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Differential Expression and Interaction Specificity of the Heterotrimeric G-Protein Family in *Brassica nigra* Reveal their Developmental- and Condition-Specific Roles

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Regular Paper

Heterotrimeric G-proteins, comprised of α, β and γ subunits, are important signal transducers across phyla. The G-proteins are well characterized in the model plants Arabidopsis and rice, and their homologs are identified in a few other plant species; however, information about the roles played by G-proteins in regulating various growth and developmental traits particularly from polyploid crops is still awaited. In this study, we have isolated one Gα (*BniB.Gα1*), three Gβ (*BniB.Gβ1–BniB.Gβ3*) and four Gγ (*BniB.Gγ1–BniB.Gγ4*) coding sequences from the paleopolyploid *Brassica nigra*, a major condiment crop of the Brassicaceae family. Sequence and phylogenetic analysis revealed that whole-genome triplication events in the *Brassica* lineage had proportionally increased the inventory of the Gβ subunit, but not of the Gα and Gγ subunits in *B. nigra*. Real-time quantitative reverse transcription–PCR (qRT–PCR) analysis showed that members of the G-protein subunit genes have distinct temporal and spatial expression patterns and were differentially altered in response to various stress and phytohormone treatments, thereby suggesting differential transcriptional regulation of G-protein genes in *B. nigra*. Interestingly, specific members of G-protein subunits were co-expressed across plant developmental stages, and in response to different elicitor treatments. Yeast-based interaction screens further predicted that the *B. nigra* G-protein subunits interacted in most of the possible combinations, although showing a high degree of interaction specificity between different G-protein subunits. Our data on physical interactions coupled with the co-expression pattern of the multiple G-protein subunit genes suggested that tissue- and condition-specific functional combinations of Gαβγ heterotrimers may exist in paleopolyploid *B. nigra*, to control diverse growth and development processes.

Keywords: *Brassica nigra* • Gene expression • Heterotrimeric G-proteins • Polyploidy • Protein–protein interaction • Signal transduction.

Abbreviations: ACC, 1-amino-cyclopropane-1-carboxylic acid; 3AT, 3-amino-1,2,4-triazole; BAP, 6-benzylaminopurine; BR, brassinosteroid; CT, cycle threshold; dpa, days post-anthesis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPCR, G-protein-coupled receptor; G-proteins, heterotrimeric G-proteins; LSD, least significant difference; mbSUS, mating-based yeast split ubiquitin system; MeJA, methyl jasmonate; MS medium, Murashige and Skoog medium; qRT–PCR, quantitative reverse transcription–PCR; SA, salicylic acid; TIPS, tonoplastic intrinsic protein-41; UBQ, ubiquitin; Y2H, yeast two-hybrid.

The sequences described in this study were deposited in the National Centre for Biotechnology Information (NCBI) database with the following accession numbers: *BniB.Gα1* (KJ451020), *BniB.Gβ1* (KJ451022), *BniB.Gβ2* (KJ451024), *BniB.Gβ3* (KJ451026), *BniB.Gγ1* (KJ451028), *BniB.Gγ2* (KJ451030), *BniB.Gγ3* (KJ451032) and *BniB.Gγ4* (KJ451034).

Introduction

Signal transduction is the fundamental biological process through which organisms perceive external signals and ultimately transmit information derived from that signal within the cell. Throughout their life cycle, plants perceive diverse environmental factors such as light and chemical signals which alter their morphology, physiology and development. Heterotrimeric G-protein (hereafter G-protein) is one of the important transmembrane signal transduction components (Gilman 1987, Temple and Jones 2007). It is comprised of three subunits, namely Gα, Gβ and Gγ. Of these subunits, Gα has the ability to bind to the guanine nucleotides, GDP and GTP. In animals, binding of a ligand to a transmembrane-localized G-protein-coupled receptor (GPCR) causes changes in its conformation that subsequently lead to exchange of GDP for GTP on the Gα subunit (Gilman 1987, Pierce et al. 2002). Binding of GTP to the Gα subunit causes the inactive heterotrimer to dissociate into two functional signaling components, namely GTP-bound Gα (Gα-GTP) and Gβγ dimer, which independently interact with other signaling molecules to perform diverse cellular functions. The paradigm of G-protein signaling in plants, however, is quite different from that of their animal counterparts (Urano et al. 2012, Urano and Jones 2013). Recent biochemical and structural evidence in plants demonstrated that the plant G-proteins are self-activating, GPCR independent and known to be regulated at a deactivation step, primarily through the seven transmembrane receptor, Regulator of G-protein

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Signaling (RGS), a class of GTPase accelerating protein. Further, within the plant kingdom, monocots lack RGS proteins, except for *Setaria italica* where a single soluble RGS protein is present (Urano et al. 2012). Interestingly, a recent report in maize suggested for the first time that a single-pass transmembrane receptor could also acts as a GPCR in plants, and activate the Gα signaling in controlling shoot meristem development (Bommert et al. 2013). These observations possibly suggest that G-protein signaling is fundamentally different even within the plant kingdom.

In metazoans, multiple copies of G-protein subunits genes have been identified, suggesting vast intricacy in signaling networks (Morris and Malbon 1999, Neves et al. 2002, Wettschureck and Offermanns 2005). In humans, 23 genes for Gα, five for Gβ and 12 for Gγ are present. In contrast, the repertoire of G-protein components in plants is quite limited, as only one Gα, one Gβ and three Gγ subunits are present in the model plant *Arabidopsis thaliana* (Botella 2012, Urano et al. 2012). Similarly, one Gα, one Gβ and up to five Gγ subunits (OsRGG1, OsRGG2, OsGS3, OsDEP1 and OsGGC2) have been reported and proposed for the rice genome, suggesting that Gγ subunits are solely responsible for providing the functional selectivity to the plant heterotrimer. In recent years, *in silico* analysis of the genome and the expressed sequence tag (EST) database of land plants clearly indicates that the repertoire of G-protein is quite complex in plants (Trusov et al. 2012, Urano et al. 2012). In some land plants, 2–4 Gα and Gβ subunits and 10 Gγ subunits have been identified (Misra et al. 2007, Bisht et al. 2011, Choudhury et al. 2011, Urano et al. 2012), thereby suggesting that the signaling networks in plants are much complex than previously believed.

G-proteins regulate various biological processes in plants such as controlling plant morphology and architecture (Ullah et al. 2001, Chen et al. 2006), hormone and sugar signaling (Ashikari et al. 1999, Fujisawa et al. 1999, Ullah et al. 2002, Chen et al. 2006, Pandey et al. 2006, Wang et al. 2006), stomatal movements (Wang et al. 2001, Chen et al. 2004), ion channel regulation (Zhang 2011) and pathogen resistance and cell death (Llorente et al. 2005, Trusov et al. 2006, Trusov et al. 2009, Trusov et al. 2012). In addition, G-proteins are also known to regulate key agronomical traits in crops such as dwarfism in rice (Ashikiari et al. 1999, Fujisawa et al. 1999), seed size and yield in rice (Fan et al. 2006, Takano-Kai et al. 2009, Haung et al. 2009, Botella 2012) and nodulation in legumes (Choudhury and Pandey 2013). With the increasing repertoire of G-protein components across plants species, it is fairly clear that functional specificity among multiple G-protein subunits exists, playing key roles in controlling various plant growth and development processes and their response to changing environmental cues (Temple and Jones 2007, Urano et al. 2013).

The genus *Brassica* is agriculturally the most important genus in the Brassicaceae family, species of which are cultivated globally as oilseeds, vegetables and condiments crops. Polyploidy has played a significant role in the evolution of the genus *Brassica*. Genetic analysis has confirmed the relationship between the six cultivated *Brassica* species. Moreover, molecular-level studies shows that the three diploid species, *B. rapa* (AA genome), *B. nigra* (BB) and *B. oleracea* (CC), formed the three amphidiploid species, *B. juncea* (AABB), *B. napus* (AACC) and *B. carinata* (BBCC), through interspecific hybridization (Parkin et al. 1995, Nagaharu 1935). For many decades, the cultivable *Brassica* species have been investigated for several agronomical traits; however, comprehensive data from any *Brassica* species on the important roles played by G-proteins in regulating fundamental growth and development processes are not available at present. Understanding of these roles has been greatly impeded because of the inherent polyploidy and genome complexity present across *Brassica* species. Over the past several years, a general consensus has been reached about the paleohexaploid ancestry of the extant diploid *Brassica* species (Lysak et al. 2005, Parkin et al. 2005, Lysak et al. 2007). Comparative mapping studies between *Brassica* species and Arabidopsis clearly suggested that the current genome structure of *Brassica* species is shaped by whole-genome triplication followed by extensive diploidization, as a consequence of which all *Brassica* species analyzed to date contain either three or six copies of orthologous genomic regions of Arabidopsis. Identification of the G-protein gene family from progenitor *Brassica* genomes is a first step towards understanding the evolution and complexity of G-protein signaling networks that exist in polyploid *Brassica* species.

In recent years, some sequencing efforts have been made towards generating genetic and genomic information in *B. rapa* (Wang et al. 2011), but such resources are greatly lacking in related *Brassica* species, including *B. nigra*, a major condiment crop which is also one of the progenitor species contributing the B-genome to the globally important allotetraploid crops, *B. juncea* (AB) and *B. carinata* (BC). A large number of cytological, biochemical and genetic studies further suggested that *B. nigra* has evolved separately from the other two diploid *Brassica* species, *B. rapa* and *B. oleracea* (Panjabi et al. 2008, Warwick 2011, Sharma et al. 2014). The present study describes the isolation of multiple G-protein subunit genes from the *B. nigra* genome; details their in-depth expression profiles across plant development including both vegetative and reproductive stages, and their transcriptional response under various environmental stress conditions; and evaluates the specificity of interactions between different G-protein subunits in the *B. nigra* genome. This work not only reveals the presence of a complex G-protein signaling network in any *Brassica* species but also suggests that both developmental and condition-specific Gαβγ combinations may exist in paleopolyploid *B. nigra* to control diverse growth and development processes.

Results

Identification and analysis of coding DNA sequences of G-protein subunit genes of *B. nigra*

Degenerate primers (**Supplementary Table S1**) based on the reported sequence of G-protein subunit genes of Arabidopsis and related *Brassica* species (Gao et al. 2010a, Gao et al. 2010b,

Gao et al. 2011) were used to isolate full-length cDNA sequences of G-protein genes from *B. nigra*. In total, one Gα (*BniB.Gα1*), three Gβ (*BniB.Gβ1*, *BniB.Gβ2* and *BniB.Gβ3*) and four Gγ (*BniB.Gγ1*, *BniB.Gγ2*, *BniB.Gγ3* and *BniB.Gγ4*) full-length coding sequences were identified and confirmed with multiple amplifications from different tissue types of *B. nigra* (**Table 1**). The G-protein subunit genes were named according to standard gene nomenclature proposed for the *Brassica* genus (Ostergaard and King 2008). Sequence alignment showed that G-protein subunit sequences of *B. nigra* have a higher level of similarity with their closest dicot model plant, Arabidopsis, compared with the monocot plant, rice (**Fig. 1**).

The open reading frame of the *BniB.Gα1* sequence was 1,152 bp (**Table 1**), and shared 92.2% identity with the coding sequence of Arabidopsis *AtGPA1* (**Supplementary Fig. S1**, **Supplementary Table S2**). The 383 amino acid long deduced BniB.Gα1 protein shared significantly high amino acid sequence identity (96.9%) with AtGPA1 compared with OsRGA1 (73.8%) from rice (**Table 1**). The five characteristics domains (G1–G5) required for guanine nucleotide binding and hydrolysis, as well as three switches (I–III) that establish the basal and active conformations of Gα protein were conserved in the canonical BniB.Gα1 protein (**Fig. 1A**). In addition, signature sequences for palmitoylation and myristoylation (MGXXCS) at the N-terminus required for plasma membrane anchoring; the conserved cholera toxin-mediated ribosylation site (R); critical threonine (T) and glutamic acid (E) contact sites for Gα and RGS-box interaction; and the conserved glutamine (Q) important for the GTPase activity of Gα proteins described by Temple and Jones (2007) were also present in BniB.Gα1, thus suggesting a high level of sequence and functional conservation of Gα proteins across the plant lineage.

The three full-length Gβ-like sequences (*BniB.Gβ1*, *BniB.Gβ2* and *BniB.Gβ3*) isolated from *B. nigra* ranged in size from 1,134 to 1,137 bp, encoding proteins of 377–378 amino acids in length (**Table 1**). The nucleotide sequences of *B. nigra* Gβ subunits showed 88.4–90.4% identity with Arabidopsis *AtAGB1* in their coding region (**Supplmentary Fig. S2**, **Supplementary Table S2**). The deduced BniB.Gβ proteins were highly conserved, sharing 91.8–94.7% and 73.8–76.5% identity with Arabidopsis AtAGB1 and rice OsRGB1 proteins, respectively (**Table 1**). The residues necessary for contact with the Gα subunit and the coiled-coil hydrophobic domain at their N-terminus, required for Gγ subunit interaction, were found to be conserved across all BniB.Gβ proteins (Temple and Jones 2007). The seven WD repeat motifs were also found to be conserved in all BniB.Gβ proteins, except for BniB.Gβ3, where aspartate is replaced by glycine at the third WD motif position (**Fig. 1B**).

We identified a total of four coding sequences of Gγ-like genes in *B. nigra*, which were highly divergent in size, ranging from 270 to 705 bp, encoding proteins of 89–234 amino acids (**Table 1**). The nucleotide sequences of *B. nigra* Gγ subunits showed 23.2–90.1% identity with Arabidopsis Gγ subunits in their coding sequence (**Supplementary Fig. S3**, **Supplementary Table S2**). Amino acid sequence alignment showed that BniB.Gγ proteins shared a comparatively higher level of sequence identity with corresponding Gγ proteins of Arabidopsis than with those from rice (**Table 1**). Sequence alignment further revealed that BniB.Gγ1 was the closest homolog of AtAGG1; BniB.Gγ2 and BniB.Gγ3 were homologous to AtAGG2; and BniB.Gγ4 was the homolog of AtAGG3, respectively (**Fig. 1C**). Further, based on a recent classification of plant Gγ proteins (Trusov et al. 2012), three *B. nigra* Gγ proteins, namely BniB.Gγ1, BniB.Gγ2 and BniB.Gγ3, were type-A proteins; whereas BniB.Gγ4 belongs to type-C Gγ proteins. The BniB.Gγ proteins were highly divergent and shared 24.6–86.1% identity among them (**Supplementary Table S3**), thereby suggesting that among plant G-protein subunits a very high level of sequence and structural divergence exists among Gγ proteins. In general, the central part of the Gγ proteins was the most conserved part, whereas the N- and C-terminal regions were highly variable both in size and in sequence (**Supplementary Fig. 1C**). Despite the presence of huge sequence divergence, the BniB.Gγ proteins had typical plant Gγ-like characteristics, such as an N-terminal helix which a forms coiled-coil structure with Gβ; the C-terminal CAAX box for isoprenylation; and the conserved DPLL motif, except for BniB.Gγ4 in which a similar type DPLI

Table 1 Inventory of heterotrimeric G-protein from *B. nigra* and their amino acid sequence identity (%) with corresponding orthologs from Arabidopsis and rice

***B. nigra* G-protein subunit genes**	**CDS (bp)**	**Protein (amino acids)**	**Amino acid identity (%) with Arabidopsis orthologs**	**Amino acid identity (%) with rice orthologs**
BniB.Gα1	1,152	383	96.9	73.8
BniB.Gβ1	1,137	378	94.7	76.5
BniB.Gβ2	1,137	378	93.1	76.0
BniB.Gβ3	1,134	377	91.8	73.8
BniB.Gγ1	270	89	74.4	52.2
BniB.Gγ2	306	101	91.1	55.3
BniB.Gγ3	303	100	87.1	52.1
BniB.Gγ4	705	234	79.1	31.8–37.0[a]

[a] Amino acid sequence identity of BniB.Gγ4 calculated with three type-C Gγ4 proteins of rice, namely OsDEP1, OsGS3 and OsGGC2.

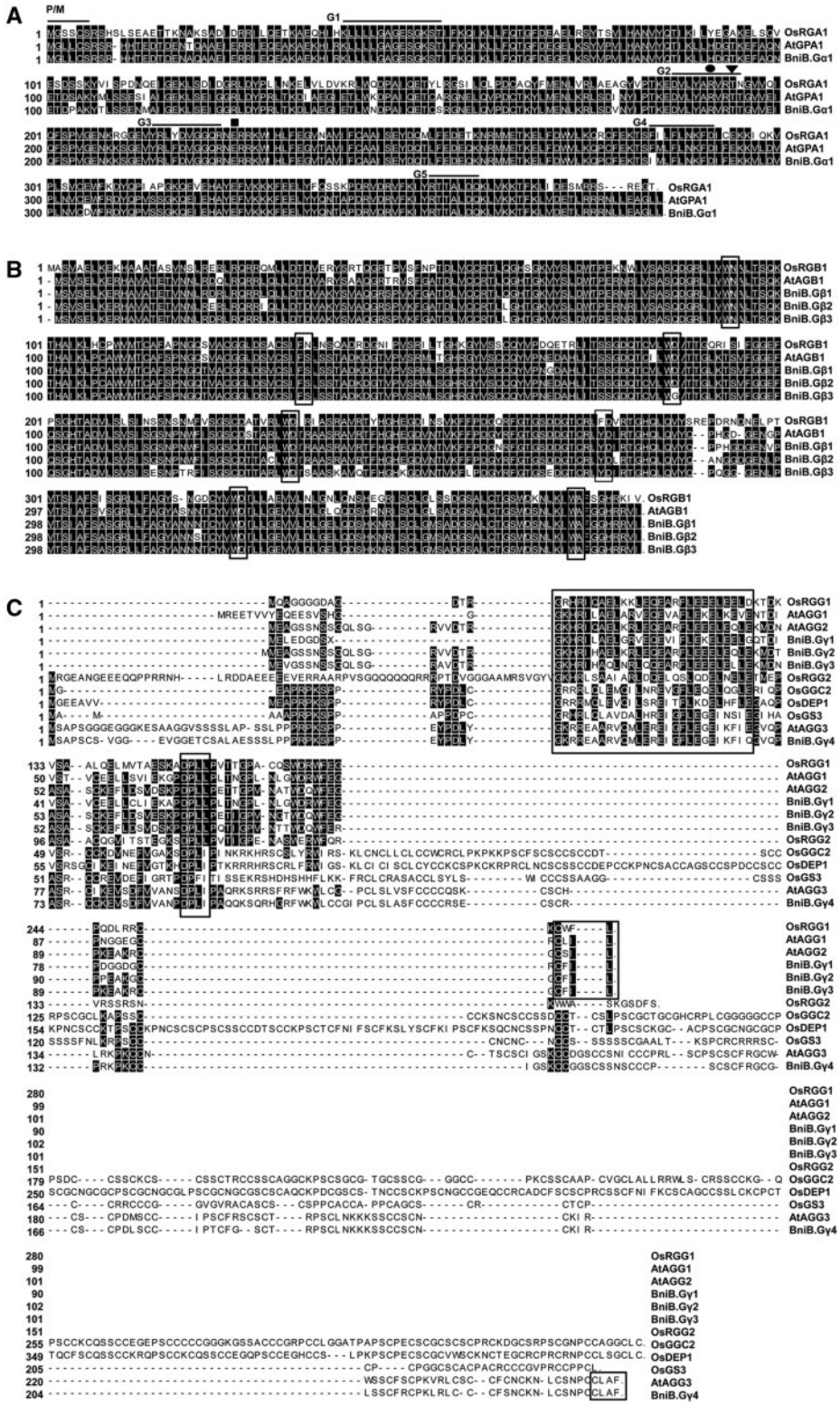

Fig. 1 Amino acid sequence alignment of *B. nigra* G-protein subunits. (A) The sequence alignment of BniB.Gα1 with rice OsRGA1 and Arabidopsis AtGPA1 was performed using Clustal W (http://www.clustal.org). Consensus sequences for GTP binding and hydrolysis are labeled with G1–G5 (Bourne et al. 1991). P/M, the predicted site for N-terminal palmitoylation/myristoylation (MGXXCS); filled circle, the conserved ADP ribosylation (R) site; filled triangle, the critical threonine (T) residue for Gα and RGS-box interaction; filled square, the conserved glutamine (Q) important for the GTPase activity of Gα proteins. (B) Amino acid sequence alignment of three BniB.Gβ proteins with rice OsRGB1 and Arabidopsis AtAGB1. Consensus sequences for the seven WD repeats conserved in Gβ proteins are marked within boxes. (C) Amino acid sequence alignment of four BniB.Gγ proteins with rice OsRGG1, OsRGG2, OsGGC2, OsDEP1 and OsGS3, and Arabidopsis AtAGG1, AtAGG2 and AtAGG3. The conserved coiled-coil motif, DPLL box and the C-terminal prenylation target (CAAX) site are marked within boxes, in series. The conserved and divergent residues are shaded in dark and white backgrounds, respectively.

motif was present. In addition, the C-terminal region of BniB.Gγ4 was very rich in cysteine residues and shared 79.1% similarity with its Arabidopsis prototype AGG3 (**Fig. 1C**, **Table 1**).

The presence of multiple copies of *B. nigra* G-protein subunit proteins, all sharing a high level of sequence identity and domain conservation, led us to estimate their evolution and phylogeny. Phylogenetic analysis of Gα proteins with publicly reported G-protein sequences from other plant species (http://www.phytozome.net) showed that BniB.Gα1 protein is evolutionarily conserved with AtGPA1, as also reflected by their high level of sequence identity and domain conservation (**Fig. 2A**). Upon phylogenetic analysis, it became evident that all three BniB.Gβ subunits also shared the same clade with AtAGB1, and these proteins had evolved from a common Gβ ancestor of the Brassicaceae family (**Fig. 2B**). The relative position and branch length of the three BniB.Gβ subunits in the phylogenetic tree further suggested that the three divergent copies of Gβ subunits could be a consequence of a whole-genome triplication event that occurred after the split of the Arabidopsis and *Brassica* lineages (Mun et al. 2009, Cheng et al. 2013). Phylogenetic analysis of Gγ proteins showed that the type-A BniB.Gγ proteins (BniB.Gγ1–BniB.Gγ3) have evolved separately from the type-C protein (BniB.Gγ4), wherein the BniB.Gγ4 protein grouped together with the recently reported Arabidopsis AtAGG3 and three agronomically important rice Gγ proteins, OsGS3, OsGGC2 and OsDEP1 (**Fig. 2C**). Interestingly, on the phylogenetic tree, AtAGG2 and BniB.Gγ3 (which form a likely a pair of orthologs) was basal to BniB.Gγ2, thereby suggesting the possibility of a primitive Gγ duplication event that pre-dated the split of the Arabidopsis and *Brassica* lineages, and this additional copy might have been lost in the Arabidopsis lineage after diverging from a common diploid ancestor. Nonetheless, a total of eight full-length cDNAs of G-protein were identified in *B. nigra*, with a possibility of 12 Gαβγ combinations, thereby making it a highly diverse G-protein signaling network compared with the model plants Arabidopsis and rice, where only three and five such combinations are possible, respectively.

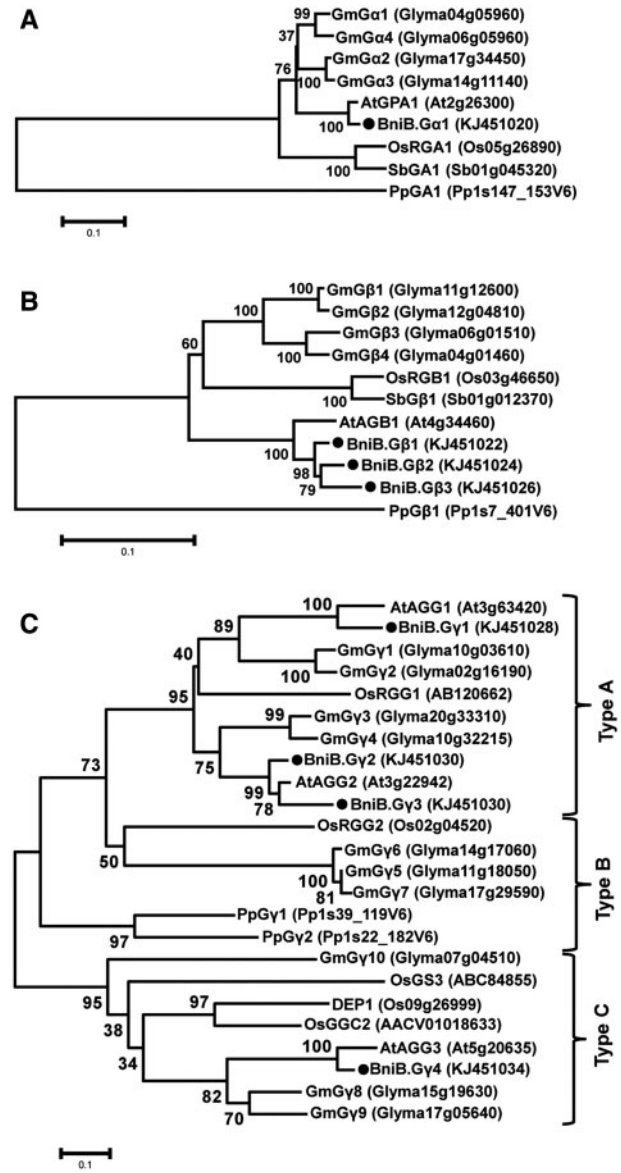

Fig. 2 Evolutionary relationship of *B. nigra* G-proteins. The phylogenetic analysis of (A) Gα, (B) Gβ and (C) Gγ proteins isolated from *B. nigra* (Bni), with their corresponding G-protein subunit proteins in *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Sorghum bicolor* (Sb), *Glycine max* (Glyma) and *Physcomitrella patens* (Pp), was performed using the Neighbor–Joining method in MEGA5.1. The names of the proteins are abbreviated followed by their locus ID or GenBank accession number. The percentage of replicate trees in which the associated proteins clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths measured in terms of the number of substitutions per site.

Expression profiling of *B. nigra* G-protein genes during plant development

The multiplicity of G-protein subunit genes led us to perform their detailed expression analysis across developmental stages of *B. nigra*. To determine the tissue-specific expression pattern of each G-protein gene in different tissue types including root, seedling, leaf, stem, shoot apex and flower, real-time quantitative reverse transcription–PCR (qRT–PCR) analysis was performed using gene-specific primers based on amplified gene sequences (**Supplementary Table S1**) and the expression was compared with reference to the root tissue (set at 1). The efficiency and specificity of gene-specific primer pairs were ascertained using a 10-fold serial dilution of the corresponding plasmid DNA. A linear correlation coefficient (R^2) of >0.99 was observed over a 1,000-fold dilution, which reflected the high efficiency of each primer pair (**Supplementary Table S4**).

All eight members of the G-protein genes were expressed across the different tissue types studied (**Fig. 3**). *BniB.Gα1* was expressed ubiquitously and maintained an almost similar expression pattern across different developing stages of *B. nigra* (**Fig. 3A**). The three *BniB.Gβ* genes were also expressed in all the tissue types tested, although they showed a differential

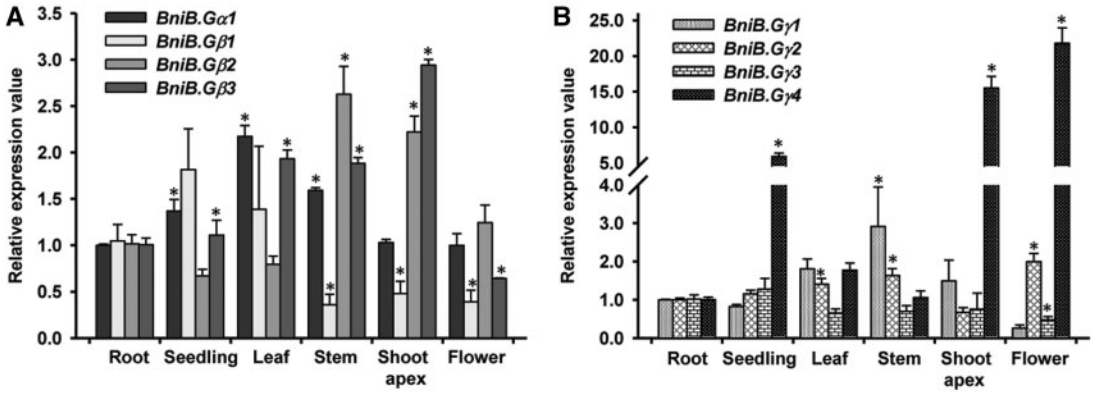

Fig. 3 Expression of G-protein genes at different developmental stages of *B. nigra*. Transcript accumulation was measured for (A) *BniB.Gα1*, *BniB.Gβ1*, *BniB.Gβ2* and *BniB.Gβ3* and (B) *BniB.Gγ1*, *BniB.Gγ2*, *BniB.Gγ3* and *BniB.Gγ4* genes. Total RNA was isolated from root, seedling, leaf, stem, shoot apex and flower; real-time qRT–PCR amplifications were performed for each target gene in triplicate and the data were averaged. The expression value was normalized against the constitutive tonoplastic intrinsic protein-41 (*TIPS-41*) gene expression level, and expression in root was set at 1. Error bars represent the SEM. An asterisk on the top indicates a significant differences at $P < 0.05$ by Fisher's LSD test.

expression pattern across plant developmental stages (**Fig. 3A**). *BniB.Gβ1* showed comparably higher expression in the seedling and young leaf, whereas *BniB.Gβ2* showed relatively higher transcript abundance in the stem, shoot apex and flower tissues. In general, *BniB.Gβ3* showed overall higher transcript abundance in all the tissue types tested, except for the flower where its level was lower. Among the *BniB.Gγ* genes, all three A-subtype genes (*BniB.Gγ1*–*BniB.Gγ3*) showed an almost comparable level of expression across plant development (**Fig. 3B**). The *BniB.Gγ1* transcript was found to be highly expressed in the stem tissue. The expression level of *BniB.Gγ4* (a type-C Gγ gene) was substantially higher in the seedling (approximately 6-fold), shoot apex (approximately 15.5-fold) and flower (approximately 22-fold) compared with the reference tissue, root. Thus, the G-protein genes showed overlapping but distinct tissue-specific expression pattern across different tissue types, thereby suggesting developmental regulation of these genes in *B. nigra*.

Expression of *B. nigra* G-protein genes during seed maturation and germination

Seeds are the most economically important tissue and are associated with both food and feed values of plants. G-proteins are known to play important roles during seed maturation and germination (Perfus-Barbeoch et al. 2004, Urano et al. 2013). We therefore investigated the detailed expression profile of individual G-protein genes using real-time qRT–PCR analysis across developing stages of seed maturation and during seed germination.

All eight G-protein genes were found to be expressed across the seed maturation stages spanning from 7 days post-anthesis (dpa) to 35 dpa in *B. nigra* (**Fig. 4A, B**). The transcript abundance of *BniB.Gα1* was almost constant throughout all stages in the developing seeds, although a significant increase (approximately 4.5-fold) was observed during later stages of seed maturation (35 dpa). Interestingly, among *BniB.Gβ* genes, a significant 8-fold increase in the abundance of the *BniB.Gβ1* transcript could be observed in early stages (14 dpa and 21 dpa) of seed maturation, and its level increased up to 29-fold during later stages of seed maturation (35 dpa), possibly suggesting that *BniB.Gβ1* could play an important role in seed maturation (**Fig. 4A**). However, the abundance of *BniB.Gβ2* and *BniB.Gβ3* transcripts was relatively less and remained almost constant throughout all the seed maturation stages. When the transcript levels of the four *BniB.Gγ* genes were compared during seed maturation stages, a differential expression pattern was observed (**Fig. 4B**). The type-A *BniGγ* genes, namely *BniB.Gγ1*, *BniB.Gγ2* and *BniB.Gγ3*, showed almost similar and constant expression up to 28 d, although their transcript abundance increased during the later phase of seed maturation (35 dpa). Interestingly, the expression level of *BniB.Gγ4*, the only type-C Gγ gene, was significantly increased up to 12-fold during the mid and later stages of seed maturation (21–35 dpa).

We also determined the relative abundance of G-protein genes during seed germination stages in *B. nigra*. Most of the G-protein genes showed increased accumulation in their transcript level during the early time point of seed germination (**Fig. 4C, D**). Within 6 h of seed germination, the expression level of *BniB.Gα1* increased significantly up to 11-fold (**Fig. 4C**), as also reported earlier in Arabidopsis and soybean (Pandey et al. 2006, Bisht et al. 2011), suggesting a key role for Gα protein during seed germination and its putative cross-talk with gibberellic acid signaling across different plant families. Of the three *BniB.Gβ* genes, *BniB.Gβ1* showed comparably higher transcript accumulation during the early time point of seed germination. Among the four *BniB.Gγ* genes, transcript levels of all type-A *Gγ* genes (*BniB.Gγ1*, *BniB.Gγ2* and *BniB.Gγ3*) were found to increase significantly at 6 h of seed germination, whereas a marginal increase in the transcript abundance was observed for *BniB.Gγ4*, a type-C *Gγ* gene, at the early time point of seed germination (**Fig. 4D**).

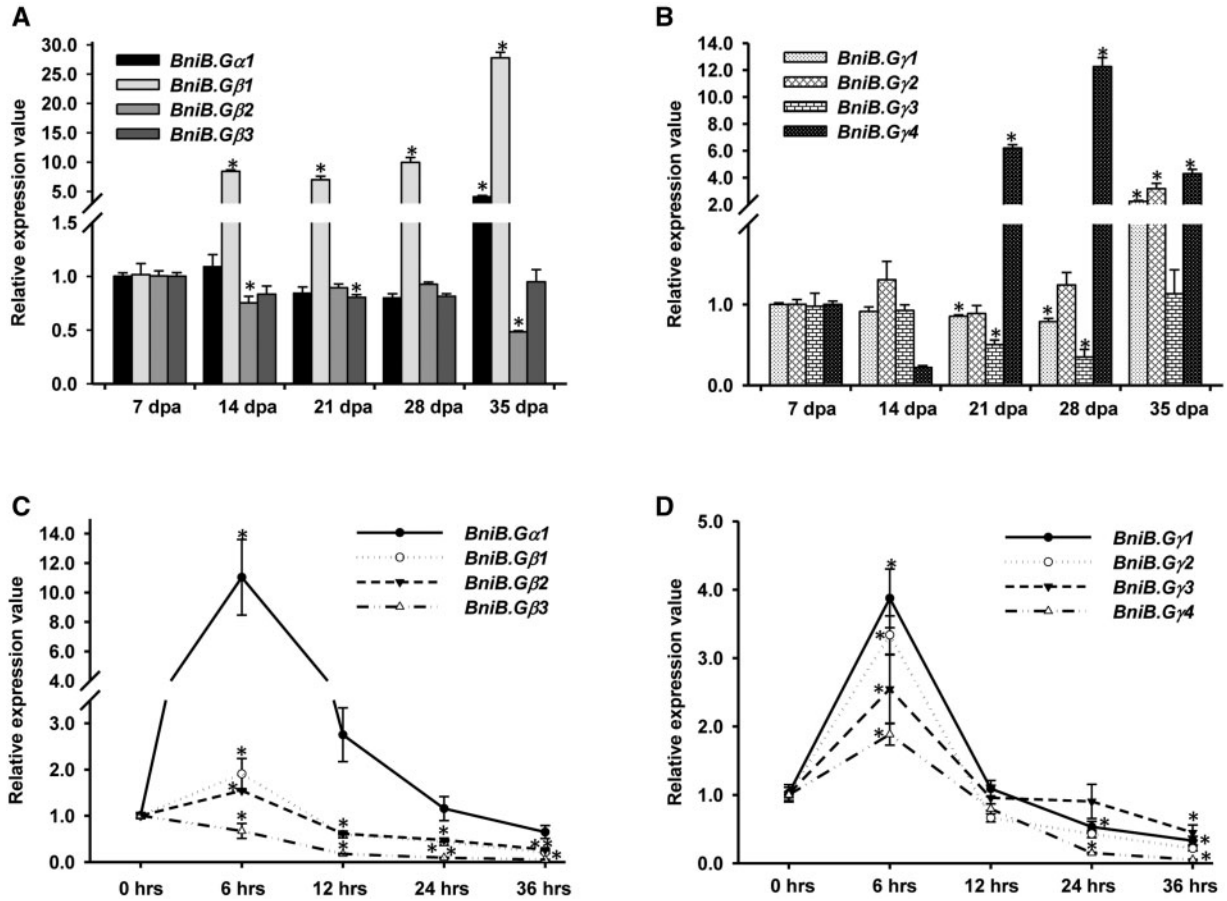

Fig. 4 Expression of G-protein genes during seed maturation and seed germination stages of *B. nigra*. The transcript accumulation was measured for (A, C) *BniB.Gα1*, *BniB.Gβ1*, *BniB.Gβ2* and *BniB.Gβ3*; and (B, D) *BniB.Gγ1*, *BniB.Gγ2*, *BniB.Gγ3* and *BniB.Gγ4* genes. In brief, total RNA was isolated from maturing seed stages (7, 14, 21, 28 and 35 dpa) and seed germinating stages (6, 12, 24 and 36 h after seed imbibition). Real-time qRT–PCR amplifications were performed for each target gene in triplicate and the data were averaged. The expression value was normalized against the constitutive *GAPDH* gene expression level. Expression in (A, B) 7 dpa seeds and (C, D) 0 h (unimbibed) seeds was set at 1. Error bars represent the SEM. An asterisk on the top indicates significant differences at $P < 0.05$ by Fisher's LSD test.

Expression of *B. nigra* G-protein genes in response to phytohormones and stress conditions

Various studies in the model plant Arabidopsis have shown that G-protein genes exhibited stress and phytohormone responsiveness. In an attempt to investigate the transcriptional regulation of *B. nigra* G-protein genes, their expression profiling was performed in 5-day-old seedlings treated with various phytohormones and elicitors mimicking biotic and abiotic stresses, during early (30 min), mid (3 h) and late (6 h) time points. Untreated seedlings harvested at each time point were used as the respective control. The expression of *Brassica* homologs of marker genes, which are commonly used as the standard for various hormones and stress conditions, was checked to validate the treatment-induced expression of *B. nigra* G-protein subunit genes (**Supplementary Fig. S4**, **Supplementary Table S5**).

The G-protein genes of *B. nigra* were altered differentially in response to the various phytohormone treatments (**Fig. 5**). In general, the expression of most of the *B. nigra* G-protein genes was unaltered or showed a significant down-regulation during early time points (30 min and 3 h) of phytohormone treatments, except for *BniB.Gγ3* which was induced within 3 h of brassinosteroid (BR) treatment (**Fig. 5A**). However, *B. nigra* G-protein genes were up-regulated during the later time point (6 h) of phytohormone treatments, although showing differential accumulation of their transcripts. For example, the level of the *BniB.Gα1* transcript increased in response to IAA and 6-benzylaminopurine (BAP) treatments after 6 h, whereas other hormones did not alter the expression of the *BniB.Gα1* gene. Among *BniB.Gβ* genes, the transcript levels of *BniB.Gβ1* and *BniB.Gβ2* were up-regulated after 6 h of gibberellic acid, ABA and BR treatments, whereas the abundance of the *BniB.Gβ3* transcript was increased only in response to IAA, even after 30 min of treatment. Similarly, among *BniB.Gγ* genes, *BniB.Gγ1* showed increased transcript accumulation after 6 h of 1-amino-cyclopropane-1-carboxylic acid (ACC) treatment, whereas the *BniB.Gγ3* transcript was induced in response to IAA, ACC and BR treatments (**Fig. 5A**). The transcripts of both *BniB.Gγ2* and *BniB.Gγ4* did not showed any induction in response to phytohormone treatments.

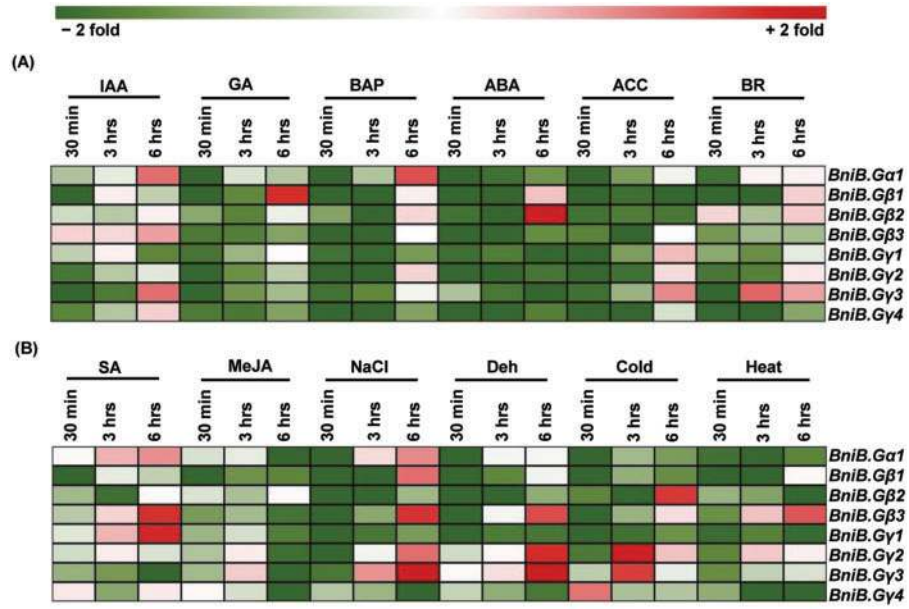

Fig. 5 Expression of *B. nigra* G-protein under various treatments. Heat map depicting (A) the effect of various hormone treatments including 100 μM IAA, 100 μM gibberellin, 100 μM BAP, 100 μM ABA, 100 μM ACC and 1 μM BR; and (B) the effect of various elicitors and abiotic stress conditions including 100 μM SA, 100 μM MeJA, 100 mM NaCl, dehydration (Deh) on the bench top, cold (4°C) and heat (42°C), on expression of *B. nigra* G-protein genes. Real-time qRT–PCR for *B. nigra* G-protein genes was conducted at three time points 30 min, 3 h and 6 h in triplicate and the data were averaged. The color bar indicates the intensity of genes showing up-regulation (≥2 fold; red), down-regulation (≤2 fold; green) and unaltered response (white). Results were normalized against the constitutive *Ubiquitin* (*UBQ*) gene expression level, and compared with the expression of G-protein genes in untreated seedlings at each time point.

The *BniB.Gα1*, *BniB.Gβ3* and *BniB.Gγ1* showed an almost similar increase in their transcript levels after 3 and 6 h of salicylic acid (SA) treatment, which mimics the biotic stress condition, thereby suggesting the possibility of condition-specific G-protein heterotrimeric interaction (**Fig. 5B**). In general, the G-protein genes were down-regulated upon methyl jasmonate (MeJA) treatment. However, in response to abiotic stress conditions, the *BniB.Gβ* and *BniB.Gγ* genes were up-regulated, although showing differential transcription regulation. For example, among *BniB.Gβ* genes, the transcript levels of *BniB.Gβ1* and *BniB.Gβ2* genes were increased in response to salt and cold treatment respectively, whereas the *BniB.Gβ3* transcript showed increased accumulation in response to salt, dehydration and heat treatments. Similarly, among *BniB.Gγ* genes, *BniB.Gγ2* and *BniB.Gγ3* showed increased transcript accumulation in response to salt, dehydration and cold treatments, whereas *BniB.Gγ1* and *BniB.Gγ4* transcripts did not show any induction in response to any of the abiotic stress conditions. All the G-protein subunit genes, except for *BniB.Gγ4*, showed up-regulation of their transcript in response to salt (NaCl) treatment.

Protein–protein interaction between *B. nigra* G-protein subunits

The presence of multiple members of G-protein subunits in *B. nigra* led us to investigate the strength and specificity of interactions that could exist between the different G-protein subunit proteins. In order to check the interaction between the canonical BniB.Gα1 and three BniB.Gβ subunits, a mating-based yeast split ubiquitin system (mbSUS) assay was performed wherein BniB.Gα1 was used as prey protein and BniB.Gβs as bait protein. The growth pattern of mated yeast cells in the mbSUS assay clearly showed that BniB.Gα1 interacted strongly with BniB.Gβ1 and BniB.Gβ3 compared with BniB.Gβ2. BniB.Gβ2, in general, showed a weak interaction with BniB.Gα1 when tested at different concentrations of methionine (**Fig. 6A**).

To determine the interaction between BniB.Gβ and BniB.Gβ subunit proteins, a yeast two-hybrid (Y2H) assay was carried out using BniB.Gβs as bait proteins and BniB.Gγs as prey proteins (**Fig. 6B**). The growth-based experiment clearly suggested that all the BniB.Gβ and BniB.Gγ subunit proteins were expressed, and showed a high degree of interaction selectivity among them, when tested utilizing different concentrations (5–100 mM) of 3-amino-1,2,4-triazole (3AT), a His3p inhibitor (**Supplementary Fig. S5**). To quantify the strength of these interactions, a quantitative β-galactosidase assay was performed, which also confirmed that BniB.Gγ1 protein interacted very strongly with both BniB.Gβ1 and BniB.Gβ2 proteins compared withBniB.Gβ3 protein which showed a very weak interaction. The other two type-A Gγ proteins, i.e. BniB.Gγ2 and BniB.Gγ3, showed very weak interaction with all three BniB.Gβ proteins under the optimal conditions used in the current study. In contrast, BniB.Gγ4 protein, the type-C Gγ protein, interacted with all three BniB.Gβ proteins, and showed a relatively higher strength of interaction with BniB.Gβ1 protein.

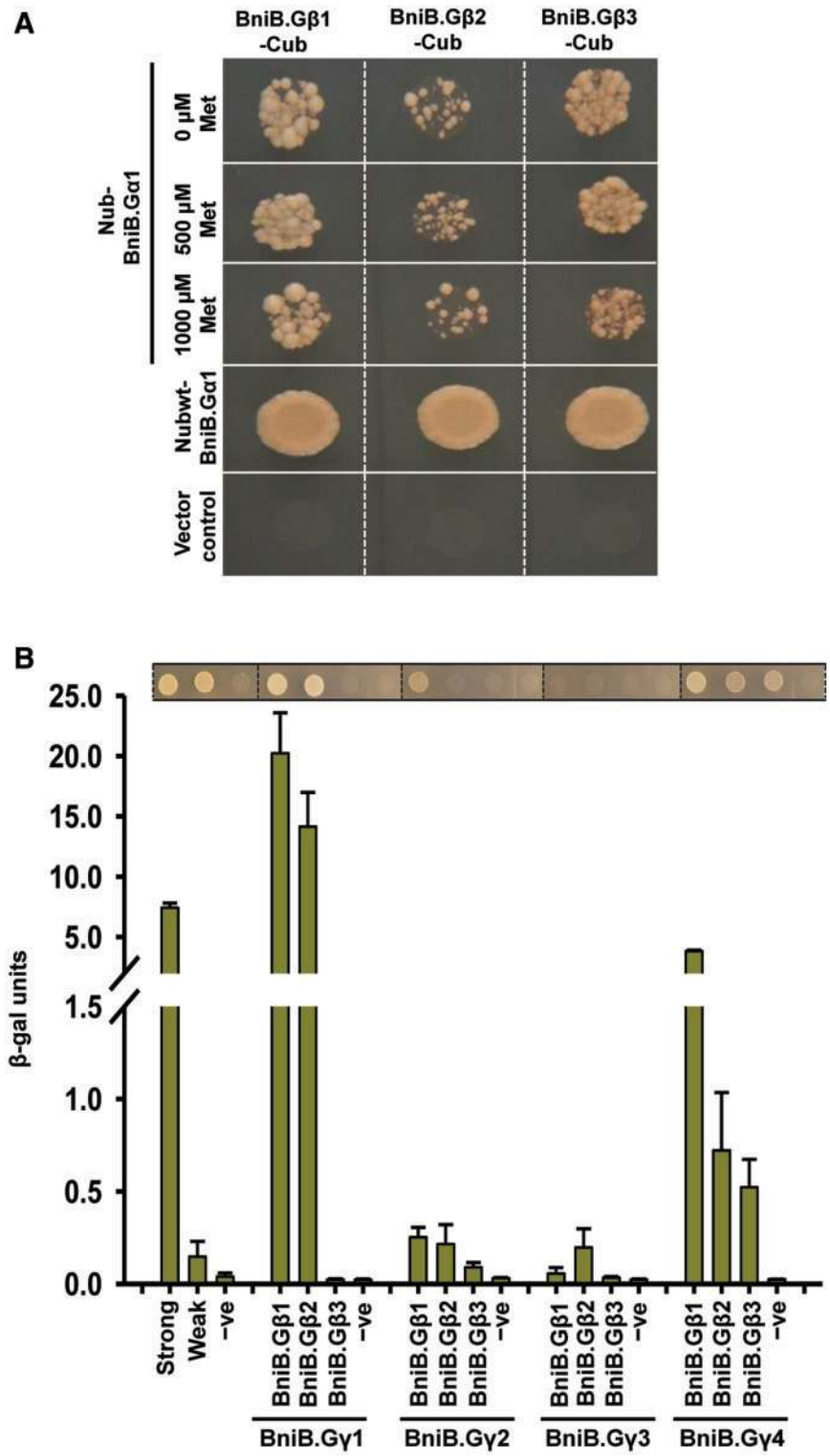

Fig. 6 Interaction of *B. nigra* G-protein subunits. (A) Interaction between BniB.Gα and BniB.Gβ subunits using the mating-based split ubiquitin system (mbSUS). BniB.Gα1 was cloned as a Nub (N-terminal half of ubiquitin) fusion, and Gβ subunits were cloned as a Cub (C-terminal half of ubiquitin) fusion, and interaction was determined by the growth ability of mated yeast on selective medium (−AHLT) supplemented with 0, 500 and 1,000 μM methionine. The NubWT−Gα fusion and NubG vector were used as positive and negative controls, respectively. Two biological replicates of the experiment were performed with identical results. (B) Interaction between BniB.Gβ and BniB.Gγ subunits using a yeast two-hybrid interaction screen. The interaction between BniB.Gβ bait protein (in pDEST32) and BniB.Gγ prey proteins (in pDEST22) was determined by performing colorimetric β-galactosidase and yeast growth-based assays. Inset: growth of yeast colonies on minimal medium lacking histidine, leucine and tryptophan (−HLT) but containing 50 mM 3AT. Strong, weak and negative refer to the interaction strength of Krev1-pDEST22 with RalGDS-wt-pDEST32, RalGDS-m1-pDEST32 and RalGDS-m2-pDEST32, respectively. Two biological replicates of the experiment were performed with identical results, and the data were averaged with error bars.

Thus, both yeast growth and quantitative β-galactosidase assays confirmed a high degree of interaction selectivity among the *B. nigra* G-protein subunit proteins.

Discussion

Heterotrimeric G-proteins are considered as one of the key signal transducers which communicate extracellular signals to activate various intracellular changes that lead to different cell behaviors. In plants, members of the G-protein family are involved in various growth and developmental process such as seed germination and maturation, stomatal opening and closure, plant defense, oxidative stress and light response (Urano et al. 2013). Whole-genome sequencing of various plant species clearly rules out the possibility of having a simple repertoire of heterotrimeric G-protein components as present in the model plants Arabidopsis and rice, but shows them to be encoded as a multigene family particularly in polyploid plants.

Gγ subunits are highly divergent among the *B. nigra* G-protein family

In this study we have identified a total of eight genes encoding one Gα (*BniB.Gα1*), three Gβ (*BniB.Gβ1*, *BniB.Gβ2* and *BniB.Gβ3*) and four Gγ (*BniB.Gγ1*, *BniB.Gγ2*, *BniB.Gγ3* and *BniB.Gγ4*) subunits from *B. nigra* (Brassica B-genome), with the possibility of 12 Gαβγ combinations, thus making it one of the most diverse G-protein families reported from the plant kingdom to date. Amino acid sequence comparison of BniB.Gα, BniB.Gβ and BniB.Gγ subunits with other plant G-proteins confirmed the presence of all three characteristic domains (**Fig. 1A–C**), thereby suggesting that the G-protein signaling is evolutionarily conserved across the plant kingdom. The canonical BniB.Gα1 protein retained a significantly high level of sequence conservation (approximately 97% identity) with Arabidopsis AtGPA1, as also reflected by their close phylogenetic relationship (**Fig. 2**), which probably suggests functional conservation of Gβ proteins within the Brassicaceae lineage during evolution. Similarly, the three BniB.Gβs were also highly conserved proteins (>90% identical), containing all the necessary Gβ signature sequences. The phylogenetic positions of BniB.Gβ proteins further suggest that BniB.Gβ1 is evolutionarily closer to Arabidopsis AtAGB1, whereas BniB.Gβ2 and BniB.Gβ3 proteins have originated from subsequent rounds of a genome duplication event of BniB.Gβ1. It is quite possible that the sequence divergence observed among BniB.Gγ proteins, although small, might have some consequences on controlling their functional specificity and/or physical interaction with other G-protein subunits and also the accessory proteins.

Amino acid sequence comparison shows that BniB.Gγ subunits are the most divergent subunits among the *B. nigra* G-protein family. Phylogenetic analysis further confirmed that the highly divergent BniB.Gγ proteins have evolved independently from a common ancestor. Recently, Trusov et al. (2012) classified the plant Gγ subunits into three distinct classes: type-A class containing Gγ subunits having a prenylation motif at their C-terminal end; type-B class lacking a prenylation motif and cysteine residues at the C-terminus; and type-C class containing an extended C-terminal domain rich in cysteine residues. Based on this classification, three of the *B. nigra* Gγ proteins, i.e. BniB.Gγ1, BniB.Gγ2 and BniB.Gγ3, fall into the type-A category, whereas only BniB.Gγ4 falls into the type-C category. BniB.Gγ1 is the closest ortholog of Arabidopsis AtAGG1; BniB.Gγ2 and BniB.Gγ3 are homologs of Arabidopsis AtAGG2, whereas the highly divergent BniB.Gγ4 (a type-C Gγ protein) paired with Arabidopsis AtAGG3. No representative of type-B Gγ subunits is found in *B. nigra*; these were also found to be absent in Arabidopsis and *B. rapa* genomes (Trusov et al. 2012), thereby confirming that the type-B genes must have been lost in the ancestral Brassicaceae.

We observed that the repertoire of the G-protein family in *B. nigra* is almost similar to that of the closest *Brassica* diploid, *B. rapa*, where nine genes encoding one Gα, three Gβ and five Gγ subunits could be amplified (our unpublished data). It is interesting to note that although whole-genome triplication events have proportionally increased the inventory of the Gβ subunit, such multiplicity was not observed for Gα and Gγ proteins across all the diploid *Brassica* species including *B. nigra* (B-genome; the current study), *B. rapa* (A-genome; http://www.brassicadb.org) and *B. oleracea* (C-genome; http://www.brassicadb.org). It is likely that some of the duplicated copies of genes encoding Gα and Gγ proteins might have been lost because of gene fractionation and genomic rearrangement events that have occurred during the evolution of extant diploid *Brassica* species (Cheng et al. 2012). Interestingly, during the evolution of *Brassica* species, genes belonging to signal transduction and transcription factors were found to be lost significantly compared with genes involved in other biological functions (Town et al. 2006). Nonetheless, the presence of multiple members of G-protein subunits, with the exception of Gα, indicates that G-protein signaling networks are more diverse and complex across all the diploid *Brassica* species, including *B. nigra*, compared with that present in Arabidopsis.

B. nigra G-protein genes are developmentally regulated and show specific co-expression patterns

The *B. nigra* G-protein genes are ubiquitously expressed in various tissue types, indicating that they may play important roles during plant growth and development. Although the canonical *BniB.Gα1* has retained an almost constant expression profile across different tissue types, the multiple members of the *BniB.Gβ* and *BniB.Gγ* subunit genes exhibit a differential spatio-temporal expression pattern across plant developmental stages (**Figs. 3**, **4**). The expression differentiation or expression subfunctionalization of multiple *BniB.Gβ* and *BniB.Gγ* subunit genes which evolved as a result of whole-genome triplication events across *Brassica* species could be quite important for directing the functional selectivity of G-protein heterodimers and heterotrimers in a tissue- or cell-specific manner across plant growth and development (**Fig. 7**). Such a tissue-specific

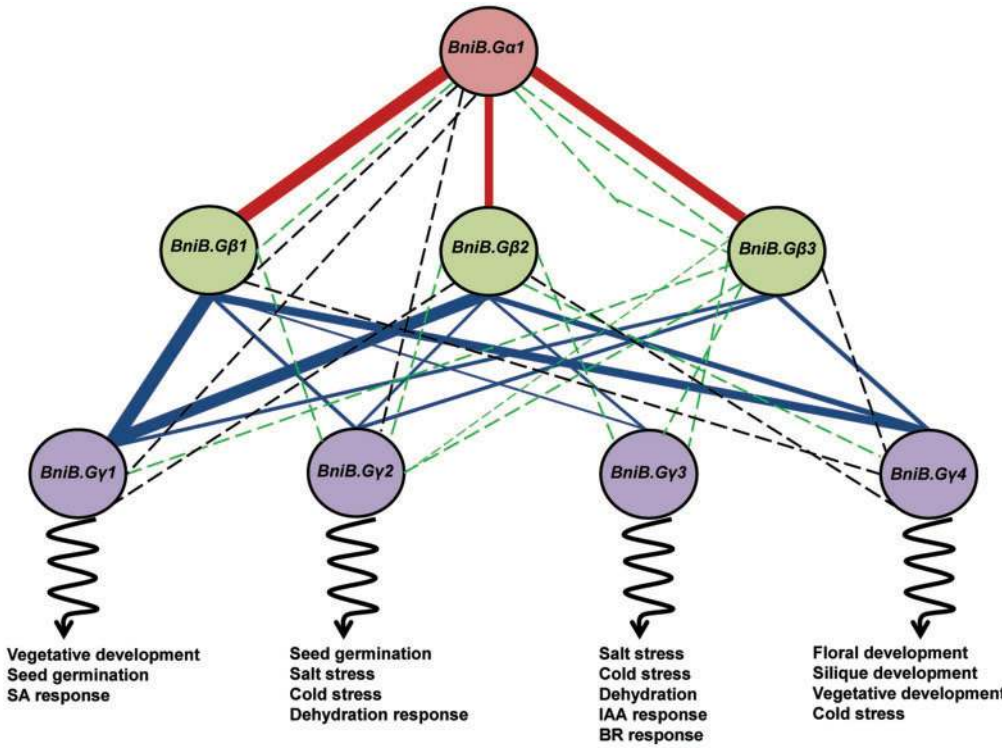

Fig. 7 Proposed model of G-protein signaling complexity in *B. nigra*. The model is based on in-depth expression and physical interaction analysis performed on eight *B. nigra* G-protein subunits. Black dotted lines indicate genes co-expressed during plant growth and development, whereas green dotted lines indicate genes co-expressed under treatment with specific elicitors (phytohormones and stress conditions). Solid lines indicate the physical interaction determined using yeast-based interaction assays, wherein the thickness of the line estimates the strength of interaction. Interactions between Gα and Gβ subunits are shown in red, whereas Gβ and Gγ interactions are shown in blue. The putative responses governed by different G-protein heterodimers and heterotrimers are given underneath.

expression pattern of G-protein genes has also been reported from the polyploid soybean, which contains multiple members of each G-protein subunit (Bisht et al. 2011, Choudhury et al. 2011).

It is interesting to note that although multiple homologs of G-protein subunit have retained a differential expression pattern across plant development, specific combination(s) of G-protein subunit genes are co-expressed in different tissue types of *B. nigra*. For example, the expression of canonical *BniB.Gα1* increases significantly during the later phase of seed maturation (35 dpa), when seed starts to dry, a process highly regulated by ABA (Pandey et al. 2006). The *BniB.Gβ1*, *BniB.Gγ1*, *BniB.Gγ2* and *BniB.Gγ4* (a type-C Gγ gene) genes also show similar transcript up-regulation during the later stages of seed maturation. The overlapping expression profiles of these subunits suggest the co-ordinated role of the BniB.G$\beta_1\gamma_4$ heterodimer during flower and silique/seed development (**Fig. 7**). Genetic evidence in Arabidopsis and rice also shows that the type-C *Gγ* genes are preferentially involved in flower development and seed maturation (Chakravorty et al. 2011, Botella 2012). The G-protein genes are also known to be involved in seed germination in Arabidopsis and soybean (Chen et al. 2004, Pandey et al. 2006, Bisht et al. 2011). In the current study, co-ordinated expression patterns of specific G-protein subunit genes are also observed during the early phases of seed germination (**Fig. 4C, D**), as well as during vegetative stages including leaf and stem tissues (**Fig. 3**). Thus differential expression observed within multiple homologs of each G-protein subunit gene coupled with the co-ordinated expression pattern of different G-protein subunits across plant developmental stages is likely to suggest that *B. nigra* G-protein genes are developmentally regulated to shape the formation of cell- and tissue-specific G-protein functional combination(s) (**Fig. 7**).

Transcriptional regulation of *B. nigra* G-proteins in response to phytohormones and stress treatments

The phenotypes of loss- and gain-of-function mutants of G-protein components leave no doubt that G-proteins cross-talk with numerous signals to regulate various types of cell behavior. Earlier reports in the model plants Arabidopsis and rice have demonstrated the involvement of G-proteins in response to multiple plant hormones, elicitors (e.g. glucose and light), and abiotic and biotic factors (reviewed in Jones and Assmann 2004, Urano et al. 2013). The expression of *B. nigra* G-protein genes is differentially regulated in response to various phytohormones and stress conditions (**Fig. 5**), thereby suggesting differential involvement of G-protein subunit members in response to various environmental cues. In general, abiotic stress conditions including salinity, dehydration and temperature stress greatly enhance the expression of specific

members of *BniB.Gβ* and *BniB.Gγ* genes (**Fig. 5B**). Misra et al. (2007) had also reported the involvement of pea *Gβ* genes under salinity and high temperature conditions.

Previous evidence obtained from Arabidopsis and rice has established that G-proteins cross-talk with various plant hormone signaling pathways. The Arabidopsis *Gβ* and *Gγ* genes are known to be involved in auxin-induced cell division and transport in roots, thereby affecting multiple developmental processes (Ullah et al. 2003, Trusov et al. 2007). In *B. nigra*, we found that all the types of heterotrimer subunit, i.e. *BniB.Gα1*, *BniB.Gβ3*, *BniB.Gγ3* and *BniB.Gγ4*, are up-regulated co-ordinately under IAA treatment (**Fig. 5**), thereby suggesting that BniB.G$\alpha_1\beta_3\gamma_{3,4}$ or BniB.G$\beta_3\gamma_{3,4}$ are functional G-protein combinations in response to auxin signaling (**Fig. 7**). Similarly, transcripts of *BniB.Gβ1*, *BniB.Gβ2* and *BniB.Gγ3* show co-ordinated induction during later time points of BR treatment, suggesting their putative cross-talk with BR signaling pathways (Tsugama et al. 2013; **Fig. 7**). However, in response to gibberellic acid treatment, only the transcript of *BniB.Gβ1* was induced, thereby showing the involvement of the said gene in gibberellic acid signaling, independently of the Gα subunit (Ueguchi-Tanaka et al. 2000).

It is interesting to note that members of G-protein subunits have somewhat overlapping and similar responses under specific treatments, as also observed across different developmental stages of *B. nigra*. For example, treatment with exogenously supplied SA, which is an inducer of systemic acquired resistance (SAR), shows an almost similar induction of the level of *BniB.Gα1*, *BniB.Gβ3* and *BniB.Gγ1* transcripts, thus suggesting that these subunits could possibly form a functional Gαβγ heterotrimer (BniB.G$\alpha_1\beta_3\gamma_1$) in plant defense (**Fig. 7**). Previously, Llorenete et al. (2005) and Trusov et al. (2006) also showed the involvement of the Gβγ dimer in plant defense in Arabidopsis. Under salinity stress, the expression of *BniB.Gα1*, *BniB.Gβ1*, *BniB.Gβ3*, *BniB.Gγ2* and *BniB.Gγ3* transcripts is induced, which is somewhat similar to what was observed for dehydration. This kind of co-ordinated up-regulation of specific G-protein subunits was also observed in response to multiple plant hormones, including IAA, BAP, ACC and BR. Such co-ordinated transcriptional regulation of G-protein subunits under different elicitor treatments probably suggests the involvement of condition-specific Gαβγ combination(s) and its interaction with other plant signaling pathways in *B. nigra* (**Fig. 7**). Nonetheless, the expression data clearly suggested that the functional selectivity of G-protein heterotrimers in *B. nigra* is largely controlled by the differential expression of multiple *BniB.Gβ* and *BniB.Gγ* genes during plant development and in a condition-specific manner. Even in the model plant Arabidopsis, wherein a canonical Gα and Gβ proteins are present, the functional selectivity is reported to be under the tight control of highly divergent Gγ proteins (Trusov et al. 2007).

Interaction specificity of *B. nigra* G-protein subunits

In metazoans, the intra- and intersubunit specificities of multiple G-protein subunits and their regulatory components are known to control diverse signaling processes (McCudden et al. 2005). Elaboration of G-protein components in the polyploid crop soybean has recently revealed the interaction complexity in plants (Bisht et al. 2011, Choudhury et al. 2011). The presence of multiple G-protein subunits with a distinct and overlapping expression profile led us to investigate the interaction specificity that exists in *B. nigra*. Although, *B. nigra* G-protein subunits interacted with each other in most of the combinations, a high degree of interaction specificity was observed between members of G-protein subunits. The canonical BniB.Gα1 protein shows a difference in its interaction with BniB.Gβ proteins (**Fig. 6A**), which could be attributed to the divergent residues present across BniB.Gβ proteins (**Fig. 1B**).

A number of studies have suggested that amino acid residues in Gβ play a crucial role in the physical interaction with effectors in Gβγ dimer-mediated signaling both in animals and in plants (Ford et al. 1998, Temple and Jones 2007, Chakravorty et al. 2011). In addition, Gγ subunits, which direct the localization of the Gβγ dimer into the cell membrane, are known to control the functional selectivity of G-protein heterodimers in the model plants Arabidopsis and rice (Trusov et al. 2007). The existence of multiple Gβ and Gγ subunits with divergent sequences can easily provide specificity for the multiple signaling pathways mediated by the Gβγ dimer in the paleopolyploid *B. nigra*. Notably, a high level of interaction specificity was also seen between members of BniB.Gβ and BniB.Gγ subunits (**Fig. 6B**; **Supplementary Fig. S5**). Both BniB.Gγ1 (a type-A Gγ protein) and BniB.Gγ4 (a type-C Gγ protein) showed stronger interaction with BniB.Gβs. In general, BniB.Gβ3 shows weak interaction with each of the BniB.Gγ proteins tested, thus indicating another level of interaction specificity across Gβγ dimers. We presume that the replacement of aspartate with glycine in the third WD domain of the BniB.Gβ3 protein (**Fig. 1B**) could have quite important consequences on directing this Gβ–Gγ interaction specificity, although a role for other divergent residues cannot be completely ruled out. Thus identifying important residues which direct the interaction specificity among divergent G-protein subunits could be undertaken in the near future to investigate the functional selectivity of multiple G-protein genes across polyploid *Brassica* species.

Taken together, we have identified a diverse G-protein family in *B. nigra*, with the possibility of 12 Gαβγ combinations. This study described the molecular basis for G-protein signal selectivity by investigating the in-depth expression profile as well as physical interaction analysis of multiple G-protein subunit genes in *B. nigra*. Our results show that specificity is provided both by spatio-temporal differences in expression patterns of *BniB.Gβ* and *BniB.Gγ* subunit genes, as well as by non-conserved amino acid residues in Gβ and Gγ proteins. Our data on physical interaction coupled with the co-expression pattern of the multiple G-protein subunit genes suggest the presence of tissue- and condition-specific functional Gαβγ combinations in *B. nigra* (**Fig. 7**). Detailed characterization of G-protein genes could be carried out to understand the consequences of whole-genome triplication events on controlling the functional diversity of such a complex G-protein signaling

network in paleopolyploid *B. nigra*. Nonetheless, identification of the G-protein inventory from *B. nigra* (B-genome) and other diploid *Brassica* species will also help us to understand the complexity of the G-protein signaling network in the agriculturally important allopolyploid species, *B. napus* and *B. juncea*.

Materials and Methods

Plant material and growth conditions

Black mustard (*B. nigra* L. cv. IC257) was grown under controlled growth conditions under a day (24°C; 10 h; approximately 300 μmol m^{-2} s^{-1}) and night (18°C; 14 h) photoperiod with 60% relative humidity. Different developmental tissues including young leaf, root, stem, shoot apical bud, flower and seeds from different stages of developing siliques (from 7 to 35 dpa) were collected, frozen immediately in liquid nitrogen and stored at −80°C. For germination experiments, seeds were imbibed on germination paper for 0 to 36 h and the germinated seedlings were collected, snap-frozen in liquid nitrogen and stored at −80°C.

Amplification and cloning of *B. nigra* G-protein subunit genes

In order to amplify the full-length coding DNA sequence of *B. nigra* G-protein subunit genes, degenerate primers were designed based on the G-protein sequences reported earlier from Arabidopsis and *Brassica* species (**Supplementary Table S1**). PCR was performed from cDNA samples of different tissue types as template, following standard amplification conditions and at an annealing temperature of 55°C (30 s). PCR products were resolved on 1% agarose gels, purified with a QIAquick Gel Extraction Kit (Qiagen), cloned into the pENTR/D-TOPO vector (Invitrogen) and sequenced to confirm the correctness of the coding sequence.

Total RNA extraction, cDNA synthesis and real-time qRT–PCR

Total RNA was isolated from approximately 100 mg of plant material using a Spectrum™ Plant Total RNA isolation kit according to the manufacturer's instructions (Sigma-Aldrich), and the quality and concentration of RNA were determined in a Nanodrop (ND-1000, Thermo Scientific). Approximately 2 μg of total RNA was used to synthesize the cDNA with both oligo(dT) and random hexamer primers (1 : 1 mix) using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions. All the cDNA samples were diluted 1 : 50 in nuclease-free water, and real-time qRT–PCRs were performed using gene-specific primers (**Supplementary Table S1**) and SYBR green PCR mix in a 7900 HT fast Real-Time PCR Detection System (Applied Biosystem) at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. *Brassica nigra* glyceraldehyde phosphate dehydrogenase (*GAPDH*), ubiquitin (*UBQ*) and tonoplastic intrinsic protein-41 (*TIP5*) genes were used as the internal control to normalize the expression data (Chandna et al. 2012).

The specificity of the reaction was verified by agarose gel electrophoresis and melting curve analysis of amplified products using qRT–PCR. Relative changes in expression value were calculated using the comparative CT method, where fold change is calculated as $2^{-\Delta\Delta CT}$. All qRT–PCRs were performed in at least three independent biological replicates. Statistical analyses were conducted by one-way analysis of variance (ANOVA) using Fisher's least significant difference (LSD) test.

Phytohormone and stress treatment

Seeds were surface sterilized, placed on 0.5× Murashige and Skoog (MS) medium containing 3% (w/v) sucrose and 0.8% (w/v) agar, and germinated for 4 d under dark and light conditions. Seedlings were then transferred and adapted into 0.5× liquid MS medium for 24 h before feeding with 100 μM IAA, 100 μM gibberellic acid, 100 μM BAP, 100 μM ABA, 100 μM ACC, 1 μM BR, 100 μM SA, 100 μM MeJA, 100 mM NaCl, cold (4°C), heat (42°C) and dehydration, each for 30 min, 3 h and 6 h, as described earlier (Chandna et al. 2012). The expression of *Brassica* homologs of commonly used marker genes for various phytohormones, elicitors and abiotic stress treatments was tested to validate the treatment conditions (**Supplementary Table S5**).

Protein–protein interaction assays

The interaction between *B. nigra* Gα and Gβ subunits was determined using the mbSUS assay (Obrdlik et al. 2004). *In vivo* cloning of full-length Gα and Gβ fragments into mbSUS vectors was carried out. *BniB.Gβ* genes were cloned into pMetYCgate (bait vector) and then transformed into the yeast haploid strain THY.AP4 (MATa). Similarly, the *BniB.Gα* gene was cloned into pNXgate32-3HA (prey vector) and then transformed into THY.AP5 (MATα). Interaction was finally determined by the growth of diploid yeast colonies on minimal media lacking adenine, histidine, leucine and tryptophan (−AHLT) but containing 0, 500 and 1,000 μM methionine.

The interaction between *B. nigra* Gβ and Gγ proteins was determined using Y2H assays (Proquest™ Two-Hybrid System, Invitrogen). Bait (Gβ subunits) and prey (Gγ subunits) constructs developed earlier in the pENTR/D-TOPO vector were mobilized into the pDEST32 vector (having a DNA-binding domain) and pDEST22 (having a DNA-activating domain), respectively. Both bait and prey constructs were co-transformed into yeast strain MaV203 according to the manufacturer's instructions. Colonies were inoculated into liquid minimal medium lacking leucine and tryptophan (−LT) and incubated overnight at 30°C. Cultures grown overnight were equalized (OD_{600} = 0.6) and 10 μl of culture was placed dropwise on the appropriate selection medium. Interaction was determined by the growth of diploid yeast colonies on minimal media lacking histidine, leucine and tryptophan (−HLT) but supplemented with different concentrations (5, 10, 25, 50, 75 and 100 mM) of 3AT), an inhibitor of the HIS3 gene product. In addition, the strength of interaction was quantified by β-galactosidase expression assay by measuring the hydrolysis of the chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG).

Phylogenetic analysis of *B. nigra* G-protein subunit proteins

To study the evolutionary relationships, amino acid sequences of G-protein subunits deduced from *B. nigra*, and those reported from Arabidopsis, *Oryza sativa*, *Sorghum bicolor*, *Glycine max* and *Physcomitrella patens* were used. The Phylogenetic tree was constructed using the Neighbor–Joining method in MEGA5.1 (Tamura et al. 2011) with 1,000 bootstrap replicates.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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