Duplicated RGS (Regulator of G-protein signaling) proteins exhibit conserved biochemical but differential transcriptional regulation of heterotrimeric G-protein signaling in *Brassica* species

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G-alpha (G\(\alpha\)) and ‘Regulator of G-protein Signaling (RGS)’ proteins are the two key components primarily involved in regulation of heterotrimeric G-proteins signaling across phyla. Unlike *Arabidopsis thaliana*, our knowledge about G-protein regulation in polyploid *Brassica* species is sparse. In this study, we identified one G\(\alpha\) and two RGS genes each from three species of *Brassica* ‘U’ triangle and assessed the effects of whole genome triplication on the divergence of gene sequence and structure, protein-protein interaction, biochemical activities, and gene expression. Sequence and phylogenetic analysis revealed that the deduced G\(\alpha\) and RGS proteins are evolutionarily conserved across *Brassica* species. The duplicated RGS proteins of each *Brassica* species interacted with their cognate G\(\alpha\) but displayed varying levels of interaction strength. The G\(\alpha\) and the duplicated RGS proteins of *Brassica* species exhibited highly conserved G-protein activities when tested under in-vitro conditions. Expression analysis of the *B. rapa* RGS genes revealed a high degree of transcriptional differentiation across the tested tissue types and in response to various elicitors, particularly under D-glucose, salt and phytohormone treatments. Taken together, our results suggest that the RGS-mediated regulation of G-protein signaling in *Brassica* species is predominantly governed by stage and condition-specific expression differentiation of the duplicated RGS genes.

Signalng through heterotrimeric G-protein (hereafter G-protein) complexes plays a fundamental role in controlling various cellular processes both in plants and animals\(^{3-12}\). The core G-protein functional complex comprises three different components i.e. G-alpha (G\(\alpha\)), G-beta (G\(\beta\)) and G-gamma (G\(\gamma\)) subunits, where only G\(\alpha\) subunit can bind and dissociate guanine nucleotides (GTP/GDP). In animals, binding of a ligand to G-protein coupled receptor (GPCR), stimulates its guanine exchange factor (GEF) activity which promotes the release of GDP for GTP from G\(\alpha\) subunit, dissociating the inactive heterotrimer into two functionally independent components i.e. G\(\alpha\)-GTP and G\(\beta\)\(\gamma\) dimer\(^{13,14}\). These two signaling units independently interact with various effector proteins which further validate their ability to participate in numerous biological functions. The intrinsic GTPase activity of the G\(\alpha\) subunit hydrolyzes the bound GTP and allows GDP-G\(\alpha\) form to reunite with G\(\beta\)\(\gamma\) dimer, subsequently making the heterotrimer inactive\(^{15}\). In addition, GAP activity of the ‘Regulator of G-protein Signaling (RGS)’ protein is also known to accelerate the GTP-hydrolysis of G\(\alpha\) subunit and deactivating the G-protein cycle\(^{16}\).

Although the core components of G-protein signaling are highly conserved across phyla, the plant and animal systems are known to have enormous diversity in their quantitative repertoire and regulation of G-protein cycle.
For example, the human genome encodes >800 GPCRs, 35 RGSs, 23 Gα, five Gβ, and 12 Gγ proteins, regulating a wide range of biological processes. In contrast, plants, in general, contain a simple repertoire of G-protein components encoding only up to four Gα and Gβ, 10 Gγ, and two RGS proteins, having no prototypical GPCR. Identification of multiple members of G-protein subunits has been attributed to inherent polyploidy in the angiosperm lineage.

In addition, the activation of plant G-protein signaling is quite contrasting to the classical G-protein paradigm present in metazoans and relies on the self-activating properties of plant Gα subunit, independent of GPCR. Structural and enzyme kinetic analysis of the Arabidopsis AtGPA1 protein plausibly explain its GPCR-independent activation. The GDP to GTP nucleotide exchange rate on AtGPA1 is approximately 100-fold faster than its rate of GTP-hydrolysis, suggesting that the plant Gα is predominantly present in the GTP-bound form. Since G-proteins are signaling molecules, it is important to turn-off the continuing activation state of Gα-GTP after stimulation in plants. Identification of RGS proteins was primarily an important finding in plant G-protein research. In plants, the RGS protein acts as a GTPase-activating protein (GAP), accelerating the rate-limiting GTP-hydrolysis of GTP-bound Gα and so neutralizing the fast nucleotide exchange rate. Interestingly, the RGS proteins reported from the plant lineage contain an N-terminal seven transmembrane (7-TM) structure which is unique and absent in their animal counterparts. The GAP activity of plant RGS proteins is shown to be confined to the ‘RGS-domain’ present at its C-terminal region. Thus, the interplay between Gα and RGS proteins is quite important in regulating overall G-protein mediated biological processes in plants. Although the plant G-protein cycle is principally known to be controlled at the deactivation step through RGS proteins; in recent years phosphorylation-dependent regulation of the G-protein cycle involving receptor-like kinases (RLKs) and their associated kinases has also been reported.

The limited G-proteins repertoire in plants can yet control wide range of biological processes encompassing plant morphology and architecture, defence responses, abiotic stress response, sugar and phytohormone response, and yield related traits. Although structurally similar across plant lineage, both Gα and RGS proteins interestingly possess distinct and species-specific functions. For example, the Gα mutation led to the dwarfing phenotype in rice d1 mutant and maize ct2 mutant, whereas the Arabidopsis mutant (gpa1) did not show any significant change in plant height. The Gα-RGS interplay is also known to regulate species-specific traits in plants, such as nodulation in soybean. Species-specific roles of Gα and RGS proteins in plant lineage could be attributed to their distinct transcriptional and biochemical properties, as well as the involvement of their upstream regulators and downstream effectors.

Brassica species play an important role in global agriculture and horticulture, and share a close relationship to the model plant Arabidopsis. The cultivable diploids (Brassica rapa, B. nigra and B. oleracea) and their natural allotetraploid (B. juncea, B. rapa and B. carinata) species belong to Brassica ‘U’ triangle have been well studied for their several agronomical traits like seed-yield, oil-quality, phyto-remediation, secondary metabolites, resistance against pests and pathogens. The Brassica species are known to possess enormous genome complexity and diverse morpho-types, shaped by lineage-specific whole genome triplication (WGT) event, allopolyploidyization and genomic rearrangements. As a result, the so-called diploid Brassica species are paleohexaploid containing three sub-genomes and possess multiple gene homologs having variable gene expression patterns, gene-silencing effects, and neo- and sub-functionalization. Although complex networks of G-protein signaling have been recently reported in few Brassica species, detailed studies on the expression, biochemical, interaction and functional variance of the Gα and RGS proteins, arising from polyploidy, are fundamentally important for a better understanding of the regulation of G-protein signaling from globally cultivated Brassica crops.

To study the RGS-mediated regulation of G-protein signaling in Brassica genus, isolation of the full-length coding sequence of Gα and RGS genes from three divergent species belonging to ‘Brassica U-triangle’ was carried out. Subsequently, the GTP-binding/hydrolysis activities of Gα-orthologs; GAP activity of Brassica RGS proteins on Gα; and interaction selectivity between Gα and RGS proteins was examined. Later, in-depth expression profiling of RGS genes in various tissue types, plant developmental stages and environmental stress conditions in the Brassica model genome, B. rapa was also investigated. This work suggests that the RGS-mediated regulation of G-protein signaling in Brassica species is highly complex and predominantly governed by stage and condition-specific expression differentiation of duplicated RGS genes to control diverse growth and development processes.

Results
Identification and sequence analysis of RGS and Gα subunit genes from diploid Brassica species.

The full-length coding DNA sequences (CDS) of RGS genes from B. rapa (A genome), B. nigra (B) and B. oleracea (C) were amplified using the degenerate primers (Table S1). Two CDS for RGS genes from each Brassica species were isolated and designated as BraA.RGS1 and BraA.RGS2 (B. rapa); BnB.RGS1 and BnB.RGS2 (B. nigra); BolC.RGS1 and BolC.RGS2 (B. oleracea), based on the standardized nomenclature adopted for Brassica genus. Full length coding RGS sequences isolated from different Brassica species ranged from 1368 to 1386 bp, encoding proteins of 455 to 461 amino acids in length, with an estimated molecular weight of approximately 52 kDa. Deduced RGS proteins of B. rapa, B. nigra and B. oleracea shared 83.8–89.3% identity with the Arabidopsis AtRGS1 (Table S2). Nucleotide sequences of Brassica RGS genes showed 87.6–91.1% identity with AtRGS1 (Fig. S1, Table S3). Sequence analysis of the deduced RGS proteins of Brassica lineage on TMHMM server revealed the presence of an N-terminal ‘seven trans-membrane domain (7-TM)’ and a C-terminal located ‘cytosolic RGS-domain’ (Fig. 1A), similar to that reported for the Arabidopsis and soybean RGS proteins. The Glu320 residue of AtRGS1 protein necessary for the GAP activity was highly conserved in all the Brassica RGS proteins. Moreover, most of the amino acid residues recently described for the plant Gα-RGS contact interface were also found to be conserved (Fig. 1A). For example, corresponding sites for Cys316, Try317, Ala318, Glu361, Asp363, Ser365, His366, Lys367, Asp389, Met392, Glu393, Leu394,
Lys396, Asp398, Leu399 and Asp402 of AtRGS1, were all found to be highly conserved across the deduced RGS proteins of *Brassica* species. Interestingly, *Brassica* lineage-specific substitution was observed at the 362nd amino acid position, wherein Leu was replaced by Val. In addition, a Met397Thr substitution was also observed for the BlnB.RGS2, localized in the Gα-RGS contact interface46.

Earlier, we reported single Gα homolog of AtGPA1 from *B. rapa* (BraA), *B. nigra* (BniB), *B. oleracea* (BolC) and *Arabidopsis* (AtRGS1) was performed using ClustalW (http://www.clustal.org). The predicted 7-TM domains are marked within the horizontal lines and RGS-domain is shown within the box. The critical Glu (E) residue for GAP activity of RGS protein is indicated with a filled circle. (B) Amino acid sequence alignment of Gα proteins from *B. rapa*, *B. nigra*, *B. oleracea* and *Arabidopsis* (AtGPA1). Position of consensus regions for GTP-binding and GTP-hydrolysis are marked within black horizontal lines (G1–G5); P/M, the predicted site for palmitoylation/myristoylation (MGXXCS); open square shows the important Thr (T) residue for RGS-Gα interaction; and the Gln (Q) residue for GTPase activity of Gα proteins is marked as filled square. The asterisks represent the important contact sites at RGS and Gα interfaces.

![Figure 1.](https://example.com/fig1.png)

**Figure 1.** Multiple sequence alignment of *Brassica* RGS and Gα proteins. (A) Amino acid sequence alignment of RGS proteins from *B. rapa* (BraA), *B. nigra* (BniB), *B. oleracea* (BolC) and *Arabidopsis* (AtRGS1) was performed using ClustalW (http://www.clustal.org). The predicted 7-TM domains are marked within the horizontal lines and RGS-domain is shown within the box. The critical Glu (E) residue for GAP activity of RGS protein is indicated with a filled circle. (B) Amino acid sequence alignment of Gα proteins from *B. rapa*, *B. nigra*, *B. oleracea* and *Arabidopsis* (AtGPA1). Position of consensus regions for GTP-binding and GTP-hydrolysis are marked within black horizontal lines (G1–G5); P/M, the predicted site for palmitoylation/myristoylation (MGXXCS); open square shows the important Thr (T) residue for RGS-Gα interaction; and the Gln (Q) residue for GTPase activity of Gα proteins is marked as filled square. The asterisks represent the important contact sites at RGS and Gα interfaces.

Evolutionary analysis of *Brassica* RGS and Gα genes. A high level of amino-acid sequence identity and domain conservation of *Brassica*-specific RGS and Gα proteins led us to investigate their evolutionary relationship with the sequences reported from other plant genomes. Phylogenetic analysis showed that all RGS and
Gα proteins belonging to Brassicaceae family were clustered together with AtRGS1 and AtGPA1, respectively (Fig. 2A,C). Interestingly, the RGS sequences identified in this study were separated into two distinct clades, named as orthologous set-I (RGS1) and set-II (RGS2), suggesting duplication of RGS genes in Brassica lineage. The RGS proteins belonging to orthologous set-I were evolutionarily closer to the AtRGS1. Further, within each orthologous set, the RGS proteins from B. rapa and B. oleracea showed a close phylogenetic relationship, compared to its B. nigra counterpart. Comparison of synonymous substitution rate (Ks) value between the duplicated RGS genes isolated from each Brassica genome showed that RGS1 orthologs have lower Ks (0.34–0.36) values than RGS2 orthologs (0.41–0.47), signifying differential divergence of the duplicated RGS proteins (Table S5).
Divergence time analysis showed that the duplicated RGS1 and RGS2 genes of each Brassica species diverged around 11.62–15.68 mya, very soon after the Arabidopsis-Brassica split event, estimated around 13–17 mya[40]. Interestingly, our data revealed that the duplicated RGS genes of B. nigra have higher Ks values compared to their B. rapa and B. oleracea counterparts. The G\(\alpha\) sequences identified in all the three Brassica species shared highly similar Ks values (0.39–0.42), estimated to diverge around 13.07–14.23 mya from the AtGPA1 (Table S5).

Based on the RGS and G\(\alpha\) coding sequences isolated in this study, we retrieved their genomic counterparts from the recently assembled Brassica database (http://brassicadb.org/brad/). The BraA.RGS1 (corresponds to Bra025181), BraA.RGS2 (Bra017336) and BraA.G\(\alpha\)1 (Bra007761) were localized onto A06, A09, and A09 chromosomes in the model B. rapa (A) genome (Table S6). Similarly, the RGS and G\(\alpha\) orthologs from Brassica ‘B’ (B. nigra) and ‘C’ (B. oleracea) were also identified and summarized in Table S6. Gene structure analysis revealed that the RGS genes contain 10 exons and 9 introns, whereas the G\(\alpha\) genes harbour 13 exons and 12 introns (Fig. 2B,D). Intron-exon organization of the G-protein orthologs present in B. rapa and B. oleracea was quite similar compared to their B. nigra counterparts, where the size of introns was found to be variable.

Brassica species are mesohexaploids, containing three sub-genomes (LF, MF and MF2) formed due to the whole-genome-triplication event[49]. Sub-genomic distribution analysis of the G-protein candidate genes in B. rapa and B. oleracea genomes (http://brassicadb.org/brad/) revealed that the G\(\alpha\) and RGS1 orthologs were present on LF (least fractionized) sub-genome, whereas RGS2 was present on MF2 (most fractionized) sub-genome (Fig. 3). Homologs of RGS and G\(\alpha\) genes were occupied within the Brassica ancestor genomic block ‘L’ and ‘I’, respectively. To get evolutionary insight into this differential gene-retention, we further analyzed the gene content within the genomic blocks ‘L’ and ‘I’, shared between A. thaliana and the three sub-genomes of sequenced B. rapa and B. oleracea genomes (Table S7). The gene-retention frequencies of ‘L’ and ‘I’ genomic blocks belonging to LF sub-genome were almost similar (12.1–17.0%), whereas within the MF1 sub-genome the gene retention of genomic block ‘L’ (19.8–20.7%) was comparably higher than genomic block ‘I’ (10.3–10.5%), thereby suggesting uneven gene-fractionation of the triplicated sub-genomes in Brassica species.

Protein-protein interaction between duplicated RGS and G\(\alpha\) proteins of Brassica species. In metazoans, where multiple members of RGS and G\(\alpha\) subunit are present, interaction specificity between various G\(\alpha\)-RGS proteins controls the kinetics of nucleotide (GTP/GDP) cycling and sensitivity of G-protein signaling[49]. In plants, studying the G\(\alpha\)-RGS interaction is quite important considering that G\(\alpha\) protein has self-activating property and so far RGS and PLD\(\alpha\)1 are the only well-studied modulators of G-protein signaling[50,51]. In this study, two divergent RGS proteins were identified each from the three diploid Brassica genomes. To analyse the interaction strength and specificity between the duplicated RGS proteins and G\(\alpha\) subunit, a mating based split G\(\alpha\) (mbSUS) assay was performed, wherein the G\(\alpha\)\(\alpha\) interaction (Fig. 4B).

In Arabidopsis, the ‘RGS-domain’ present at the C-terminal region of RGS protein is known to interact with the AtGPA1[49]. Therefore, to further validate our observation, we analysed the interaction of the C-terminal cytosolic domain containing the RGS-box of the duplicated RGS proteins of Brassica origin with their cognate G\(\alpha\) protein by utilizing the conventional GAL4 based yeast two hybrid (Y2H) system. The RGS-domains of the two RGS proteins of B. rapa showed strong interaction with their respective G\(\alpha\) subunit in both the orientations, whereas RGS2 protein could interact only in one orientation of G\(\alpha\) (as a C-terminal fusion of Nub, Nub-G\(\alpha\)) when tested on three different concentrations of Met (Fig. 4A.C). The duplicated RGS proteins of B. nigra interacted with its cognate BniB.G\(\alpha\)1 specifically in one orientation (Nub-G\(\alpha\)), wherein the BniB.RGS2 showed relatively weak interaction (Fig. 4B).

GTP-binding/hydrolysis activity of G\(\alpha\) protein and GAP activity of duplicated RGS proteins in Brassica species. Among the different G-protein core components, the biochemical properties of G\(\alpha\) subunit are contrasting between plants and animals thereby making the paradigm of G-protein signaling quite interesting. In order to get a primary insight into the regulation of G-protein in Brassica species, recombinant G\(\alpha\) proteins containing N-terminal His-tag were purified using Ni-NTA affinity chromatography (Fig. S4A) and an in-vitro activity assay of recombinant G\(\alpha\) proteins was carried out using BODIPY-GTP FL fluorescent dye in a real time fluorescent assay. The rates of GTP-binding (increase in fluorescence) and GTP-hydrolysis (decrease in fluorescence) of all the three Brassica G\(\alpha\) proteins was found to be highly comparable, and similar to that observed for the Arabidopsis AtGPA1 (Fig. 5A). In general, the intrinsic GTP-hydrolysis activity of the Brassica G\(\alpha\) proteins was found to be very slow.

Slow GTP-hydrolysis activities of G\(\alpha\) proteins indicate the important role of RGS proteins in regulating G-protein cycle in genus Brassica. Due to a high level of sequence similarity between RGS1 and RGS2 orthologs across Brassica species, we initially selected duplicated RGS proteins of the model Brassica genome, B. rapa, and for biochemical characterization of the same. The C-terminal region containing RGS-domain of the B. rapa RGS proteins i.e. BraA.RGS1-box (296–417 amino acids) and BraA.RGS2-box (284–415 amino acids) containing N-terminal His-tag were heterogeneously expressed in E. coli and purified using Ni-NTA based affinity chromatography (Fig. S4B). Real-time assays using BODIPY-GTP FL showed that both BraA.RGS1 and BraA.RGS2 accelerated the GTP-hydrolysis of their cognate BraA.G\(\alpha\)1, and the GTPase (GAP) activity was found to
be somewhat similar for both of the duplicated BraA.RGS1/2 proteins (Fig. 5B). Likewise, the GAP activity of the duplicated RGS proteins of *B. nigra* and *B. oleracea* also showed similar trend on their cognate Gα proteins (Fig. S5A,B). Further, to determine an accurate rate of GAP activity of the duplicated BraA.RGS proteins, steady

**Figure 3.** Comparison of gene organization in the genomic blocks 'I' and 'L' of *A. thaliana, B. rapa* and *B. oleracea*, as obtained from BRAD database (http://www.brassicadb.org/). Gene arrangement of *B. rapa* and *B. oleracea* syntenic orthologs of 25–30 representative *A. thaliana* genes flanking each side of (A) *AtGPA1* within genomic block 'I' and (B) *AtRGS1* within genomic block 'L'. The syntenic position of *AtGPA1* (*At2g26300*) and *AtRGS1* (*At3g26090*) are marked within green boxes in (A) and (B), respectively. Syntenic genes shared between the three species are represented with black circle; *Brassica* lineage specific genes are marked with red circle showing their gene ID. The line nomenclature describes the 'subgenome-genomic block-linkage group-*Brassica* species'.
state kinetics of Pi release was carried out using 1 μM of BraA.Gα1 and 0.1–2.0 μM of BraA.RGS proteins. BraA.Gα1 showed a marginal difference in its rate of Pi release when tested using the BraA.RGS1 (Km 0.244 ± 0.03; Vmax 2.70 ± 0.09) and BraA.RGS2 (Km 0.311 ± 0.04; Vmax 2.55 ± 0.11) proteins (Fig. 5C). Overall, the presence of highly similar GTP-binding/hydrolysis activities of Gα proteins, and somewhat similar GAP activities of the duplicated RGS proteins, in all possibility, suggest that the RGS-mediated G-protein regulation is biochemically conserved in Brassica lineage.

Transcript expression and sub-cellular localization of B. rapa duplicated RGS proteins. Further, to investigate the transcriptional regulation of the duplicated RGS genes, we carried out gene expression analysis in the model Brassica species, B. rapa. Real-time qRT-PCR analysis revealed that both the B. rapa RGS genes (paralogs), resulting from WGT event, were expressed, showing a contrasting difference in their expression patterns. In general, BraA.RGS1 had higher expression compared to BraA.RGS2 in most of the tissue types, except seedlings (Fig. 6A). The expression of BraA.RGS1 was also found to be higher than BraA.RGS2 during all stages of seed development (Fig. 6B). Interestingly, the expression of both BraA.RGS1 and BraA.RGS2 was found to be up-regulated during later stages of seed maturation (35 dap, days-after-pollination) suggesting their important during seed development in B. rapa. Sub-cellular localization studies in transgenic Arabidopsis hypocotyls revealed that the C-terminal YFP-fusion of both BraA.RGS1 and BraA.RGS2 proteins were localized in the plasma-membrane, along with FM4-64 (red), a dye used for staining cell membrane (Fig. 6C). Moreover,

Figure 4. Interaction between Gα and RGS proteins of Brassica species namely (A) B. rapa, (B) B. nigra, and (C) B. oleracea using mating based split ubiquitin system. Gα subunit was cloned in both the orientation of Nub vector (containing N-terminal half of ubiquitin), and RGS proteins were cloned in Cub vector (C-terminal half of ubiquitin), as fusion proteins. The interaction was examined by the growth of mated diploid yeast cells on selection plates (SD-AHLT) containing 0, 500 and 1000 μM Met. The NubWt-Gα protein and empty NubG vector were used as positive and negative control, respectively. Two biological replicates of the experiment were performed with identical results.
transient expression studies in *N. benthamiana* epidermal cells also established that the duplicated RGS proteins of *B. rapa* are localized in the plasma-membrane (Fig. S6), as also reported for the soybean RGS proteins.

Expression analysis of *B. rapa* duplicated RGS genes under various elicitor treatments. So far, information about the roles and regulation of plant RGS genes under various developmental and environmental cues is sparse and mostly limited to the model plant *A. thaliana*. To get an initial insight into the transcription regulation of the duplicated RGS genes, in-depth transcript expression profiling was carried out in *B. rapa*. Five-day-old uniform seedlings of *B. rapa*, grown on 0.5 × Murashige and Skoog (MS) medium containing 3% sucrose, were subjected to different elicitor treatments, including D-glucose, phytohormones, abiotic and biotic stress conditions for 1, 3, 6, 12 and 24 hours (h) as described previously. qRT-PCR analysis showed a significant up-regulation of *BraA.RGS2* transcript compared to *BraA.RGS1* when treated with D-glucose for all the tested time points (Fig. 7A), thereby suggesting a differential transcriptional response of the *B. rapa* duplicated RGS genes under glucose treatment. The *BraA.RGS* genes also showed differential expression patterns in response to exogenously supplied phytohormones. Expression of *BraA.RGS1* transcript was, in general, found to be up-regulated during most of the tested time points of phytohormone treatments, including IAA, GA, BAP, ABA and BR (Fig. 7A). However, the transcript abundance of *BraA.RGS2* was found to be up-regulated only

Figure 5. Biochemical characterization of *Brassica* Gα and RGS proteins. (A) GTP-binding/hydrolysis activity of the recombinant Gα protein using BODIPY fluorescent dye in real-time fluorescence assays; (B) Effect of the recombinant BraA.RGS1 and BraA.RGS2 domains (expressed as C-terminal region containing RGS-box) on GTP-hydrolysis of BraA.Gα1 using BODIPY fluorescent dye in real-time fluorescence assays; and (C) *In-vitro* Pi release activity of BraA.Gα1 in the presence of different concentration of BraA.RGS1 and BraA.RGS2 domains. Inset table shows the kinetic parameters of Pi release from GTP-bound Gα protein, in the presence of RGS domains of the duplicated *B. rapa* RGS proteins. Experiments were carried out three times and data was averaged. Error bars represent the mean (±)SE. Data were analyzed using GraphPad Prism version 6.0.
during early time points for most of the phytohormone treatments. The duplicated RGS genes also showed distinct expression patterns in response to various abiotic and biotic stress conditions. A profound up-regulation of both BraA.RGS1 and BraA.RGS2 was observed under SA treatment, whereas these transcripts showed significant down-regulation under heat and cold treatments (Fig. 7B). Further, differential transcriptional regulation of the duplicated RGS genes was also observed in response to both NaCl and MeJA treatments, wherein the BraA.RGS1 transcript showed a significant up-regulation upon these treatments.

Brassicaceae species are known to be the heavy metal accumulators and are globally used for phyto-remediation purposes. We, therefore, studied the expression patterns of the duplicated RGS genes under various heavy-metal ion toxicity in B. rapa. Heavy metal treatment showed substantial parallelism in the expression patterns of the duplicated RGS genes (Fig. 7C). Interestingly, we found a high accumulation of BraA.RGS1 and BraA.RGS2 transcript under Cu stress during all the tested time points (1 to 24 h). Likewise, up-regulation of the duplicated RGS
genes was also observed under Cd, Mn and As stress although, showing a time-dependent transcript accumulation. Under Zn and Pb treatments, we found differential transcript responses of the duplicated RGS genes during different time points. The profound up-regulation of the duplicated RGS genes in all possibility suggests their key involvement during heavy-metal ion toxicity in *Brassica* crops.

**Discussion**

Over the recent years, it has become increasingly clear that plants have a unique mechanism of G-protein signaling, owing to limited repertoire of core G-protein components, fast GTP-binding with very slow intrinsic GTPase activity of Gα proteins, and most importantly the absence of functional GPCRs in plants. Among the various G-protein signaling components reported across the plant lineage, the physical interaction and biochemical activities of Gα and RGS proteins are quite crucial for regulating the G-protein cycle

**Brassica** genomes encode highly conserved Gα and RGS proteins. Various comparative genomics and genome sequence studies have unequivocally reported the existence of a WGT event in *Brassica* lineage, after its split from the model plant *Arabidopsis*, dating around 13–17 mya, as a result of which the so-called diploid *Brassica* species are paleohexaploid containing three sub-genomes (LF, MF1 and MF2)39–41. Although three copies of each of the *Arabidopsis* ortholog are quite expected, in the current study only one Gα and two RGS genes were identified in each of the three *Brassica* genomes, present in LF and MF2 sub-genomes only (Fig. 3, Table S7). The uneven expansion of the candidate G-protein genes in *Brassica* species could be attributed to the biased gene fractionation (gene-loss) frequency across the three sub-genomes41,48. Notably, within the sub-genome MF2, the genomic block containing Gα (I) encountered higher gene-loss than the RGS containing genomic block (L). Our observation was quite in agreement with earlier reports describing the uneven expansion of key signaling genes in *B. rapa*, particularly involved in G-protein and 14-3-3 signaling pathways10,32.

Sequence and phylogenetic analysis of both RGS and Gα proteins suggest that these proteins are evolutionarily conserved in *Brassica* lineage (Figs 1 and 2), wherein the Gα orthologs have significantly high sequence conservation compared to the duplicated RGS proteins. This in all possibility suggests that the canonical Gα proteins might have retained highly conserved biochemical activities vis-à-vis biological functions during the evolution of extant *Brassica* species. In such scenario, the presence of divergent RGS or other regulatory proteins could play an important role in regulating the G-protein cycle and signaling in these mesohexaploid *Brassica* species. The differential synonymous base substitution (Ks) rates observed for the duplicated RGS proteins suggests their differential functional specificity and interaction selectivity with the Gα and other effector proteins, which needs detailed investigation.

**Brassica** Gα and RGS proteins display differential interaction specificity. Expansion of the repertoire of G-protein components revealed the presence of complex signaling network in plants7. These components tend to interact in various combinations to govern the functional selectivity in various cell and/or tissue types. Various studies in animal systems, clearly established the significance of RGS and Gα interactions in regulating the GAP activity54. Our data suggest that during evolution, the canonical Gα protein present in each *Brassica* species has retained strong interaction with the ancestral RGS1 protein compared to the recently evolved RGS2 protein (Figs 4, S3). Divergent residues present between the duplicated RGS proteins (Fig. 1) could govern this differential interaction specificity with the canonical Gα protein. Interestingly, among the RGS2 orthologs present
across Brassica species, the B. nigra BniB.RGS2 showed comparatively weaker interaction with its cognate BniB.
Gα1. A comparably higher Ks value of the BniB.RGS2 and various invariant residues, preferentially present at the C-terminally located RGS-domain (including Met397Thr substitution), might alter the stability and interaction of the Gα-Gβγ contact interface86, which needs further investigation. The predominance of BniB.RGS1-BniB.
Gα1 interaction in all possibility suggested that the regulation of G-protein signaling is quite distinct in B. nigra, and somewhat different from the Brassica A/B genomes. Noteworthy, the gene-structure analysis in our study also revealed that the intron-exon attributes of both Gα and RGS genes in B. nigra varied somewhat from their B. rapa and B. oleracea counterparts (Fig. 2). These observations could be best explained by the fact that B. nigra has evolved separately from the B. rapa/B. oleracea lineage in the tribe Brassicaceae85. Nonetheless, the differential RGS-Gα interaction specificity suggests distinct RGS-mediated regulation of G-protein signaling in Brassica lineage. Detailed functional studies using gain- and loss-of-function strategies of G-protein genes in each of these Brassica species need to be undertaken to uncover the significance of this differential interaction.

The canonical Gα and duplicated RGS proteins display similar G-protein activities in Brassica lineage. The unusual self-activating property of Gα protein makes the plant G-protein signaling unique from that of metazoans. The plant Gα proteins posses fast rate of GTP-binding and slow GTP-hydrolysis ability, which are quite contrasting to their animal counterparts2. In this study, we observed that the canonical Gα orthologs of three Brassica species are biochemically active and display highly similar GTP-binding and GTP-hydrolysis activities, similar to the Arabidopsis AtGPA1 (Fig. 5). This indicates that during the evolution of extant Brassica species, changes in few amino acid residues do not seem to impart any significant differences on the activities of these Gα proteins, at least under the tested in-vitro conditions. In plants, GTP-hydrolysis of Gα is the rate limiting step of G-protein cycle and is modulated by the GAP activity of RGS proteins82. Our in-vitro data based on fluorescence assays and steady state kinetics experiments show that the cytosolic RGS-domain of the duplicated RGS proteins increases the rate of GTP-hydrolysis of the cognate Gα, and are active GAP proteins (Fig. 5). Further, comparable GAP activities of the active RGS-box of duplicated RGS proteins in Brassica lineage suggest that the RGS-mediated regulation of G-protein cycle in Brassica crops is biochemically conserved, although other modes of regulation may be expected.

Our observation is different from that reported in allotetraploid soybean genome, where the four Gα and duplicated RGS protein exhibit distinct G-protein activities8,45. The distinct activities of G-protein regulatory elements can be best explained by the fact that the soybean genome has experienced two rounds of whole genome duplication (WGD) events dating around 58–60 mya (ancient duplication) and a recent duplication ca. around 13 mya56. Since the paralogs created during the ancient WGD event are expected to diverge out considerably, the G-protein members in soybean have retained distinct G-protein activities and functional divergence to regulate various plant growth and developmental traits37. However, multiple homologs formed in Brassica lineage have resulted from a very recent WGT event (~13–15 mya), as a result of which the duplicated/triplicated genes (paralogs) could have retained comparably higher sequence identity vis-à-vis similar biochemical activities and biological functions in the extant Brassica species, as evident from our current study.

Differential transcriptional regulation of duplicated RGS genes under plant developmental stages and elicitor treatments. Over time, the duplicated genes formed as a result of WGD and WGT events in polyploids are known to alter their gene expression, as a result of which these genes undergo different evolutionary fates including neo-functionalization, sub-functionalization and pseudogenization57,58. The transcriptional differentiation of multiple gene homologs is well documented in polyploid plant species59. In this study, the duplicated RGS genes of B. rapa showed a high degree of transcriptional bias across various developmental stages wherein BraA.RGS1 was found to be transcriptionally more active compared to Braa.RGS2 (Fig. 6). Considering only one canonical Gα subunit, and conserved biochemical activities of both Gα and duplicated RGS proteins present in each species, it is quite possible that the RGS-mediated G-protein regulation could be dependent on the differential transcriptional response of duplicated RGS genes to regulate various assets of plant growth and development, as also proposed for soybean45. Moreover, in Brassica lineage where WGT is an inherited norm the sub-genome dominance also shapes the expression and functional dominance of paralogous gene within a gene family42,32. The sub-genome dominance effect is also evident in this study, wherein the highly expressed BraA.RGS1 is localized in the transcriptionally active least-fragmentized (LF) sub-genome of B. rapa.

Both loss- and gain-of-function studies in plants under phytohormones and other elicitor treatments show the significance of G-proteins in controlling various assets of plant growth and development. The Arabidopsis G-protein mutants exhibit differential phenotypic response to auxin and ABA treatments. For example, gpa1 mutant displayed hypersensitivity towards ABA during seedling and root development86, whereas, it showed wild-type like inhibition of seedling growth; and hyposensitivity in lateral root development to auxin treatment26,28. Among the two RGS1 genes of B. rapa, a higher up-regulation of Braa.RGS1 during IAA and ABA treatments is quite evident and might suggest its preferential cross-talk with auxin and ABA-mediated seedling development. The Arabidopsis G-protein mutants show hypersensitive response to D-glucose during early stages of plant development like seed germination, seedling development and root growth1. In recent years, it has been well documented that upon D-glucose and NaCl treatments, AtRGS1 undergoes endocytosis from the plasma-membrane to the endosomes which lead to physical uncoupling of AtRGS1 from AtGPA1, thereby allowing the AtGPA1 to self-activate51,62,63. Among the duplicated RGS genes, the up-regulation of Braa.RGS1 and BraA.
RGS2 expression in response to NaCl and D-glucose treatment, respectively, suggest their specific roles in regulating various salt and sugar-responsive phenotypes in B. rapa. The involvement of G-protein components in plant defence is quite established in Arabidopsis26,27,29,34. In addition, the up-regulation of the duplicated BraA. RGS genes under SA treatments possibly suggests their coordinated involvements in defence signaling in B. rapa.
Quite interestingly, both the duplicated RGS genes are highly up-regulated in response to Cu and Cd treatments, suggesting their potential roles during heavy-metal toxicity. Recently, Kunihito et al.\textsuperscript{63} also showed the involvement of G-proteins in conferring Cd tolerance in yeast and Arabidopsis. Thus RGS-mediated G-protein signaling could represent a novel pathway for phyto-remediation of heavy-metal ions in Brassica species, although other, yet unknown, mechanism may also exist, which warrants further investigation.

Overall, our study shows that the transcriptional differentiation of the biochemically conserved RGS proteins could be quite important to condition-specific Go-\textsuperscript{α}RGS interaction vis-à-vis biological functions in the Brassica lineage. A detailed characterization of Go and RGS genes could be carried out to integrate the multiple molecular connections that co-ordinately regulate the strength and/or duration of G-protein signals in controlling the various assets of plant growth and development in the globally important Brassica crops.

Materials and Methods
Plant material and growth conditions. Three Brassica species namely, B. rapa L. (cv. YID1), B. nigra L. (cv. IC257) and B. oleracea L. (cv. Golden Acre) used in the present study were grown under controlled growth conditions at day (24 °C; 10 h; ca. 300 \( \mu \)mol m\(^{-2}\) s\(^{-1}\)) and night (18 °C; 14 h) photoperiod with 55–60% relative humidity. Tissue types representing different developmental stages of B. rapa including five-day old seedlings, fully developed leaves, root, stem, flower and different stages of developing siliques (7 to 35 days after pollination) were collected and stored at –80 °C.

Amplification and cloning of Go and RGS CDS from Brassica species. The standard PCR amplification conditions were deployed with an annealing temperature of 55 °C (30 sec) to obtain the full-length coding DNA sequence of Go and RGS genes from B. rapa (A genome), B. nigra (B genome) and B. oleracea (C genome). The cloning of Go CDS from B. rapa and B. nigra has been reported in our earlier studies\textsuperscript{29,43}, whereas B. oleracea was performed in the present study. For cloning RGS genes, the primers were designed based on the Arabidopsis ortholog (AtRGS1) and annotated B. rapa genes available in the phytozone database (Locus ID: Brara.F03296.1 and Brara.I02169.1) (Table S1). Subsequently, PCR products were cloned into the pENTR/D-TOPO vector (Invitrogen, USA) and sequenced to confirm their fidelity. At least, three independent PCR amplifications were carried out to confirm the gene sequences.

Sequence alignment, phylogenetic and divergence analysis. Phylogenetic analysis of the deduced RGS and Go protein sequences isolated from B. rapa, B. nigra, B. oleracea, and those retrieved from different plant species (https://phytozome.jgi.doe.gov/pz/portal.html) was carried out using the maximum likelihood method in MEGA5.1 with 1,000 bootstrap iterations in MEGA5.1.\textsuperscript{64} The full-length genomic sequences and the chromosomal attributes of the G-protein candidate genes of Brassica origin was retrieved from BRAD database (http://brassicadb.org/brad/). To estimate the divergence time, ClustalW was used for the pairwise alignments of coding DNA sequences of Brassica-specific RGS and Go genes with their Arabidopsis orthologous counterparts. Ks (synonymous substitution rate) and Ka (non-synonymous substitution rate) were calculated using the DnaSP v5 program bases on the multiple sequence alignment. The divergence time (T) was calculated using the equation: 

\[
T = \frac{\lambda K_s}{2N_{\lambda}} \approx 1.5 \times 10^{-8} \text{ substitution per site per year for Brassica genes}\textsuperscript{65}.
\]

Go and RGS protein-protein interaction assays. Mating based split ubiquitin system (mbSUS) was utilized to study the interaction between Go subunit and RGS proteins\textsuperscript{66}. Full-length CDS of Brassica Go proteins were cloned in both the orientation of Nub vector (N- and C-terminal of Nub vector) and RGS proteins in CUB vector. Nub-Wt and empty Nub-vector were used as positive and negative controls, respectively and transformation and mating were performed as described\textsuperscript{29,43}. Finally, strength and selectivity of Brassica Go and RGS subunit protein interactions were determined by the growth of mated yeast cells on the selection medium lacking adenine, histidine, leucine and tryptophan (SD–AHLT), having 0, 500 and 1000 μM of methionine.

The interaction between Go subunit and the cytosolic RGS-domain (RGS-box + C-terminal) of RGS proteins was tested using GAL4 based yeast two hybrid system. Full length CDS of Brassica Go and RGS-domain were cloned into pENTR/D-TOPO entry vector. Thereafter, Brassica Go proteins (bait) and RGS-domain (prey) were mobilized in pDEST-GBK77 gateway (containing DNA binding domain) (ABRC stock: CD3-764) and pDEST-GAD77 gateway (containing activation domain) (ABRC stock: CD3-763) vectors\textsuperscript{67}, respectively using gateway based cloning strategy. The independent sets of bait and prey plasmids were then co-transformed post inoculation, having different concentration of 3-amino-1,2,4-triazole (3-AT).

Expression and purification of recombinant Go and RGS proteins. The coding regions of AtGPA1, Braa.Goa1 (Go subunit of B. rapa), BrniB.Goa1 (Go subunit of B. nigra), BoIc.Goa1 (Go subunit of B. oleracea), Braa.RGS1box + Ct and BraA.RGS2box + Ct (cytosolic RGS-domain with C-terminal region) were cloned into pET28a expression vector (Novagen, USA) and transformed into E. coli Rosetta-gami2 (DE3) cells (Novagen, USA). The N-terminal His-tagged recombinant Go proteins were purified by Ni\textsuperscript{2+}-NTA affinity chromatography\textsuperscript{68}. Under the condition described for Go protein purification, the recombinant RGS2-domain (RGS2box + Ct) was accumulated in the inclusion bodies (IBs). In order to maintain the purification similarities, the recombinant RGS-domain of duplicated RGS proteins were purified under denaturation condition from inclusion bodies. The pellet fraction containing the expressed protein was resuspended in extraction buffer (50 mM Tris-HCl pH 7.5; 8 M Urea; 1 mM DTT; 1 mM PMSF) and kept for 60 min at room temperature for solubilization. Solubilized
protein was diluted 10 fold with extraction buffer and dialyzed overnight in wash buffer (50 mM Tris-HCl, pH 7.5; 200 mM NaCl; 1% Triton X-100 and 1 M Urea). Dialed protein was pooled and centrifuged at 12,000 rpm for 30 min at 4 °C and the supernatant was purified using Ni2+-NTA affinity chromatography similar to Go proteins.

G-protein activity assay of recombinant Go and RGS proteins. In-vitro G-protein activity assay of the purified Go proteins was carried out using 4,4-difluoro-4-bora-3a,4a diaza-s-indacene-GTP Fluorophore (BODIPY-GTP FL, Invitrogen) dye in real time fluorescent assays as described previously. Further to determine the GAP activity of the recombinant RGS-domain, in-vitro Pi release activity was also carried out using ENZchek phosphate assay kit (Invitrogen) as described previously. Briefly, BraA.Go1 protein (1 µM) was pre-loaded with GTP (1 mM) and incubated with 0.1 to 2 µM of purified RGS-domain of BraA.RGS1 and BraA.RGS2 proteins. Phosphate (Pi) release was measured as the absorbance at 360 nm using a spectrophotometer (FLUOstar Optima, BMGLab Technologies).

Total RNA isolation, cDNA synthesis and real-time qRT–PCR. Total RNA isolation from different developmental tissues of Brassica species, first strand cDNA synthesis and real-time qRT-PCR were performed as described previously. CDNA samples representing various growth and developmental stages were diluted 1:25 in nuclease-free water, and real-time PCRs were performed using gene-specific primers (Table S1).

Sub-cellular localization of B. rapa RGS genes. In order to study the sub-cellular localization, the full-length coding regions of BraA.RGS1 and BraA.RGS2 were mobilized into destination binary vector pEarleyGate101 (ABRC stock CD3-683) from Gateway entry vector pENTR/D-TOPO using LR recombination strategy (Invitrogen, USA). Thereafter, stable Arabidopsis lines (Col-0 background) were generated independently expressing BraA.RGS genes fused to C-terminal YFP tag using Agrobacterium-mediated transformation. Hypocotyl cells of four-day old seedlings (T2 generation) grown under continuous dark conditions on 0.5 × (MS) medium without any exogenous sugar source were used to study the localization of BraA.RGS proteins. Localization of B. rapa RGS proteins was also tested in Nicotiana benthamiana epidermal cells using Agro-infiltration, as described previously. The overnight grown culture of Agrobacterium was pelleted down and resuspended in infiltration buffer (10 mM MgCl2; 10 mM MES and 100 mM Acetosyringone, pH 5.6) to an OD600 of 0.6. Thereafter, infiltration was carried out on the abaxial side of 4-weeks old N. benthamiana leaves. Infiltrated plants were kept under dark for 24 h followed by 18 h light and 6 h dark cycle for 2 days in a plant growth chamber. Fluorescence was detected using following parameters: YFP (λ ex514 nm, λ em530-560) and FM4-64 (λ ex543 nm, λ em560). Confocal images were analysed using LAS AF Lite software (Leica Microsystems). At least two independent lines were tested to establish the localization.

Elicitor treatments. Seeds of B. rapa were sterilized using 0.05% HgCl2 and washed thoroughly using sterile distilled water. Sterile seeds were then transferred on 0.5 × (MS) medium containing 0.8% (w/v) agar and 3% (w/v) sucrose. Seeds were germinated under controlled Brassica growth conditions for four days under light and dark. Subsequently, uniformly grown seedlings were then adapted for 24h in 0.5Xliquid MS medium containing 1% sucrose (except for D-glucose treatment) before feeding with different phytohormones (100 µM IAA, 100 µM GA, 100 µM BAP, 100 µM ABA, 100 µM ACC and 1 µM BR), D-glucose (3%); stress and elicitors (42°C heat, 4°C cold, 200 mM NaCl, 200 mM MeJA, 200 mM SA, 300 mM Cu2+ as CuCl2, 500 mM Zn2+ as ZnCl2, 500 mM Mn2+ as MnCl2, 80 µM Cd2+ as CdCl2, 300 mM Pb2+ as PbCl2, and 300 µM As (V) as Na3HAsO4) each for 1, 3, 6, 12 and 24h as described previously. The untreated seedlings of each time points served as respective controls.

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**Author Contributions**

R.K. and N.C.B. planned and designed the research; R.K. performed experiments, analyzed and interpreted data; R.K. and N.C.B. wrote and approved the manuscript.

**Additional Information**

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