Four-wk-old plants were used for experiments.

Plant stress treatments

Four-wk-old uniform tobacco plants were used for stress treatments. For SA or ABA treatments, seedlings were sprayed with 2 mmol L^{-1} SA or 100 μmol L^{-1} ABA on the surface of the leaves. Stress treatments to induce water-logging, PEG and saline stress were achieved by floating tobacco leaves on the following solutions respectively, distilled water, 250 mmol L^{-1} NaCl and 25% (m/v) PEG. Wounding was performed by cutting leaves with forceps. Cold stress was carried out by placing seedlings in cold room at (4±2)°C. Heat stress treatments were conducted by placing seedling in an incubator at (42±2)°C. Drought stress was performed by leaving the seedlings without watering for given time in a growth chamber with 50% humidity and 16-h-light period. Before each treatment, untreated leaves were harvested as control samples. The treated leaves were harvested at given time post-treatment, frozen in liquid nitrogen, and stored at -80°C for later use.

RNA Isolation and RT-PCR reactions

Total RNA was isolated from roots, stems, and leaves by using Trizol reagent (Invitrogen) following the manufacturer's instruction. Approximately 2 μg of total RNA was used in each RT-PCR reaction to reverse transcribe using oligo (dT) primer and SuperScript II reverse transcriptase (Promega, USA). The information of four cDNAs (GU991537, GU991538, GU991539, and GU991540) including sequences and corresponding primers were submitted to the Genbank (www.ncbi.nlm.nih.gov).

Northern blot analysis

Total RNA for Northern blot analysis was extracted from leaves of control and stress-treated plants as per the protocol described previously (Logemann *et al*. 1987). Four specific probes were obtained from 3-untranslated regions of the four cDNAs by PCR amplification or by digestion with restriction enzymes. Northern blot analysis was performed by standard protocols (Sambrook and Russell 2001). 20 μg of total RNA was electrophoresed on a 1% agarose gel made in 1 × morpholinopropanesulp honic acid (MOPS) and 1.2% formaldehyde. RNA was transferred to the nylon membrane and subsequently cross-linked with UV irradiation. Hybridization was performed in PerfectHyb Plus hybridization buffer (Sigma, USA) for 16 h at 68°C. The membranes were then washed once for 10 min with 2°C × SSC and 0.5% SDS, twice for 20 min with 0.5 × SSC and 0.1% SDS, and then once for 20 min with 0.1 × SSC and 0.1% SDS at 68°C, and exposed to X-ray films at -80°C.

RESULTS

Isolation and identification of four cDNAs from *N. tabacum*

According to the four described *NsRGPs* (GenBank accession no. D16204, D16205, D26182, and D28862), we designed four primer pairs and isolated four matured cDNAs (GU991537, GU991538, GU991539, and GU991540) from *N. tabacm*, named as *NtRGP-1a*, *NtRGP-1b*, *NtRGP-2*, and *NtRGP-3*, respectively, by RT-PCR analysis.

The gel electrophoresis results showed the alternatively spliced mRNAs presented both in *NtRGP-1a* and *NtRGP-1b* (Fig. 1). It is very interesting that these spliced mRNAs of the two genes presented not only in leaves but also in roots and stems, which suggested that alternatively splicing did not occur in tissue-specific manner in tobacco cultivar. These three alternatively spliced mRNAs (Fig. 1-B, a) as well as four fully spliced mRNAs were cloned to pUCm-T vector and sequenced at Shanghai Sangon, China. Sequences analysis revealed minor difference between matured cDNA sequences in *N. tabacum* and *N. sylvestris*. Variation in *RGP-1a* occurred as a two base difference between *N. sylvestris* and *N. tabacum*, a T in *N. sylvestris* changed to a C in *N. tabacum* at 372nd position of the coding region, and a T insertion at the 3′-untranslated region of *N. tabacum*. In *RGP-3*, one base difference existed at the 210th position of coding region (a C in *N. sylvestris* changed to a T in *N. tabacum*). However, no difference was found in coding region and untranslated regions of *NtRGP-1b* and *NtRGP-2*. Subsequently, the

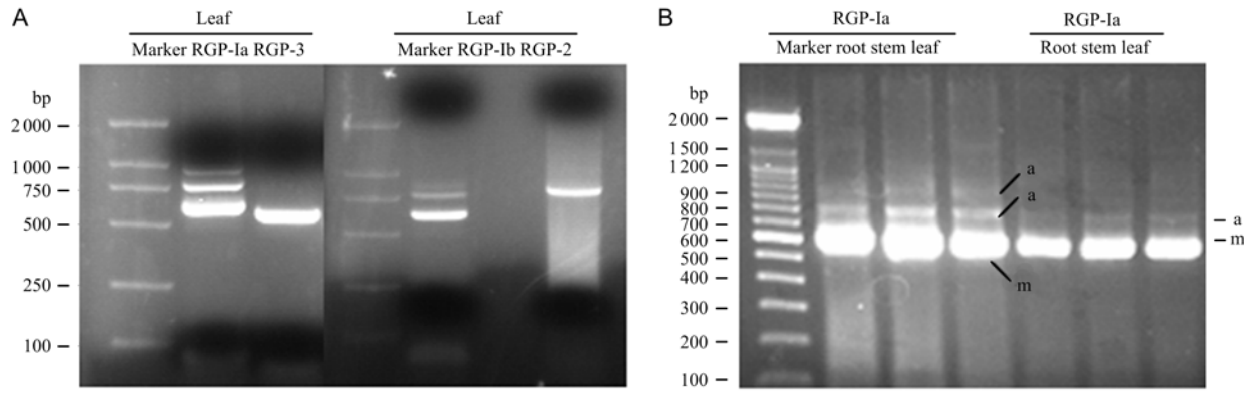

Fig. 1 Detection of mRNA species from the *NtRGP-la*, *-lb*, *-2*, and *-3* by RT-PCR. A, cDNAs profiles from leaf. B, detection of alternatively spliced mRNA in tobacco leafs, stems and leaves. m for matured mRNA; a for alternatively spliced mRNA.

four deduced proteins were predicted by EXPASY proteomics server (www.expasy.org). Analysis showed that these proteins are typical glycine-rich RNA-binding proteins and show 100% identity with the corresponding proteins in *N. sylvestris*, indicating that point difference in *NtRGP-1a* or *NtRGP-3* was synonymous mutation. This result suggested that glycine-rich RNA binding proteins in tobacco were quite conserved and might play important roles in biology process.

Homology analysis of the deduced NtRGPs

Based on their structural features, the GR-RBPs can be grouped into several different classes. For instance, proteins from the first class show a RRM conserved motif at the N-terminus and followed by a GD with GGYGG repeats. GR-RBPs in the second class show similar structure, but present zinc-finger motif inside their GD. Other classes exist with a cold-shock domain at N-terminus, or with the repeats of GGGY or GXGX in GD (Sachetto-Martins *et al*. 2000; Fusaro *et al*. 2001). To further analyze the homology of the four NtRGPs with other four GR-RBPs from monocots and dicots, including NgRBP (AF005359), RZ-1 (BAA12064), AtGRP2 (NM179046), and ZmGRP (AF034945), we performed a multiple alignment by ClustalX (Fig. 2).

NtRGP-la shared 80.4% identical amino acids with NtRGP-1b, 93.6% with NgRBP, 73.1% with ZmGRP, and shared lower identity (<50%) with the rest. There were 54.2% identical amino acids between NtRGP-2 and NtRGP-3. These four proteins have significant high glycine content in glycine-rich region, which were 64.7, 71.4, 33.8, and 44.4%, respectively. The alignment showed that N-terminal short peptide was present in NtRGP-2, 3 and AtGRP-2, and absent in other proteins (Fig. 2). The motif of GGYGG repeat was frequently found in glycine-rich domain in NtRGP-1a, NtRGP-1b, NgRBP, and ZmGR. Except for the existence of CCHC Zinc-finger motif in RZ-1, none of other proteins contained zinc-finger motif (Fig. 2). It can be observed that three proteins (NtRGP-1a, NgRBP, and ZmGRP) contained RGG motif which has been proposed as a predictor of RNA binding activity, and an important domain for protein functions (McBride *et al*. 2009). The typical GGGY array was also present in NtRGP-2, 3 and AtGRP-2.

Phylogenetic analysis of four *NtRGPs*

To investigate the phylogenetic relationships of *NtRGPs*, both coding regions of cDNAs and deduced amino acids sequences were compared by ClustalW with a set of 69 GR-RBPs in the genbank, including animals, plants, moss, fungi, and prokaryotes. *Anabaena variabilis* rbpA1 was used as outgroup. The trees based on both nucleotides and amino acid sequences appeared quite similar (Fig. 3, data not shown). Considering that nucleotide might contain more information than deduced amino acid, the phylogenetic tree based on the coding region cDNA sequences was used for illustration.

Phylogenetic analysis revealed that 69 GR-RBPs genes were grouped into three larger groups. Obviously,

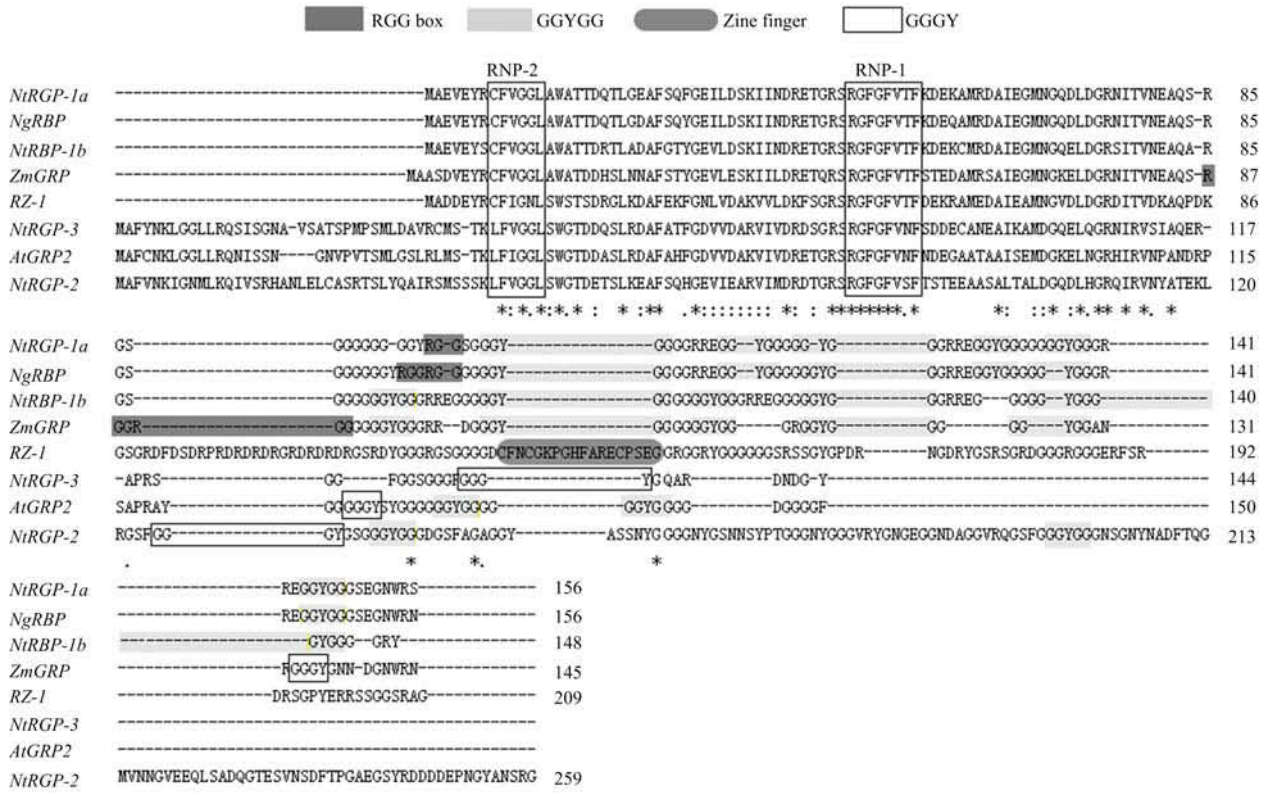

Fig. 2 Homology analysis of the deduced NtRGPs. Alignment of deduced amino acid sequences of *NtRGP-1a*, *-1b*, *-2*, and *-3* with other typical GR-RBPs by ClustalX. Different shapes were used to represent typical domains or motifs.

group I was basal group and consisted of all prokaryotes. Group II was clustered into two clades, clade I and clade II. Clade I contained genes in gramineous plants, while clade II contained *NtRGP-1a* and *NtRGP-1b* in this study as well as several genes in other herbaceous plants including *Catharanthus roseus*, *Nicotiana glutinosa*, *Solanum tuberosum*, *Euphorbia esula*, *Ricinus communis*, *Prunus avium*, *Sinapis alba*, *bassica napus*, *Daucus carota*, *Glycine max*, and *Medicago sativa*. Group III was clustered into clade III and clade IV. Seven animal genes were clustered in clade III with perfect support. The *NtRGP-2* and *NtRGP-3* in this study belonged to clade IV together with five *Arabidopsis* genes (*AtGRP2*, *3*, *4*, *5*, and *6*), and several genes in *Pisum sativum* (*PsGRBP*), *Oryza sativa* (*OsGRP2*), and *Physcomitrella patens* (*PpGRP*3) (Fig. 3, group III).

It was shown that four *NtGRPs* were divided into two different groups. *NtRGP-2* and *NtRGP-3* were evolutionarily closest to *Arabidopsis* genes and closely related to animal genes. In contrast, *NtRGP-1a* and *NtRGP-1b* were closest to *NgRBP* and closely related to *StGRP*, *CrGRP1*, *CrGRP2*, *CrGRP3*, *EeGRRBP1*, and *EeGRRBIP2* (Fig. 3).

Expression patterns of *NtRGPs* under different stresses

To further understand the possible roles of *NtRGPs* in plant growth under normal conditions and stress conditions, the expression of four *NtRGPs* in different organs and under various stresses was detected by Northern blot analysis. As shown in Fig. 4, the transcripts of four *NtRGPs* were largely accumulated in roots and stems but a little in leaves (Fig. 4-A, the last three lanes). Although the mRNA level of four *NtRGPs* was different under stresses, patterns of accumulation for the individual *NtRGPs* transcripts were very similar (Fig. 4). More striking responses of these genes were observed when tobacco leaves were treated with water-logging and wound. During water-logging treatment, the mRNA accumulated significantly at 4 h, peaked at 8 h, and reduced to normal level at 24 h (Fig. 4-B). NaCl solution had reduced the level of transcript in cor-

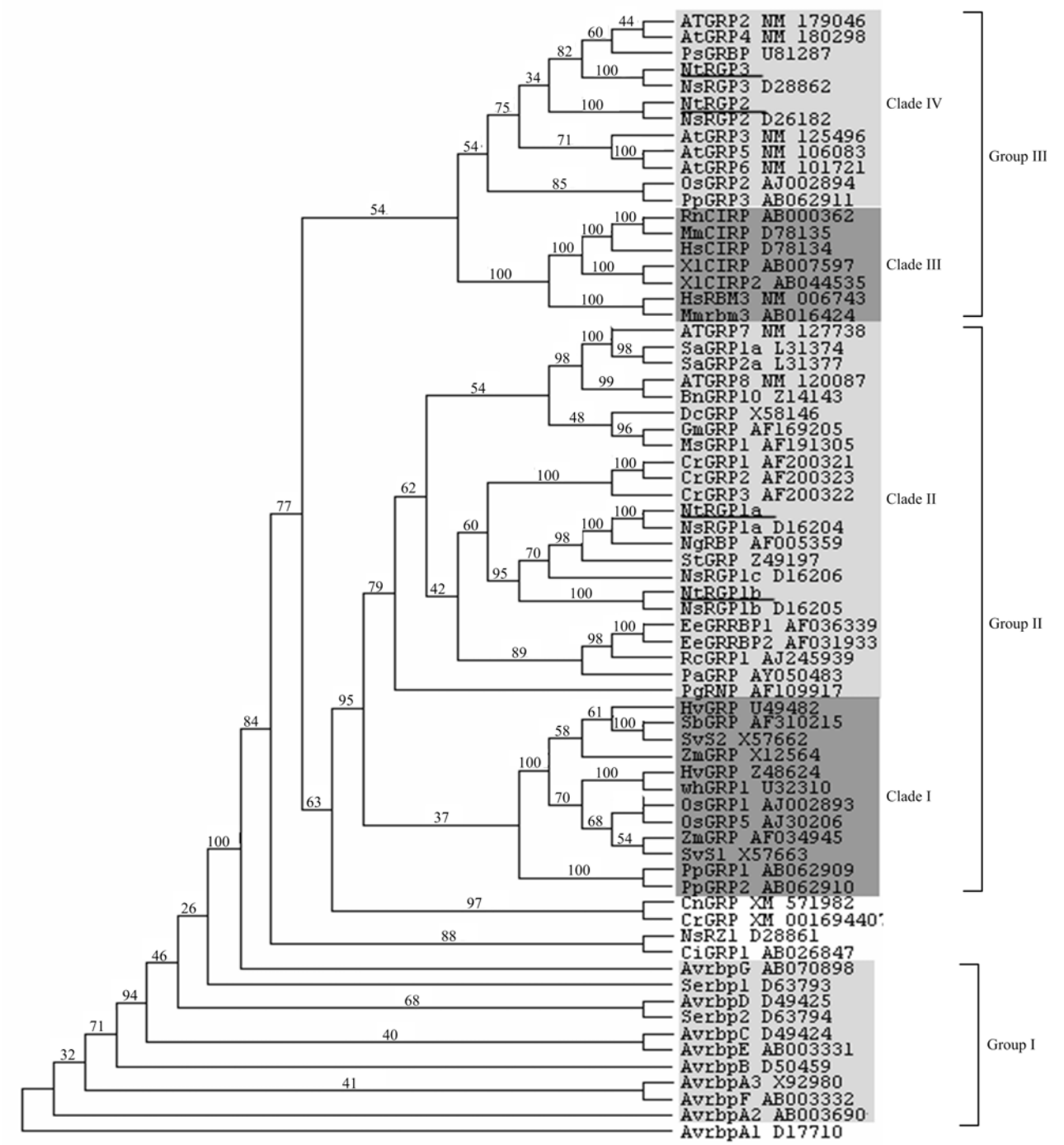

Fig. 3 Phylogenetic analysis of GR-RBPs genes from various species. Neighbor-joining tree was generated from the coding region of sequences from 69 GR-RBPs by PAUP 4.0, and AvrbpA1 from *Anabaena variabilis* was used as outgroup. Bootstrap values from 1 000 replications for each branch are shown above the branches. Four *NtRGPs* in this study are underlined.

responding time compared with that in water-treated leaves, suggesting the presence of NaCl appeared to counteract the effect of water logging (Fig. 4-A). The expressions of *NtRGP-1a* and *NtRGP-3* were rapidly up-regulated at 1 h after wound treatment, and increased continuously until reached the peak at 4 h, and then reduced gradually (Fig. 4-B). As ABA or SA was dissolved in water for stress treatment, water-spraying treatment was used as the control. It was observed that ABA had no effect on the transcript accumulation of four *NtRGPs* (Fig. 4-A), and *NtRGPs* showed a slight increase at 8 and 24 h during SA treatment (Fig. 4-B).

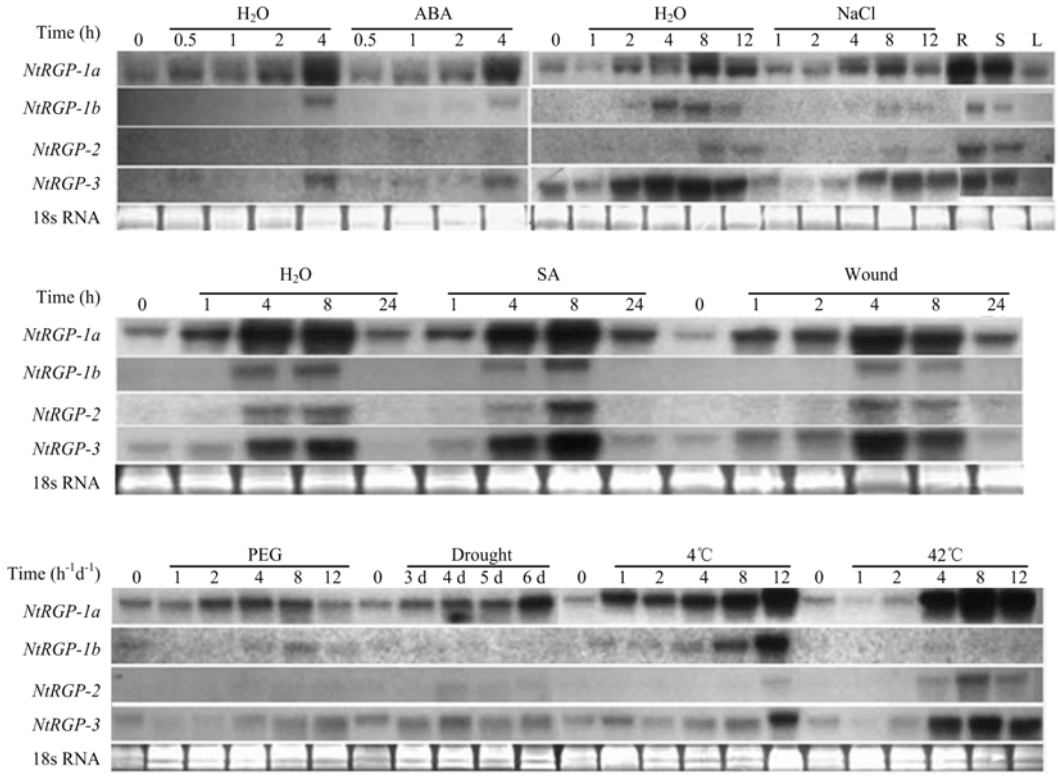

Fig. 4 Expression patterns of *NtRGP-1a*, *-1b*, *-2*, and *-3* during abiotic stresses. Water-treated leaves were made for contrast in ABA, NaCl and SA treatment. The expression pattern in roots (R), stems (S), and leaves (L) were shown on the last three lanes. Ribosomal 18S RNA was used as the control for RNA loading.

PEG had moderately regulated the accumulation of the *NtRGP-1a* mRNA at 2, 4, and 8 h, followed by a moderate reduction at 12 h, whereas other three *NtRGPs* were stable or showed minor change (Fig. 4-C). Under drought treatment, the *NtRGP-1a* transcript was continuously accumulated from 3 to 6 d (Fig. 4-C), however, the plants died when the drought time prolonged beyond 6 d. Under cold treatment, the expression levels of *NtRGP-1a*, and *-3* were up-regulated at 1 h, followed by a mild reduction at 2 h, and then showed an upward trend continuously after 4 h treatment. When normal plants were transferred to high temperature condition, a remarkable increase in *NtRGP-1a* and *NtRGP-3* transcript was detected at 4 h, and the expression peak arrived at 8 h, while the expression of *NtRGP-1b* was unaffected by high temperature treatment (Fig. 4-C).

DISCUSSION

We isolated four cDNAs from *N. tabacum*, named *NtRGP-1a*, *-1b*, *-2*, and *-3*, which were highly identical with the corresponding cDNAs in *N. sylvestris*. The four deduced proteins shared 100% identical amino acids with NsRGPs in *N. sylvestris* reported earlier by Hirose *et al*. (1993, 1994) and Moriguchi *et al*. (1997). The point mutation in the coding regions of *NtRGP-1a* and *NtRGP-3* were synonymous mutation, implicating that these conserved GR-RBPs in tobacco may play crucial roles in plant growth process.

The alternatively spliced mRNAs of *NtRGP-1a* and *NtRGP-1b* were detected in roots, stems, and leaves, which was not consistent with the previous research in which alternatively spliced mRNAs existed only in roots (Hirose *et al*. 1993). Our findings suggested that alternatively splicing occurred in a non-tissue-specific manner in a tobacco cultivar, *N. tabacum cv*. Xanthi. Further more, we found that these alternatively spliced mRNAs as well as the matured mRNAs were regulated by abiotic stresses. For example, cold stress and water stress increased the amount of alternatively spliced

mRNAs (data not shown). The same manner was reported for *Arabidopsis UBA*2 gene, which was considered to be involved in wound signal transduction pathway (Bove *et al*. 2008). The previous study demonstrated that both the alternatively spliced mRNAs and matured mRNAs are translatable (Hirose *et al*. 1993). So, it was indicated that these alternatively spliced mRNAs might be essential for some physiological processes.

GR-RBPs containing a conserved RNA-recognition motif and a diverse glycine-rich domain are consistent with the character of DNA barcoding, which can be used to identify different species (Hebert 2003). In our phylogenetic analysis of various species of GR-RBPs cDNA sequences, GR-RBPs in all species were clustered into several classes including prokaryotes, gramineae, animals, and some other species, suggesting GR-RBPs may be used as DNA barcoding to classify and identify species. Similarly, *NtRGP-2* and *NtRGP-3* were evolutionarily closest to *Arabidopsis* genes (*AtGRP2*, *3*, *4*, *5*, and *6*) and closely related to animal genes. As mentioned above, *AtGRP2* in cold stress and *AtGRP4* in salt or drought stress affected seed germination and seedling growth of *Arabidopsis*, and the expression of *NtRGP-3* induced by cold and drought, which suggested that *NtRGP-3* may also have impact on seed germination or seedling growth. On the other hand, *NtRGP-1a* and *NtRGP-1b* were closest to *NgRBP* which is possibly involved in plant pathogen-interaction (Naqvi *et al*. 1998), suggesting that *NtRGP-1a* and *NtRGP-1b* may serve as functional members similar to *NgRBP*.

Expression analysis of target gene is one of the most effective ways to uncover the function of gene. Various GR-RBPs were reported to be regulated by biotic or abiotic stresses (Table). In our study, the *NtRGPs* in *N. tabacum* were mainly expressed in root, moderately expressed in stem, and slightly expressed in leaf, suggesting that *NtRGPs* may play important roles in root-related physiological activity. According to the expression profiles of *NtRGPs* in various stresses, the expression patterns of *NtRGPs* were very similar (Fig 4). Four *NtRGPs*, especially *NtRGP-1a* and *NtRGP-3*, were induced strongly by stresses including water, wound, cold, and high temperature, moderately induced by PEG, drought, while reduced by NaCl (Fig. 4). Different stresses to plant may result in similar responses at the cellular and molecular level, because different stressors can trigger similar strains and downstream signal transduction chains. A good example is the reaction to stressors which induce water deficiency, e.g., drought, salinity, and cold, especially frost (Beck *et al*. 2007). At the metabolic level, osmotic adjustment by synthesis of low-molecular osmolytes (carbohydrates, betains, proline) can counteract cellular dehydration and turgor loss (Beck *et al*. 2007). The fact that all of these abiotic stresses in our experiments including water, NaCl, wound, drought, cold, PEG, and high temperature affected the water balance and resulted in osmotic stress on cellular, suggests that NtRGPs are a kind of osmotic stress-related proteins, and might play a key role in response to osmotic stress. Signal transduction path-

Table The GR-RBPs regulated by stresses

Species	Name	Regulation	Reference
Homo sapiens	HsCIRP	Cold shock	Nishiyama *et al*. (1997b)
Mus musculus	MmCIRP	Cold shock	Nishiyama *et al*. (1997b)
Xenopus laevis	XlCIRP	Cold shock	Matsumoto *et al*. (2000)
Rattus norvegicus	RnCIRP	Cold shock	Xue *et al*. (1999)
Picea glauca	PgRNP	Cold, circadian rhythm, wound, JA, drought	Richard *et al*. (1999)
Arabidopsis thaliana	AtGRP1, 4, 7	Cold, dehydration, salt	Kwak *et al*. (2005)
	AtGRP2	Cold , dehydration	Kwak *et al*. (2005)
	AtGRP3, 6	Cold, salt	Kwak *et al*. (2005)
	AtGRP5	Cold	Kwak *et al*. (2005)
	AtGRP8	Cold, circadian rhythm	Kwak *et al*. (2005)
Nicotiana glutinosa	NgRBP	Hypersensitive response, SA, copper sulfate	Kwak *et al*. (2005)
Sinapis alba	SaGRP1a, 2a	Cold, circadian rhythm	Heintzen *et al*. (1994)
Daucus carota	DcGRP	Wound	Sturm (1992)
Euphorbia esula	EeGRRBP1, 2	Cold	Horvath and Olson (1998)
Zea mays	CHEM2	Mercuric chloride, cold, water, wound	Didierjean *et al*. (1992)
	MA16	ABA, dehydration	Gomez *et al*. (1988)
Hordeun vulgare	HvGRP	Light/dark, cold shock, fungal infection	Molina *et al*. (1997)

ways involved in abiotic stresses are well known to be cross-linked (Mahajan and Tuteja 2005). In other words, the result that the expression of *NtRGPs* were influenced by various stresses suggests that *NtRGPs* in tobacco may act as a key node in signal transduction network.

Sensitivity to abiotic stresses is a very complex phenomenon and there are intricate signaling pathways which enable plants to tolerate and adapt during abiotic stress. Phytohormones, such as jasmonic acid (JA), ethylene, and ABA, regulate the responses of plants against biotic and abiotic stresses via signaling crosstalk. It has been known that both ABA-dependent and ABA-independent signaling pathways involved in osmotic stress (Himmelbach *et al*. 2003; Fujita *et al*. 2006; Chen *et al*. 2009). In this study, two methods of ABA treatment, leaf-spraying and ABA treatment with the root immersed in 100 μmol L^{-1} ABA solution for time course (data not shown), were used to test the expression change of *NtRGPs*. The results showed that no transcript accumulation in leaf was observed (Fig. 4), indicating that the NtRGPs family may play a role in tobacco osmotic stresses through a specific signal transduction pathway without ABA-dependent pathway.

CONCLUSION

Four cDNAs, *NtRGP-1a*, *1b*, *2*, and *3*, were isolated from *N. tabacum*. The expression patterns indicated that NtRGPs in tobacco are crucial osmosis-related proteins, and may function as a key node in signal transduction with ABA-independent pathway under abiotic stresses.

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