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Identification of the genes involved in heterotrimeric G-protein signaling in mulberry and their regulation by abiotic stresses and signal molecules

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Abstract

Heterotrimeric guanine-nucleotide-binding proteins (G-proteins) play important roles in signal transduction and regulate responses to various stresses. Although G-protein signaling pathways have been extensively identified and characterized in model plants, there is little knowledge in non-model and especially in woody plants. Mulberry is an economically and ecologically important perennial tree, which is adaptable to many environmental stresses. In this study, we identified and cloned six G-protein genes including one *Gα*, one *Gβ*, two *Gγ*, one *RGS* (regulator of G-protein signaling protein) and one *RACK1* (receptor for activated C kinase 1) involved in G-protein signaling. Sequence and phylogenetic analysis revealed that *Morus* G-proteins are evolutionarily conserved compared with those of other plants. A real-time quantitative reverse transcription polymerase chain reaction analysis showed that *Morus* G-protein signaling genes were ubiquitously but differentially expressed in various tissues. The expression of all of these genes was affected by abiotic stresses and signal molecules, which indicates that *Morus* G-protein signaling may be involved in environmental stress and defense responses.

Additional key words: biotic stress, expression profile, *Morus atropurpurea, Morus notabilis.*

Introduction

Heterotrimeric guanine-nucleotide-binding proteins (G-proteins) are key signaling intermediates that regulate fundamental signaling pathways involved in sensory perception, signal transduction, hormone perception, and immunity-related cues in all eukaryotes (Roy Choudhury and Pandey 2013). The G-protein complex comprises three subunits, namely Gα, Gβ, and Gγ. The functions of G-proteins has been well understood in animals (Wettschureck *et al*. 2015). In animals, activation of G-protein coupled receptors (GPCRs) causes a conformational change of the Gα subunit that leads to the exchange of GDP for GTP. Then, G-proteins dissociate into Gα-GTP and a Gβγ dimer, and both of these components can interact with downstream effectors to perform diverse cellular functions. Regulators of G-protein signaling (RGS) proteins, a class of GTPase-accelerating proteins, can enhance the GTPase activity of Gα and inactivate G-protein signaling (Chen *et al*. 2003, Urano *et al*. 2013, Urano and Jones 214).

In plants, the first subunit of G-proteins was cloned from *Arabidopsis* in 1990 (Ma *et al*. 1990). The functions of G-proteins were elucidated in *Arabidopsis* and rice (*Oryza sativa*) by loss or gain-of-function analyses of mutants, as well as by molecular and biochemical assays, and they are quite different from those of animals (Perfus-Barbeoch *et al*. 2004, Urano and Jones 2014). On one hand, the genomes of most plants encode only single Gα and Gβ subunits and a few Gγ subunits

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Abbreviations: ABA - abscisic acid; 3-AT - 3-amino-1,2,4-triazole; CDS - coding sequence; G-proteins - guanine-nucleotide-binding proteins; MAPK - mitogen-activated protein kinase; MeJA - methyl jasmonate; PEG - polyethylene glycol; RACK1 - receptor for activated C kinase 1; RGS - regulator of G-protein signaling protein; RT-qPCR - reverse transcription quantitative PCR; SA - salicylic acid; Y2H - yeast two-hybrid.

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(Urano *et al*. 2013), which is much less than those of animals or humans (23 Gα, 5 Gβ, and 12 Gγ subunits in humans). On the other hand, recent studies demonstrated that plants have evolved a distinct mechanism to regulate the activity of G-proteins compared with animals. The plant Gα protein is self-activated because its rate of guanine nucleotide exchange is approximately 100 times faster than its rate of GTP hydrolysis (Urano and Jones 2014). The regulator of G-protein signaling protein (RGS protein), comprising a seven-transmembrane domain, serves as the regulatory point of G-protein activation and stimulates the rate-limiting GTPase activity of the Gα subunit (Chen *et al*. 2003, Jones *et al*. 2011). In *Arabidopsis*, the endocytosis of AtRGS1 can uncouple *Arabidopsis* G-protein α subunit (AtGPA1) from AtRGS1 and leads to the subsequent sustained activation of G-protein signaling (Urano *et al*. 2012).

The G-protein signaling in plants is involved in many aspects of the plant life, including morphology, seed germination, cell proliferation, defense, stomatal movements, channel regulation, sugar sensing, and responses to hormones and environmental stresses (Perfus-Barbeoch *et al*. 2004, Klopffleisch *et al*. 2011, Urano *et al*. 2013). Furthermore, G-proteins also regulate some key agronomical traits in crops (Huang *et al*. 2009, Takano *et al*. 2009, Roy Choudhury *et al*. 2014a, Roy Choudhury and Pandey 2013, 2015).

Recently, the receptor for activated C kinase 1 (RACK1) was identified as the scaffold protein that connects heterotrimeric G-protein signaling with a mitogen-activated protein kinase (MAPK) cascade to form a unique signaling pathway in plant immunity (Cheng *et al*. 2015). RACK1 was originally identified as a receptor for activated protein kinase C in mammals and is structurally similar to Gβ protein (Mochly-Rosen *et al*. 1991, Ron *et al*. 1994, Dell *et al*. 2002, Chen *et al.* 2006). In plants, RACK1 was first cloned from tobacco BY-2 cells as an auxin-induced gene (*arcA*) (Ishida *et al*. 1993). Thereafter, the *RACK1* gene was isolated from a range of plant species, and it plays important roles in hormone responsiveness, developmental processes, and innate immunity (Chen *et al*. 2006).

Mulberry is a deciduous and economically important perennial tree, and its leaves are the main source of food for silkworms. In addition, *Morus* species have multiple uses in ecology, pharmaceutics, and traditional Chinese medicine (He *et al*. 2013). *Morus* adapts well to many different environmental stresses, including drought, salinity, water logging, and metal stresses (Ramachandra Reddy *et al*. 2004), but there is little research into the mechanisms of its adaptation. The aim of this study was to identify the putative genes involved in the G-protein signaling pathway in the *M. notabilis* and *M. atropurpurea* genomes and to examine the expression patterns of these genes in different organs, as well as their responses to various abiotic stresses and signal molecules.

Materials and methods

Plants and treatments: Seeds from *Morus atropurpurea* Roxb. cv. Guiyou No.62 were germinated in Petri dishes and grown in a *PQX* type plant incubator (*Ningbo Southeast Instrument Corporation*, Ningbo, China) with a day/night temperatures of 24/22 °C, relative humidity of 80 %, a 16-h photoperiod, and an irradiance of 400 μmol m^{-2} s^{-1}. After 14 d, the seedlings were treated with high temperature (40 °C), low temperature (4 °C), salt [0.6 % (m/v) NaCl], drought [30 % (m/v) PEG6000], 400 μM abscisic acid (ABA), 2 mM salicylic acid (SA), 10 mM hydrogen peroxide, or 4 mM methyl jasmonate (MeJA). The seedlings were sampled at 0, 1, 3, 6, 12, and 24 h post-treatment, respectively. Seedlings without treatments were used as control. Finally, the harvested seedlings were preserved at -80 °C for total RNA extraction.

Cloning and sequencing identification of G-protein signaling genes: The protein sequences of G-protein signaling genes from other plant species were downloaded from *GenBank* (http://www.ncbi.nlm.nih.gov/Genbank/) and were used as queries in *BLAST* search against the *M. notabilis* genome database (http://morus.swu.edu.cn/morusdb/). This yielded non-redundant full mRNA sequences. The candidate genes were identified using *BLASTN* and *SMART* (http://smart.embl-heidelberg.de/). Gene-specific primers (Table 1 Suppl.) were designed to amplify the full-length coding sequences (CDSs) of the candidate genes involved in G-protein signaling pathway from *M. notabilis* and *M. atropurpurea*, respectively. The purified PCR products were cloned into the pMD19-T simple vector (*TaKaRa*, Shiga, Japan) and they were confirmed by sequencing.

Sequence alignments and phylogenetic analysis: The amino acid sequences of proteins involved in the G-protein-mediated signaling were downloaded from the *National Center for Biotechnology Information* (*NCBI*; Table 2 Suppl.) and used for alignment with putative protein sequences of *Morus* signaling elements given by *ClustalW* (http://www.clustal.org), and the results were displayed using *GeneDoc*. A phylogenetic tree was constructed using the neighbor-joining method in *MEGA 5.0* with 1 000 replicated bootstrap values.

Protein-protein interaction assays: The interaction between the Gα and Gβ or RGS proteins was performed

using the *DUALmembrane* system (*Dualsystems Biotech*, Schlieren, Switzerland) according to the manufacturer's instructions. Full-length CDS of *Gα* from *M. atropurpurea* was cloned into the pPR3-N vector (prey vector), whereas the full length CDSs of the *Gβ* and *RGS* genes were cloned into the pBT3-STE vector (bait vector). The recombinant plasmid constructs were transformed into yeast strain NMY51 (MATα). Three clones were chosen from minimal media synthetic dropout (SD)-Trp-Leu plates and incubated in SD/-Trp-Leu liquid medium at 30 °C until the absorbance at 600 nm reached 0.6 - 0.8 and 1 mm^3 of culture was placed dropwise onto SD/-Trp-Leu-His-Ade plates containing 1.5 mM 3-amino-1,2,4-triazole (3-AT) and 40 mg dm^{-3} 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-gal).

An interaction between Gβ and Gγ proteins was determined using the *Clontech* two-hybrid system (*TaKaRa*). Bait (Gβ subunit) and prey (Gγ subunits) were cloned into the pGBKT7 and pGADT7 vectors, respectively. The bait and prey vectors were transformed into yeast strains Y2HGold (MATα) and Y187 (MATα), respectively. The clones from Y2HGold and Y187 containing the corresponding vector were placed into the same tube containing 2× yeast extract-peptone-dextrose-adenine liquid medium and incubated at 30 °C for 24 h, and the culture was plated onto SD/-Trp-Leu plates. Finally, the yeast colonies from the SD/-Trp-Leu plates were transferred onto SD/-Trp-Leu-His-Ade plates containing 40 mg dm^{-3} X-α-gal.

Quantification of transcripts using real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR): Total RNA was extracted with the *RNAiso Plus* kit (*TaKaRa*), and the first-strand of cDNA was synthesized using the perfect real time version of the *PrimeScript*™ RT reagent kit (*TaKaRa*). Ten-fold diluted cDNA was used in the RT-qPCR. The primers (Table 1 Suppl.) used for RT-qPCR were designed based on the gene sequences obtained from *M. atropurpurea*. The RT-qPCR procedure was the same as that described in our previous study (Wei *et al*. 2014). The *MaACTIN3* (HQ163775.1) gene was used as an internal control, and the relative expression was defined as $2^{-[Ct(target\ gene)\ Ct(control\ gene)]}$. All RT-qPCRs were performed in at least three independent biological replicates. Statistical analysis of the RT-qPCR data was conducted using *Excel* (*Microsoft*, Redmond, WA, USA) and *SPSS Statistics 17.0* software (*SPSS Inc.*, Chicago, IL, USA). The means were compared by Student's *t*-test.

Results

In this study, we identified six putative G-protein signaling genes in *M. notabilis,* using bioinformatic methods, including one Gα (*MnGα*), one Gβ (*MnGβ*), two Gγ (*MnGγ1* and *MnGγ2*), one RGS (*MnRGS*), and one RACK1 (*MnRACK1*) coding sequences (CDS) (Table 3 Suppl.). *MnGα* has a 13 exon/12 intron structure; *MnGβ* has a five exon/four intron structure; both *MnGγ1* and *MnGγ2* have a four exon/three intron structure; *MnRGS* has an 11 exon/10 intron structure, and *MnRACK1* has a two exon/one intron structure (Fig. 1 Suppl.). These genes have also been isolated from *M. atropurpurea* (Table 3 Suppl.).

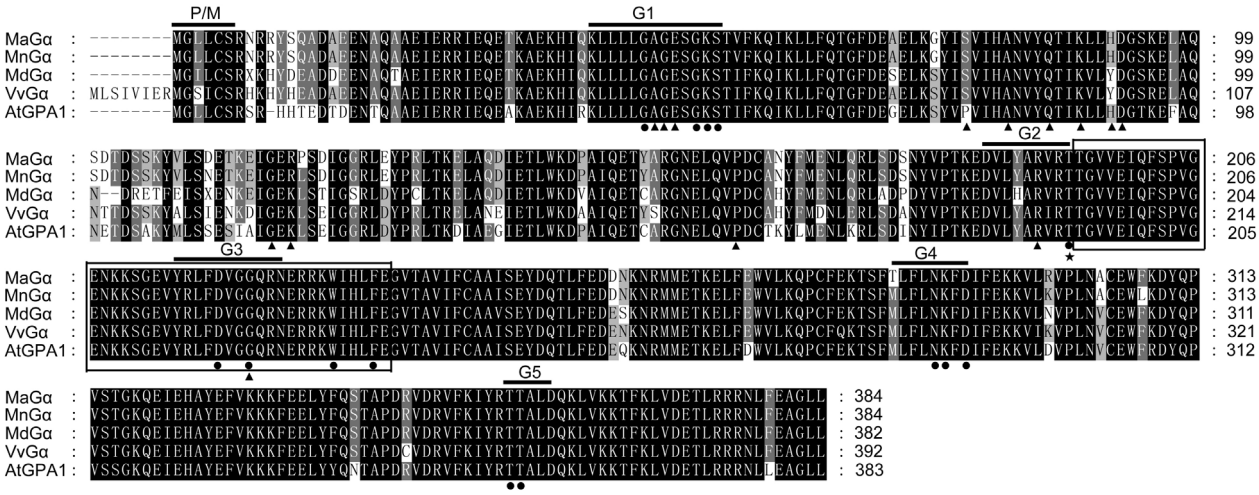

Fig. 1. Amino acid sequence alignments of *Morus atropurpurea* (Ma) and *Morus notabilis* (Mn) Gα protein with Gα proteins from other plant species (Vv - *Vitis vinifera*, Md -*Malus domestica*, At - *Arabidopsis thaliana*). Consensus sequences for GTP binding and hydrolysis are labeled G1 to G5; P/M represents the predicted sites for palmitoylation/myristoylation; ● indicates the predicted sites for GTP/Mg^{2+} interaction; ▲ indicates the GoLoco motif; the amino acid residues for the β/γ subunits are marked within boxes; ★ indicates residue for Gα and the RGS-box interaction.

The *Gα* gene isolated from *M. notabilis* and *M. atropurpurea* has a full-length CDS of 1 155 bp and it encodes 384 amino acids (Table 3 Suppl.), and the amino acid sequence identities between MnGα and MaGα is 99 %. Multiple sequence alignments revealed that the *Morus* Gα subunit contains a conserved sequence for myristoylation and palmitoylation (MGXXCS) at its amino-terminal end. The signature motifs required for guanine nucleotide binding and hydrolysis (G1-G5) are highly conserved in *Morus* Gα compared with the Gα subunits from other species. Within these domains, there are some predicted sites for GTP/Mg^{2+} interaction. The sequences of amino acids ranging from 195 to 235 are essential for β/γ subunit interaction. Thr194 was proposed to be critical for its regulation by RGS (Fig. 1).

The *Gβ* gene isolated from *M. notabilis* and *M. atropurpurea* has a CDS of 1 134 bp, corresponding to a protein with 377 amino acids (Table 3 Suppl.), and the Gβ CDS of both species share 99 % amino acid sequence identity. Multiple sequence alignments showed that Gβ protein contain seven tryptophan-aspartic acid (WD) repeats and the coiled-coil hydrophobic domain required for Gγ subunit interaction (Fig. 2 Suppl.).

We identified two *Gγ* genes in *Morus*. Multiple sequence alignments of the Gγ1 and Gγ2 amino acids revealed that their sequence identities are 55 and 66 % in *M. notabilis* and *M. atropurpurea*, respectively. The sequence identities were very high between MnGγ1 and MaGγ1 (97 %) and between MnGγ2 and MaGγ2 (95 %). Multiple sequence alignments showed that both Gγ subunits clustered into type A, and no Gγ in *Morus* belonged to the other two types. *Morus* Gγ1 and Gγ2 contain the DPLL box and the coiled-coil hydrophobic domain required for Gβ subunit interaction. Additionally, the prenylation target (CAAX) site, which does not exist in type B and type C Gγ, was also found in the C-terminus (Fig. 3 Suppl.).

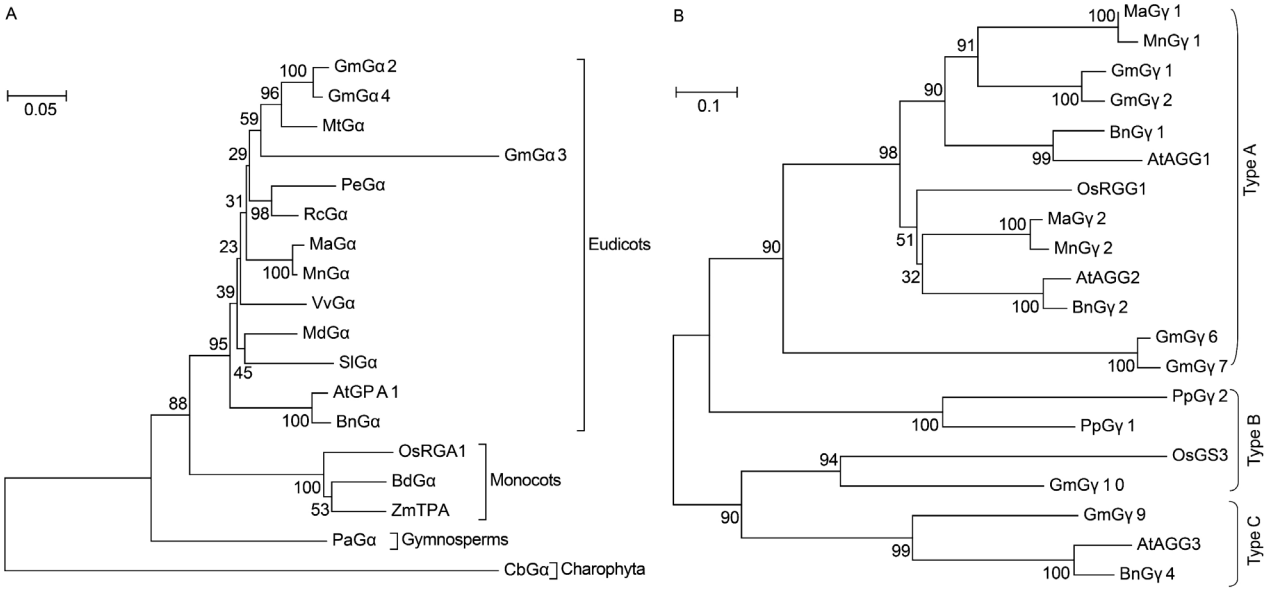

Fig. 2. Phylogenetic analysis of Gα (*A*) and Gγ (*B*) isolated from *Morus atropurpurea* (Ma) and *Morus notabilis* (Mn) and corresponding G-proteins from other plant species. The amino acid sequences were analyzed with *ClustalW* and the phylogenetic tree was constructed with *MEGA 5.0* using a bootstrap test of phylogeny with a minimum evolution test and default parameters. Gm - *Glycine max*, Mt- *Medicago truncatula*, Pe - *Populus euphratica*, Rc - *Ricinus communis*, Vv - *Vitis vinifera*, Md - *Malus domestica*, Sl - *Solanum lycopersicum*, At - *Arabidopsis thaliana*, Bn - *Brassica napus*, Os - *Oryza sativa*, Bd - *Brachypodium distachyon*, Zm - *Zea mays*, Pa - *Picea abies*, Cb - *Chara braunii*, Pp - *Physcomitrella patens.*

The *RGS* gene isolated from *M. notabilis* and *M. atropurpurea* has a CDS length of 1 386 bp, and it encodes 461 amino acids (Table 3 Suppl.). The RGS from both two species share 99 % amino acid sequence identity, and they show approximately 45 - 90 % sequence homology with RGS proteins from other plants. Seven transmembrane domains were found in the N-terminus (amino acids ranging from 13 to 247) of RGS, and the sequences of amino acids ranging from 294 to 412 were predicted to be the RGS box. Glu319 is the key site for endocytosis of the RGS protein. Ser433, Ser437, and Ser438 were predicted to be responsible for G-proteins activation (Fig. 4 Suppl.).

The amino acid sequences of G proteins from monocotyledons, eudicotyledons, gymnosperms, and *Charophyta* plants were used in a phylogenetic analysis. The phylogenetic trees showed that Gα, Gβ, and RGS from *M. notabilis* and *M. atropurpurea* clustered into the eudicotyledons group and were more closely related to the corresponding proteins from *Vitis vinifera*, *Ricinus*

communis., and some *Rosales* plants, and are more distantly related to other examined plants. These findings are consistent with the evolution of land plants (Hackenberg *et al*. 2013) (Fig. 2*A*; Fig. 5 Suppl.). A phylogenetic analysis of Gγ proteins showed that *Morus* Gγ1 and Gγ2 clustered into the type A Gγ family, which is consistent with the classification based on the differences in the C-terminal sequences of Gγ (Fig. 2*B*).

In this study, we identified a *RACK1* gene in *M. notabilis* and *M. atropurpurea*. *Morus RACK1* has a CDS length of 993 bp, and it encodes 330 amino acids (Table 3 Suppl.). Like Gβ, RACK1 is also a member of the WD40 family, and it contains seven WD repeat motifs (Fig. 6 Suppl.). *Morus* RACK1 shares approximately 67 - 91 % sequence homologies with RACK1 proteins from other plants, and it was closely related to RACK1 from *Vitis vinifera* and *Glycine max*, while it was distantly related to RACK1 in monocotyledons and *Charophyta* (Fig. 5 Suppl.).

To confirm the physical interactions between individual subunits, as well as between Gα and RGS, membrane-based split ubiquitin system and yeast two-hybrid (Y2H) assays were performed. The growth pattern of mated yeast cells in this assay showed expected strong interactions between MaGα and MaGβ and between MaGα and MaRGS (Fig. 7*A* Suppl.). The results also showed that MaGβ interacted with both MaGγ1 and MaGγ2 (Fig. 7*B* Suppl.), which indicated that there are two βγ dimers in *Morus*. These results support the reliability of the genome-wide identification of G-protein signaling genes in *Morus*.

Expression of G-protein signaling genes was detected in different tissues: root, stem, bark, petiole and leaf. The results revealed that all G-protein signaling genes were ubiquitously expressed in each of the tissue types tested, but they showed different expression patterns (Fig. 3).

To explore the responses of G-protein signaling genes to abiotic stresses, *M. atropurpurea* seedlings were subjected to high temperature, low temperature, PEG, and NaCl treatments. The results showed that the expression of all of the G-protein signaling genes was significantly down-regulated after 1 h of high temperature (40 °C) stress. Under low temperature (4 °C), the transcript abundances of *MaGα* and *MaGβ* were mostly

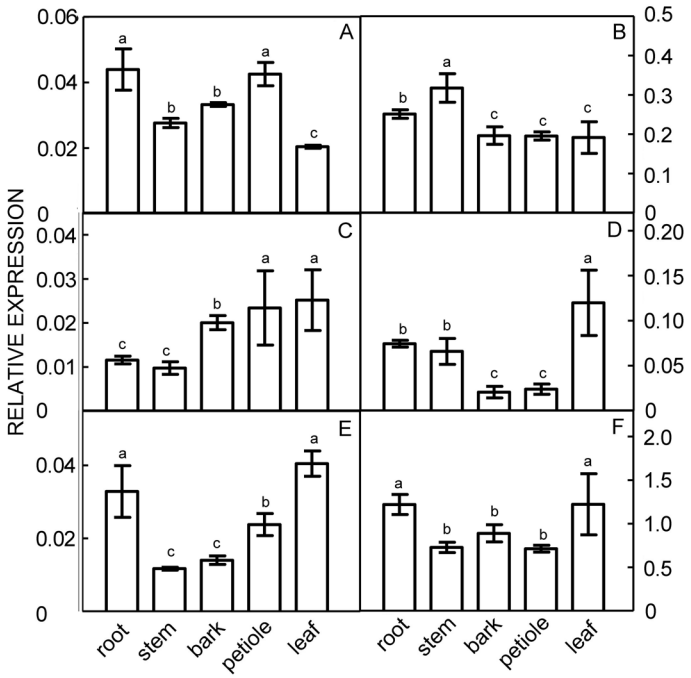

Fig. 3. Expression of G-protein signaling genes [*MaGα* (*A*), *MaGβ* (*B*), *MaGγ1* (*C*), *MaGγ2* (*D*), *MaRGS* (*E*), and *MaRACK1* (*F*)] in various *Morus atropurpurea* tissues including root, stem, bark, petiole, and leaf. Means ± SEs, $n = 3$. Significant differences ($P < 0.05$) are marked with different letters.

up-regulated, although *MaGβ* expression was suppressed at 3 h. *MaGγ1* expression was up-regulated at 1 and 6 h, and *MaGγ2* expression was up-regulated at 3 and 24 h. The expression of *MaRGS* and *MaRACK1* was suppressed, although *MaRGS* expression was up-regulated at 1 h. Under drought stress, the expression of *MaGβ*, *MaGγ2*, and *MaRGS* was up-regulated after 1 h of drought stress. The expressions of *MaGα, MaGγ1*, and *MaRACK1* were significantly down-regulated, although *MaGα* and *MaGγ1* expressions were up-regulated at 3 and 24 h, respectively. Additionally, the expression patterns in response to salt stress were detected, and the expressions of all of these genes, except *MaRACK1*, were significantly up-regulated (Fig. 4). These results indicate that G-protein signaling genes can be induced by a variety of abiotic stresses.

In this study, the expression patterns of G-protein signaling genes in response to plant signal molecules, ABA, SA, H_2O_2 and MeJA were explored. The expressions of all of the genes were mostly up-regulated by ABA treatment, although *MaRGS* and *MaRACK1* expressions were down-regulated at 1 and 24 h, respectively. The expressions of all of the G-protein signaling genes, except *MaRACK1*, were significantly up-regulated by SA, whereas *MaRACK1* expression was down-regulated. Under H_2O_2 stress, *MaGβ*, *MaRGS*, and *MaRACK1* expressions were mostly up-regulated, but *MaRGS* expression was down-regulated at 24 h. *MaGα* and *MaGγ2* expressions were mostly down-regulated by H_2O_2, although *MaGα* expression was up-regulated before 6 h of treatment. The expression of *MaGγ2* did not change in response to H_2O_2. *MaGα* and *MaGβ* expressions were significantly up-regulated by MeJA treatment, while *MaGγ1* and *MaRACK1* expressions were down-regulated. *MaGγ2* and *MaRGS* also responded to MeJA treatment (Fig. 5).

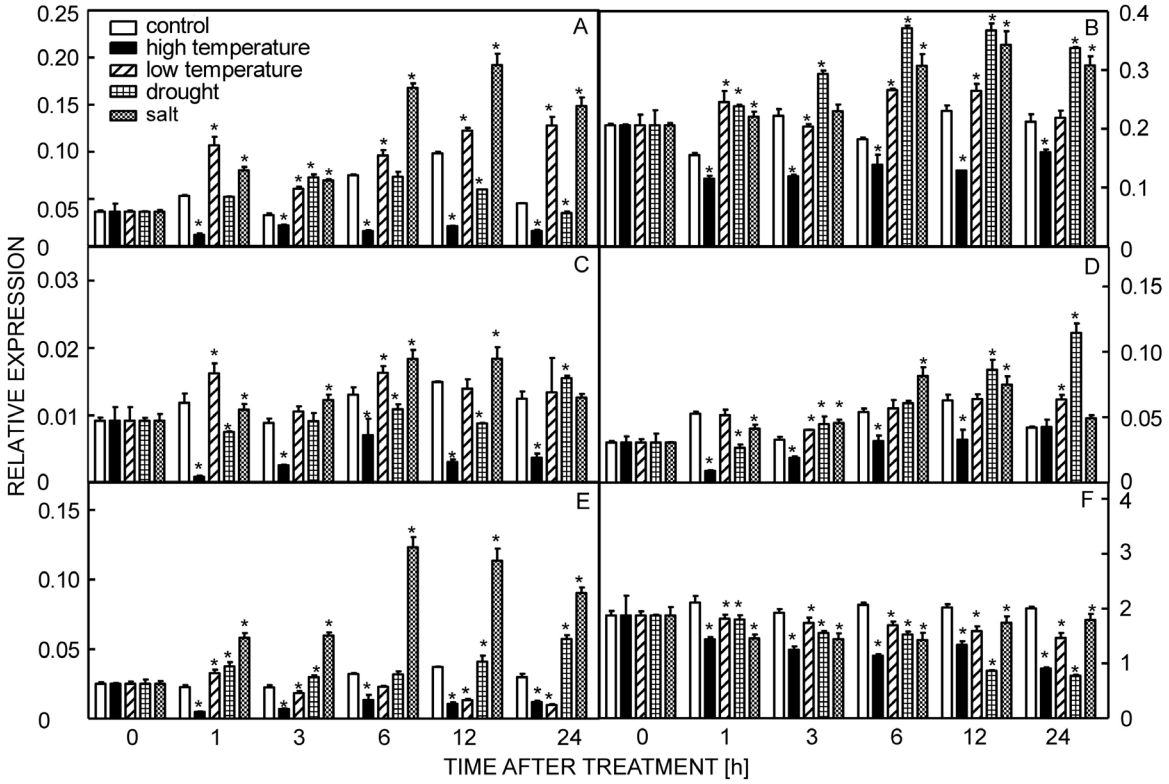

Fig. 4. Expression profiles of G-protein signaling genes [*MaGα* (*A*), *MaGβ* (*B*), *MaGγ1* (*C*), *MaGγ2* (*D*), *MaRGS* (*E*), and *MaRACK1* (*F*)] in response to abiotic stress treatments. *Morus atropurpurea* seedlings that were subjected to high temperature of 40 °C, low temperature of 4 °C, drought stress (30 % PEG6000), or salt stress (0.6 % NaCl). Means ± SEs, $n = 3$. Significant differences ($P < 0.05$) among treatments are marked with *asterisks*.

Discussion

Heterotrimeric G-proteins play important roles in transferring extracellular signals to bring about various intracellular changes that regulate several biological functions in plants. The functions of the G-protein signal pathway in model and some other herbaceous plants were clearly explored, and they are involved in many aspects of the plant life cycle, including seed germination, seedling development, stomatal movement, plant defense, and responses to biotic and abiotic stresses (Ullah *et al*. 2003, Pandey *et al*. 2006, Huang *et al*. 2009, Oki *et al*. 2009, Lee *et al*. 2013). However, there is little knowledge regarding these pathways in woody plants.

In the present study, six genes were identified in mulberry as being associated with G-protein signal pathway using bioinformatics tools, including one *Gα* gene, one *Gβ* gene, two *Gγ* genes, one *RGS* gene, and one *RACK1* gene (Table 3 Suppl), and the numbers of these genes are similar to those in other plant species (Table 4 Suppl.). In general, the *Morus* G-protein signaling proteins are evolutionarily conserved compared with those of other plants. In addition, the exon-intron organization of the *Morus* G-proteins was relatively similar to G-protein genes identified in *Arabidopsis*, *Brassica rapa*, and *Glycine max* (Roy Choudhury *et al*.

2011, 2014b, Arya *et al*. 2014).

A multiple sequence alignment analysis showed that *Morus* Gα, Gβ, and RGS shared high similarity with those in other species except *Chara braunii*, with sequence identities greater than 77 %. In addition, all of the signature motifs and domains that are required for proper function were found in the *Morus* Gα and Gβ subunits (Fig. 1, Fig. 2 Suppl.). The interactions between MaGα and MaGβ and between MaGα and MaRGS were confirmed in yeast (Fig. 7*A* Suppl.). These results indicate that heterotrimeric G-proteins are evolutionarily conserved in higher plants.

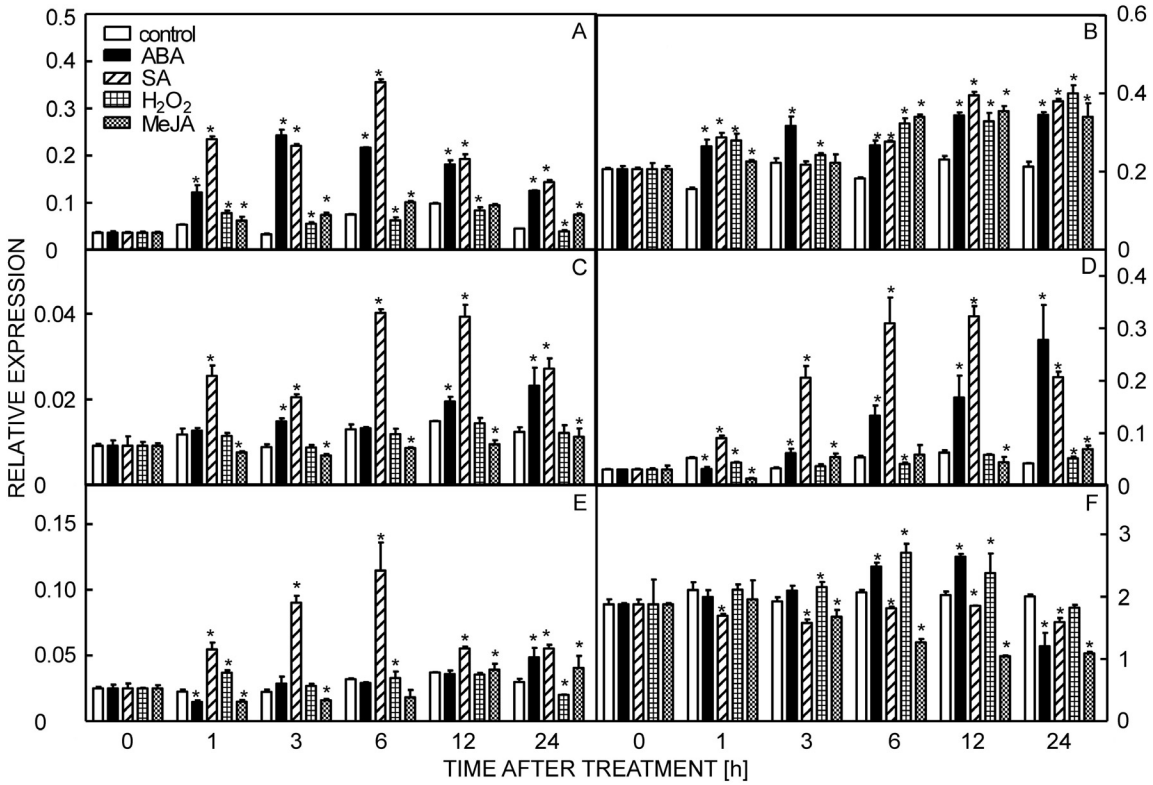

Fig. 5. Expression profiles of G-protein signaling genes [*MaGα* (*A*), *MaGβ* (*B*), *MaGγ1* (*C*), *MaGγ2* (*D*), *MaRGS* (*E*), and *MaRACK1* (*F*)] in response to signaling molecule treatments. The *Morus atropurpurea* seedlings were subjected to 400 μM abscisic acid (ABA), 2 mM salicylic acid (SA), 10 mM hydrogen peroxide, or 4 mM methyl jasmonate (MeJA) for 1 - 24 h. Means ± SEs, $n = 3$. Significant differences ($P < 0.05$) among treatments are marked with *asterisks*.

Plant Gγ subunit sequences can be classified into three types based on differences in their C-terminal sequences (Trusov *et al*. 2012). The distribution of Gγ is diverse in plants, and all three Gγ types were found in soybean, while no type B Gγ was found in *Arabidopsis* and *Oryza sativa*. In this study, two Gγ genes were characterized in *Morus* using bioinformatics methods, and they clustered into type A, whereas no type B and type C Gγ genes were found in *Morus* (Fig. 3 Suppl.). A Y2H analysis showed that the products of these two Gγ interacted with Gβ (Fig. 7*B* Suppl.), which indicates that there are two different βγ dimers in *Morus*.

Many studies confirmed that G-protein signaling plays an important role in regulating various abiotic stresses. Loss-of-function analyses of mutants and functional characterization revealed that G-proteins play important roles in regulating plant responses and tolerance to salt, drought, heat, and cold stresses (Colaneri *et al*. 2014, Xu *et al*. 2015, Zhang *et al*. 2015, Jangam *et al*. 2016). It has been reported that G-protein signaling is involved in different plant signaling pathways, such as SA-, MeJA-, H_2O_2-, ethylene-, and ABA-mediated signaling (Wang *et al*. 2001, Trusov *et al*. 2009, Shi *et al*. 2015). In this study, the results showed that the expressions of *Morus* G-protein-encoding genes were induced by low temperature, PEG and NaCl stresses, and all these genes were down-regulated by high temperature stress (Fig. 4); similar results have been observed in *Brassica napus* and *Oryza sativa* (Gao *et al*. 2010a,b, Yadav *et al*. 2013, 2014). *MaRGS* expression was up-regulated by NaCl and suppressed by low and high temperatures (Fig. 4), which contradicts the result in *Brassica napus* (Chen *et al*. 2014). This indicates that *MaRGS* may play distinct roles in responses to abiotic stresses in *Morus*. Besides, *Morus* G-protein signaling genes were found to be significantly regulated by different signaling molecules including ABA, SA, H_2O_2, and MeJA (Fig. 5), as has been reported in *Brassica*

species (Arya *et al*. 2014, Kumar *et al*. 2014). The function of *Morus* G-protein signal pathways in ABA, SA, H_2O_2, and MeJA signaling requires further investigation.

Furthermore, the expression patterns of *Morus* G-protein signaling genes under abiotic stresses and signal molecules suggest a working model for G-protein signaling pathway in response to abiotic and biotic stresses (Fig. 8 Suppl.). When *Morus* encounters salt and drought stresses, G-protein signaling pathways are activated. Previous studies indicated that the activation of G-proteins increases salt tolerance in plants (Colaneri *et al*. 2014). However, the roles of G-proteins in drought stress still need to be clearly elucidated. The expression data also suggest that ABA, SA, H_2O_2, and MeJA may also lead to the activation of G-protein signaling pathway. It is interesting that *Morus* G-protein signaling genes were suppressed by high temperature, which suggests that the inactivation of G-proteins may protect plants from high temperature. How G-proteins activate downstream proteins remains to be studied. A protein interactome analysis *AGIdb*, (http://bioinfolab. unl.edu/AGIdb). indicated that G-proteins may mediate stress responses by interacting with their effector proteins in *Arabidopsis*, including protein kinases, transcription factors, and several stress response proteins. Previous studies suggested that some proteins, MAPK3, MAPK6, and a bZIP protein (VIP1), are associated with G-proteins to regulate plant development and stress responses (Tsugama *et al*. 2013, Xu *et al*. 2015). Recent study showed that the RACK1 protein links the MPK3/MPK6 cascade to the upstream heterotrimeric G-protein to mediate Pvds-regulated endoprotease (PrpL) and endoproteinase ArgC triggered immunity (Cheng *et al*. 2015). Our previous study indicated that *Morus MAPK* genes can respond to different stresses (Wei *et al*. 2014), and the overexpressions of these genes in *Arabidopsis* suggested that *MAPK* genes may play pivotal roles in response to stresses (unpublished data). These results suggest that RACK1 may link G-protein signaling with the MAPK kinase pathway in response to various biotic and abiotic stresses in *Morus*. However, much more work is needed to investigate the mechanism of G-protein signaling pathways in response to environmental stresses.

In conclusion, an analysis of the *Morus* genome data characterized six genes that are associated with G-protein signal pathway, including one *Gα* gene, one *Gβ* gene, two *Gγ* genes, one *RGS* gene, and one *RACK1* gene. Bioinformatic analysis suggests that *Morus* heterotrimeric G-proteins are evolutionarily conserved. A yeast-based analysis confirmed the interaction between *Morus* G-protein subunits and RGS. A RT-qPCR analysis showed that *Morus* G-protein signaling genes were ubiquitously and differentially expressed in various tissues. The expression of all of the *Morus* G-protein signaling genes was induced by various stresses, which provides new insights into the functions of G-protein signaling in response to environmental changes.

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