

Genome-wide Characterization of GRAS Transcription Factors and Their Potential Roles in Development and Drought Resilience in Rose (*Rosa chinensis*)

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Abstract

In the past few years, plant-specific GRAS transcription factors (TFs) were reported to play an essential role in regulating several biological processes, such as plant growth and development, phytochrome signal, arbuscular mycorrhiza (AM) symbiosis, environmental stress responses. *GRAS* genes have been thoroughly studied in several plant species, but unexplored in *Rosa chinensis* (rose). In this study, 59 rose *GRAS* genes (*RcGRAS*) were identified. Phylogenetic analyses grouped *RcGRAS* genes into 17 subfamilies, of which subfamily *Rc2* was *Rosaceae* family-specific. Gene structure analyses showed that most of the *RcGRAS* genes were intronless and were relatively conserved. *Cis*-element analyses suggested that *RcGRAS* genes may involve in distinct biological processes and responsive to diverse abiotic stresses. Most of the genes were localized in the nucleus, except for a few in the cytoplasm. Gene expression analysis was also performed in various tissues, during gibberellin (GA) and drought stress treatment. The expression patterns of *RcGRAS* genes during GA treatment and in response to drought stresses suggested the potential functions of these genes in regulating stress and hormone responses. In summary, a comprehensive exploration of the rose *GRAS* gene family was performed, and the generated information can be utilized for further functional-based studies on this family.

1. Introduction

The plant-specific *GRAS* transcription factors (TFs) have been widely explored in the past decade and were found to be involved in cell signaling, hormone signaling, arbuscular mycorrhiza (AM) symbiosis, biotic and abiotic stress tolerance, etc., (Bolle, 2004; Davière and Achard, 2013; Di Laurenzio et al., 1996; Fode et al., 2008; Greb et al., 2003; Guo et al., 2019; Heckmann et al., 2006; Zhang et al., 2020). The abbreviation *GRAS* was taken from its first three identified members, i.e., gibberellic acid insensitive (GAI), repressor of GA1–3 mutant (RGA), and a scarecrow (SCR) (Pysh et al., 1999). *GRAS* proteins are generally composed of 400-770 amino acid residues and have conserved carboxyl (C)- terminal and highly variable N terminal (Hakoshima, 2018). The C-terminal contains the following five conserved motifs: leucine-rich region I (LHRI), VHII D, leucine-rich region II (LHRII), PFYRE, and SAW; these motifs are involved in the protein-protein interactions (Itoh et al., 2002). Whereas, variable N-terminal domain provides specificity to *GRAS* proteins, which helps *GRAS* proteins to participate in diverse biological processes (Bolle et al., 2000; Greb et al., 2003; Hakoshima, 2018; Tian et al., 2004). Previously, the *GRAS* genes have been identified in more than 30 plant species including the following important plant species, *Arabidopsis*, rice, soybean, cassava, barley, tea, etc. (Ma et al., 2010; Shan et al., 2020; Tian et al., 2004; To et al., 2020; L. Wang et al., 2020), for more detail see Table S1.

In *Arabidopsis* and rice, the *GRAS* genes were grouped into eight subfamilies, including- (i) DELLA, (ii) Hairy meristem(HAM), (iii) Light signaling through interactions light-responsive transcription factor PIFs (LISCL), (iv) Lateral suppressor (LS or LAS), (v) Phytochrome A signal transduction1 (PAT1), (vi) Scarecrow-like3 SCL3, (vii) SCR, and (viii) short-root (SHR) (Lee et al., 2008; Tian et al., 2004). However, in

other plant species, the number of subfamilies were reported higher than 8; for instance, 13 subfamilies were identified in the case of *Populus trichocarpa* (Liu and Widmer, 2014), *Camellia sinensis* (Wang et al., 2018) and castor bean (Xu et al., 2016), 14 subfamilies were identified in strawberry (Chen et al., 2019) and cotton (Zhang et al., 2018) and 16 subfamilies were identified in bottle gourd (Sidhu et al., 2020). However, a combined phylogenetic analysis using eight different angiosperm species, regrouped GRAS genes into 17 different subfamilies (Cenci and Rouard, 2017).

Several GRAS TFs have functionally characterized in plants for their functional role during growth, developmental and signal transduction processes. For instance, two GRAS genes SCR and SHR form complex and play a key role in asymmetric cell division and radial root patterning by differential expression in the quiescent centre and stelar tissue (Nakajima et al., 2001; Sabatini et al., 2003). The SCR and SHR genes were also involved in osmotic and salinity stress regulation (Bolle, 2016). DELLA, a major class of GRAS genes play a substantial role in GA signaling in a negative manner (Achard and Genschik, 2009; Davière and Achard, 2013; Fukazawa et al., 2014). GAI and RGA are degraded via the E3 SCFSLY complex in the presence of GA. Degradation of DELLA activates the downstream genes positively involved in plant growth. DELLA gain of function mutant showed GA insensitivity and resulted into dwarf phenotype and *vice-versa* (Cheng et al., 2004; Wild et al., 2012). In addition to GA signalling, DELLAs were also involved in abscisic acid (ABA), auxin, brassinosteroids (BRs) and cytokinins (CKs) signalling, thereby regulating a range of growth and developmental processes in plants (Davière and Achard, 2016; Dolgikh et al., 2019). Genes including HAM, LAS, and MOC1 are reported to develop and maintain shoot apical and axillary meristem (Greb et al., 2003; Stuurman et al., 2002). In rice, MOC1 is mainly expressed in the axillary buds and regulates tillering process (Tong et al., 2009). Similarly, another gene in rice, GS6 belongs to DLT subfamily of GRAS TFs negatively regulates grain size (Sun et al., 2013). GRAS genes also found to be involved in AM symbiotic association and phytochrome A signal transduction. For example, RAM1 (GRAS gene) was found as a master regulator for AM symbiotic association (Hartmann et al., 2019; Pimprikar et al., 2016). Phosphorylate CYCLOPS interact with RAM1 as well as DELLA to induce hyphopodia formation. More recently, GRAS TF, NSP2 (*AhNSP2*) was shown to control the nodulation in cultivated peanuts (Peng et al., 2021). The PAT1 and SCL21 GRAS TF members considered positive regulators of phytochrome A signal transduction in plants (Bolle et al., 2000; Muntha et al., 2019; Torres-Galea et al., 2013).

Rose, the most important ornamental crop, has high demand in different industries like perfumery, cosmetics and pharma, etc. Being small size genome (560Mb) and diverse ploidy (2x-10x), the rose has been considered as a model plant for evolutionary and polyploidization studies (Zhang et al., 2013). After the release of whole genome sequences (Nakamura et al., 2018; Raymond et al., 2018; Saint-Oyant et al., 2018), a genome-wide study for identifying important gene families have been taken place in rose in the last two years. Some important examples include the identification of AP2/ERF (Li et al., 2020), WRKY (X. Liu et al., 2019), Rdr1 (Menz et al., 2020), R2R3-MYB (Han et al., 2019) gene families and their potential role in different biological process i.e., growth and development, phenylpropanoid pathway, abiotic stress tolerance, and tolerance against biotic stresses like Botrytis, gray mold and black spot. However, a comprehensive study of rose GRAS genes is still lacking.

This study identified 59 GRAS genes in rose using the latest rose genome database (Saint-Oyant et al., 2018). We performed phylogenetic, conserved motif, gene structural (exon/introns) and cis-acting regulatory elements analyses of *RcGRAS* genes. The comparative analysis for the *GRAS* genes from rose, *Arabidopsis* and rice was also conducted. Furthermore, expression profiles of the *RcGRAS* genes were investigated in different tissues and responses to GA and drought stress treatments using qRT-PCR. Altogether, our study provided some insights that could be used in future functional validation of *RcGRAS* genes and may be beneficial for rose improvement.

2. Materials And Methods

2.1. Plant materials, gibberellin and stress treatments

The rose genotype belong to *Rosa chinensis* were used in this study. Uniform growing plants in plastic pots containing a 1:2:1 soil/peat/sand mix and in greenhouse conditions (photoperiod =14h light/10h dark and temperature = $22 \pm 2^\circ\text{C}$) were used for expression profiling of *RcGRAS* genes. The leaf, stem and flower buds samples were collected from two pots (considered as two biological replication) for tissue-specific expression patterns analysis under normal conditions. Gibberellin (GA) treatment was given according to Randoux et al. (Randoux et al., 2012) with slight modifications. Rose plants were sprayed once with GA₃ (30 μM) and maintained for one-week and then plants were pruned. Subsequently, the pruned plants were sprayed with GA₃ (30 μM) three times a week (on alternative days) for 34 d. After treatment, the leaf, flower bud, and stem tissues were collected (two biological replications). Drought stress (DS) was imposed according to Niu and Rodriguez (Niu and Rodriguez, 2009), the irrigation was withheld for 30 days. The DS was determined through visual stress sign (wilting). The leaf tissues were collected (two biological replications) from control and DS treated plants. All samples were collected in liquid nitrogen and stored at -80°C till further use.

2.2. Identification of GRAS family genes in rose

To identify the rose GRAS family genes, hidden markov model (HMM) profile of the GRAS domain (PF03514) was downloaded from the Pfam database (Mistry et al., 2021). The HMM file was used to examine all protein sequences in the rose genome using *Rosa chinensis* Homozygous Genome v2.0 (Saint-Oyant et al., 2018). Domain composition analysis was performed using NCBI-conserved domain database (Marchler-Bauer et al., 2012) and SMART server (Schultz et al., 2000). Molecular weight (MW), isoelectric point (pI), and an amino acid number of rose GRAS proteins were determined using the ExPasy program (<http://www.expasy.org/>).

2.3. Exon-Intron and conserved motif analysis

The gene structures of rose GRAS TFs were illustrated using TBtools (Chen et al., 2020) with the GFF3 file of the rose genome. MEME v5.1.1 suite (Bailey et al., 2009) was used to identify conserved motifs of rose GRAS proteins with default parameters and the maximum number of motifs was 10. The MEME-motifs and Seq Logos were visualized on TBtools.

2.4. Phylogenetic analysis and classification of RcGRAS genes

The GRAS protein sequences of rose (59), Arabidopsis (33) available at TAIR database (<https://www.arabidopsis.org/>), rice (52) available at Rice Genome Annotation Project (<http://rice.plantbiology.msu.edu/>), strawberry (54) available at GDR database (https://www.rosaceae.org/projects/strawberry_genome/tools_) were aligned using MUSCLE program available at MEGA vX (Stecher et al., 2020). The phylogenetic tree was built with MEGA vX software's help using Poisson model, pair-wise deletion, and neighbour Joining (NJ) algorithm with 1000 bootstraps. The RcGRAS proteins were also divided into different sub-families based on their grouping with characterized Arabidopsis and rice GRAS proteins.

2.5. Chromosomal localization and synteny analysis of RcGRASs

Using *Rosa chinensis* Homozygous Genome v2.0, the identified rose GRAS genes were mapped and displayed using TBtools. The synteny relationships of GRAS genes among rose and other species (Arabidopsis and rice) were performed using MCScanX software (Wang et al., 2012). Protein sequences and genome annotation file gff3 for *Rosa chinensis* 'Old Blush' were downloaded from (<https://lipm-browsers.toulouse.inra.fr/pub/RchiOBHm-V2/>), for rice and Arabidopsis protein sequences and gff3 file were downloaded from (<http://rice.plantbiology.msu.edu/>) and (<https://www.arabidopsis.org/>), respectively.

2.6. Cis-acting regulatory elements (CARE) analysis of RcGRASs

The upstream 2 kb sequences of the *RcGRAS* genes were fetched from the *Rosa chinensis* Homozygous Genome v2.0 database. The fetched sequences were then submitted to the PlantCARE server (Lescot et al., 2002) to identify the conserved CAREs.

2.7. Sub-cellular localization, NLS prediction and miRNA target site prediction

To predict the subcellular locations of RcGRAS proteins, Plant-mPLOC in Cell-PLOC 2.0 server (<http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLOC-2/>) was used (Chou and Shen, 2008). The Nuclear localization signal (NLS) prediction of *RcGRAS* gene sequences was performed with cNLS Mapper with default parameters and prediction scores >5.0 (Kosugi et al., 2009). The psRNATarget tool (Dai et al., 2018) was used to identify the microRNA which targets *RcGRAS* genes.

2.8. RNA isolation and qRT-PCR analysis

Total RNA was isolated using PureLinkTM RNA Mini Kit (Invitrogen) as per manufacturer protocol. The Verso cDNA Synthesis Kit (Thermo Scientific) was used to convert total RNA to cDNA. Quantitative RT-PCR (qRT-PCR) was conducted using SYBR green master mix (Applied Biosystems) in StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, USA). The primers of the selected *RcGRAS* genes were designed using Primer Express[®] Software ver3.0 (Applied Biosystems) and listed in Table S2. The housekeeping gene *RcActin* was used as internal control. Each reaction was conducted with three

technical replications. Relative expression of genes were measured using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

3. Results

3.1. Genes encoding GRAS transcription factors (TFs) in the rose genome

A total of 59 genes that encoding GRAS TFs were identified from the rose genome. The basic descriptions of all RcGRAS TFs were listed in Table 1 and Table S3, including the gene nomenclature, chromosomal position, coding sequence (CDS) length, the protein length, the protein MW, theoretical pI, conserved domains, the predicted subcellular localization. All of these 59 RcGRAS proteins had a conserved GRAS domain (PF03514); however, three RcGRAS members (RcGRAS7, RcGRAS23 and RcGRAS25) also possessed a DELLA domain (PF12041). The protein length and molecular mass of the RcGRAS TFs showed enormous variations, with lengths ranged from 160 to 832 amino acids, molecular weights ranged from 19.08 to 93.79 kDa and isoelectric points ranged from 4.63 to 9.62 (Table 1). We named these GRAS candidates genes from *RcGRAS1* to *RcGRAS59* based on their coordinate position (from top to bottom) on seven different rose chromosomes.

3.2. Chromosomal locations

Based on genome annotation, the *RcGRAS* genes were positioned on the rose chromosomes (Fig. 1). We observed that 59 *RcGRAS* genes were unevenly distributed on the seven rose chromosomes (Chr1 to Chr7), for instance Chr5 had 25 genes (42.4%), followed by Chr2 (12, 20.3%), Chr1 (6, 10.1%), Chr3 and Chr7 (5, 8.5% on each), Chr6 (4, 6.8%) and Chr4 (2, 3.4%). Intriguingly, of the 21 *RcLISCL* genes, 19 genes were located in a cluster on Chr5 (Fig. 1). Further, any correlation between the chromosome length [ranges from 49.74 (Chr3) to 89.95 Mb (Chr5)] and the number of *RcGRAS* genes was not found.

3.3. Phylogenetic and syntenic analyses of GRAS genes

To identify the evolutionary relationships of the GRAS TFs in rose and to classify them into different subfamilies, a total of 198 full-length GRAS proteins, comprising 59 from rose, 33 from Arabidopsis, 52 from rice, 54 from strawberry, were used to construct a phylogenetic tree (Fig. 2). The GRAS proteins of the four species were grouped into 17 different subfamilies. Out of 17, 16 subfamilies (PAT1, LISCL, SCL3, SCL32, DELLA, SCR, DLT, HAM, LAS, SCL4/7, SHR, SCLA, RAD1, RAM1, NSP1, NSP2) were designated according to previous studies (Cenci and Rouard, 2017; Sidhu et al., 2020; Tian et al., 2004). A remaining Rosaceae-specific subfamily (contains only rose and strawberry GRAS proteins) which is not grouped into any of the earlier reported subfamilies was named as *Rc2* subfamily. It suggests that *Rc2* subfamily had been gained in the rose and strawberry lineage after divergence from the most recent common ancestors (Arabidopsis and rice). Further analysis revealed that LISCL was the largest subfamily which accounted for 29% (58) of the total GRAS genes, while RAM1 and SCLA were the smallest subfamilies and each consist of three members (Fig. 2). It was also observed that in all clades

strawberry GRAS were closer to rose GRAS proteins than other two species (Arabidopsis and rice) used in this study.

To further understand the evolutionary traces for the GRAS genes, we identified orthologous genes between rose and Arabidopsis and rose and rice. Total 26 and 20 GRAS orthologous gene pairs were identified between rose/Arabidopsis and rose/rice, respectively (Fig. 3 and Table S4). We observed that each Arabidopsis GRAS gene had one to three rose orthologous genes, for instance AT5G59450 showed an ortholog relationship with the three rose genes (*RcGRAS12*, *RcGRAS14* and *RcGRAS38*), it showed that some GRAS TFs in rose underwent duplication events. More number of orthologous genes between rose/Arabidopsis as compared to rose/rice emphasized the close relationship of rose and Arabidopsis. We also calculated K_a/K_s (non-synonymous substitution/synonymous substitution) to examine the molecular evolution of orthologous GRAS genes (Table S4). We found that most of the orthologous gene pairs had $K_a/K_s < 1$, indicating that the GRAS genes underwent purifying selection during the evolution.

3.4. Motif configurations and gene structure analysis of RcGRAS Family

Motif composition analysis of RcGRAS proteins showed that most of the motifs were located C-terminus as compare to N-terminus (Fig. 4). In general, it was also observed that RcGRAS protein belongs to a similar subfamily had similar motif composition and orders. For instance, the LISCL subfamily contained all the ten types of motifs. Motifs 3, 9 and 10 were not found in HAM, motifs 3 and 10 were not found in NSP2 and LAS, motifs 8 and 10 were not found in DLT, motifs 6 and 7 were not found in RAD1 and motifs 2 and 9 were not found in *Rc2* (Fig. 4 and Table S5). Further, motifs 1, 4, and 5 were found in all the 17 GRAS subfamilies, advocating significant roles in the GRAS genes' conserved function in rose. The variations in motif number and localizations on GRAS subfamilies suggested that GRAS proteins' function likely diverged during its expansion in plants.

Exon-intron pattern analysis of rose GRASs showed that *RcGRAS* genes have one to three exons. Most of the RcGRAS (48, 81.3%) do not have introns and only 11 (18.7 %) genes with 1–2 introns (Fig. 4). Generally, members of the same subfamily had similar gene structures and domains. For example, RcGRAS7, RcGRAS23 and RcGRAS25 belonged to the DELLA subfamily, and each of them consists of single exon and DELLA domain (Fig. 4 and Table S3).

3.5. Cis-acting regulatory elements (CARE) in GRAS genes

The cis-acting **regulatory** elements (CAREs) play an important role in the transcriptional regulation of the genes. We also explored for the CAREs in the upstream sequences (2 kb) of identified *RcGRAS* genes. In total 47 types of CAREs were identified in the promotor region of *RcGRAS* genes (Fig. 5). These CAREs were classified into four categories according to their biological function, including (1) stress response elements, (2) development-related elements, (3) light-responsive elements, and (4) hormone response elements. Among these CAREs, the stress-responsive elements were most abundant (1175) and included ARE, CAAT, DRE-core, F-box, LTR, MBS, TC-rich repeats, W box, GC-motif. Stress-responsive elements were followed by light response (571), hormone-related (541) and development-related (74) elements (Fig. 5).

These results indicated that *RcGRAS* genes diversely respond to various abiotic and biotic stresses and might regulate different biological processes.

3.6. NLS prediction and sub-cellular localization

Generally, the intracellular distribution of TFs dynamically trades between the nucleus and cytoplasm. The NLS at the C-terminal of various TFs are required to regulate this localization (Heerklotz et al., 2001). The NLS prediction in the GRAS TFs revealed that out of 59 rose GRAS TFs, 31 members have either bipartite or monopartite or both types of NLS (Table S3). For instance, 25 GRAS TFs were predicted with bipartite NLS. Monopartite NLS was predicted in five GRAS TFs (*RcGRAS24*, *RcGRAS25*, *RcGRAS26*, *RcGRAS24*, *RcGRAS58* and *RcGRAS59*). One GRAS TFs (*RcGRAS56*) was predicted with both NLSs (Table S3). Further, subcellular prediction of rose GRAS TFs showed that most of these were confined into the nucleus or cytoplasm. Forty-four *RcGRAS* genes were located in the nuclei and the remaining 15 were located in the cytoplasm (Table S3).

3.7. MicroRNA target analysis

According to earlier studies, GRAS TF members are regulated by miRNA171 in plants (Guo et al., 2019; Ma et al., 2014). Thus, we searched for putative target sites of miR171 on rose GRAS TFs. Our results showed that two *RcGRAS* genes (*RcGRAS4* and *RcGRAS57*) belonging to HAM subfamily has targeted by miR171 (Fig. 1 and Fig. S1).

3.8. Tissue-specific expression of *RcGRAS* genes

Expression profiling of total 22 GRAS genes were conducted on three different tissues including leaf, stem and flower bud. These genes belonged to 13 different subfamilies of GRAS. We observed different expressions the genes belonged to the same sub-family (Fig. 6). For instance, three genes (*RcGRAS57*, *RcGRAS9*, *RcGRAS4*) of subfamily HAM showed a different pattern of expression in three tissues. No significant differences were observed in expression for gene *RcGRAS57* in leaf, stem and flower bud, however, *RcGRAS9* significantly upregulated in stem than the leaf and bud, and in the case of *RcGRAS4* significant downregulation was observed in flower bud as a comparison to leaf. Similarly, in case of DELLA, three genes (*RcGRAS7*, *RcGRAS23*, *RcGRAS25*) showed different expression patterns in three tissues. No expression difference was observed for *RcGRAS23*; however, *RcGRAS7* and *RcGRAS25* significantly down- and up-regulated in stem tissues respectively. *RcGRAS30* of SCL3 subfamily showed 20- and 18-times higher expression in stem and flower bud compared to leaf, respectively; however, *RcGRAS1* of SCL3 significantly down-regulated in stem and flower bud (Fig. 6).

Altogether, four GRAS genes [*RcGRAS9* (HAM), *RcGRAS22* (LISCL), *RcGRAS19* (SCR) and *RcGRAS8* (SCL4/7)] were showed unique expression in stem. *RcGRAS9*, *RcGRAS19* and *RcGRAS8* showed enhanced expression and *RcGRAS22* showed decreased expression in stem tissue. None of the GRAS genes showed unique expression in a flower bud. However, total five GRAS genes [*RcGRAS7* (DELLA), *RcGRAS30/RcGRAS1* (SCL3), *RcGRAS50* (LAS), *RcGRAS53* (Rc2)] showed leaf specific

expression. *RcGRAS7* and *RcGRAS1* were upregulated while *RcGRAS30*, *RcGRAS50*, and *RcGRAS3* were down regulated in leaf tissues. Significant differences in expression level of two GRAS genes (*RcGRAS16* and *RcGRAS8*) were observed in all the three tissues (Fig. 6).

3.9. Expression profile of *RcGRAS* genes to exogenous GA treatment

Under GA treatment, downregulation was observed for most of the GRAS genes (Fig. 7A). We examine the expression of 11 GRAS genes under GA treatment in three tissues leaf, stem and flower bud. These genes belonged to seven sub-families. No expression was detected for GRAS genes belonging to four sub-families, including DELLA, LISCL, HAM, and SCL32 in any tissue under exogenous GA treatment. In the case of *RcGRAS1* (SCL3), significant downregulation was observed in stem and flower bud under GA treatment; however, GA treatment did not affect the *RcGRAS1* expression in leaf. Different expression patterns were observed in case of two genes (*RcGRAS6* and *RcGRAS16*) of PAT1 sub-family. *RcGRAS16* was significantly down regulated under GA treatment, however, *RcGRAS6* was significantly up-regulated in leaf, downregulated in stem under GA treatment. No effect of GA was observed in the expression of *RcGRAS3* (*Rc2*) gene (Fig. 7A).

3.10. Expression profile of *RcGRAS* genes under drought stress

Expression of five GRAS genes (*RcGRAS15*, *RcGRAS18*, *RcGRAS28*, *RcGRAS32*, *RcGRAS56*) of four sub-families (SCR, RAM1, PAT1, DLT) were examined under drought stress condition (Fig. 7B). We observed that four genes *RcGRAS15* (SCR), *RcGRAS18* (RAM1), *RcGRAS28/ RcGRAS32* (PAT1) significantly downregulated under drought stress conditions; however, drought had no significant effect on the expression of the *RcGRAS56* (DLT) gene.

4. Discussions

GRAS genes are reported to play various role in plant growth and development, phytohormone and phytochrome signaling, AM symbiosis and stress responses (Bolle, 2004; Davière and Achard, 2016; Fode et al., 2008; Greb et al., 2003; Guo et al., 2019; Heckmann et al., 2006; Zhang et al., 2020). They are hence promising targets that could be used for plant and crops species improvement. GRAS gene family has been identified and characterised in more than 30 plant species (Table S1); however, associated studies on the rose GRAS gene family have not been performed. We identified and characterized 59 GRAS TF family members in the rose genome. All the identified *RcGRAS* genes were randomly distributed on seven rose chromosomes (Chr1-Chr7) (Fig. 1). Markedly, Chr5 had the highest number of genes (25 genes), which were mainly belong to LISCL (19), PAT1 (3), SCL3 (1), RAD1 (1) and LAS (1) subfamilies. The number of GRAS members in rose is higher than the Arabidopsis (33 members), cabbage (35 members) and sacred lotus (38 members), close to that in barrel clover (59), strawberry (54), and pepper (50), and less than that in mustard (88), rapeseed (92), soybean (117), cotton (150), apple (127) for detail see Supplementary Table S1. This variation in GRAS gene numbers might be associated with genome size or gene duplication events during the evolution of a particular plant species (Grimplet et al., 2016).

Interestingly, LISCL subfamily members in rose were found in a higher number as compared to other members, in total 35.6 % (21 of 59 genes) of GRAS genes belongs to the LISCL subfamily. This number is significantly higher than plant species, such as cotton (13%), tea plant (19%), castor beans (15%), Arabidopsis (21%) and rice (20%) but similar to strawberry (35%) belongs to family Rosaceae. These observations indicated an apparent expansion of LISCL subfamily during the Rosaceae family's evolution.

The phylogenetic analyses showed that the GRAS proteins of rose, Arabidopsis (model eudicot plant species, rice (model monocot plant species), and strawberry (model plant for Rosaceae family) were grouped into 17 different subfamilies (Fig. 2). Interestingly, three of these subfamilies (SCLA, RAM1, and RAD1), do not have members in Arabidopsis; however, in the case of rice, members of these three subfamilies were available. These results suggested that these three subfamilies existed before the divergence of dicots and monocots, however, somehow lost in Arabidopsis. We also found one subfamily *Rc2*, appeared to be a Rosaceae-specific family, because GRAS members belong to Arabidopsis or rice were not present in this family. Similar specific families were also reported in other plant species, such as Rc-GRAS in castor beans (Xu et al., 2016), Pt20 family in populus and tomato (Huang et al., 2015; Liu and Widmer, 2014), G-GRAS in cotton (Zhang et al., 2018), and *Fve39* in strawberry (Chen et al., 2019). The species-specific families may be evolved from common ancestors but after the divergence of monocot and eudicot. To further explore the evolutionary relationships of GRAS members, we also carried out the synteny analyses. We observed that the number of orthologous gene pairs between rose and Arabidopsis were more than that between rose and rice (Fig. 3). It further suggested that rose is genetically closer to Arabidopsis as compared to rice.

As earlier studies showed that the GRAS family in the plant has been evolved from the prokaryotes via horizontal gene transfer (HGT) along with gene duplications (Zhang et al., 2012). Results of gene structure analysis of *RcGRAS* genes showed that most (81%) of them were introless (Fig. 4), which is a typical feature in prokaryotes (Roy and Penny, 2007). It suggested that *RcGRAS* genes originated from the prokaryotes *via* HGT. Similar results were also observed in other species such as Arabidopsis (Tian et al., 2004), soybean (L. Wang et al., 2020), orange (Zhang et al., 2019), buckwheat (M. Liu et al., 2019), maize (Guo et al., 2017) and cassava (Shan et al., 2020), where, most of the GRAS genes lack introns (60-80%) or have only single intron (30-40%). But, some of the LISCL members (5) in rose had introns suggested the recent evolution of LISCL subfamily in rose.

Further, our NLS prediction and subcellular localization analyses showed that most of the *RcGRAS* genes had either bipartite or monopartite or both, and are localized in the nucleus or cytoplasm (Table S3). These results are in agreement with earlier studies (Guo et al., 2019; Song et al., 2014; N. Wang et al., 2020) and suggested that the function of these GRAS genes may be conserved in plants.

Earlier, it was reported that *miR171* post-transcriptionally regulates some HAM subfamily genes (Guo et al., 2019; Ma et al., 2014). Interestingly, in the present study, two *RcGRAS* genes (*RcGRAS4* and *RcGRAS57*) belonging to HAM subfamily were found to have a putative binding site for *miR171* (Fig. S1).

The miR171-GRAS module has been reported to involve in regulation meristem development and maintenance in plants (Fan et al., 2015; Huang et al., 2017). Our results also indicated that miR171-GRAS module might also be involved in meristem development and maintenance in rose.

The CAREs are involved in the regulation of several biological processes in plants, i.e., growth, development and stress responses (Bilas et al., 2016). Promoter analysis of *RcGRAS* genes revealed that the CAREs associated with stress (ARE, CAAT, GC-motif, MBS, DRE-core, F-box, LTR, TC-rich repeats, W box), light response (MRE, ACE, AE-box, ATCT-motif, GATA, I-box, CAG-motif, BoxII) and hormone-related (ABRE, CGTCA-motif, ERE, P-Box, TATC-box, TCA-element) were broadly existed (Fig. 5). The presence of a similar type of CAREs in the promoter region of GRAS genes in other plant species has also been documented (Li et al., 2019; Shan et al., 2020; L. Wang et al., 2020). These results indicated that *RcGRAS* genes may also be involved in various stresses and regulation of different biological processes in rose.

To explore the expression patterns of *RcGRAS* genes in different tissues, we examined the relative expression of GRAS genes in leaf, stem, and flower bud, and identified the tissue specific expression of GRAS genes, which suggested the possible involvement of GRAS gene in different growth and development processes in case of rose also. For instance, *RcGRAS9* (HAM) showed significantly higher expression in stem tissue. HAM protein is well known for the maintenance of indeterminate shoot growth in flowering plants (Engstrom, 2012). The interactions between *HAM*, *Wuschel* (*WUS*) and *CLAVATA3* (*CLV3*) maintain the indeterminate stem growth (Engstrom, 2012). Further, DELLA proteins are the negative regulator of GA signaling. In presence of GA, DELLA degraded and resulted into activation of downstream genes involved in plant growth particularly stem elongation. It has been shown that DELLA form a complex with IDD (DELLA/IDD complex) and upregulate the *SCL3* (a positive regulator of GA) (Yoshida et al., 2014). During present study, significantly downregulation of *RcGRAS7* (DELLA) and upregulation of *RcGRAS30* (*SCL3*) in stem and flower bud as comparison with the leaf were good concurrence with earlier reports, and suggested possible interaction of DELLA and *SCL3* in case of rose also. In *Arabidopsis*, *SHR/SCR* regulatory complex has been characterized for the development of endodermis in shoot, root and leaves (Cui et al., 2007; Dhondt et al., 2010; Yoon et al., 2016). During the present study, the similar expression pattern of *RcGRAS19* (*SCR*) and *RcGRAS11* (*SHR*) in three tissues of rose also support the formation of a regulatory complex of *SHR/SCR* and their role in plant development and growth. During the present study, different expression patterns of GRAS genes of the same or other family suggested the multitasking role of GRAS genes in plant growth and development in the case of rose also.

GA is an important phytohormone that promotes seed germination, elongation of root and shoot, fruit ripening, etc. GRAS genes are well characterized to be involved in GA signaling in different plant species and act as a repressor of GA response in plants (Park et al., 2013; Tyler et al., 2004). Application of exogenous GA led to downregulation of GRAS genes particularly DELLA, subsequently activation of downstream genes involved in plant growth. In *Brachypodium*, the expression of most of the GRAS genes declined to zero under exogenous GA treatment (Niu et al., 2019); however, some GRAS genes showed different expression in different tissues. Similarly, in *Citrus sinensis*, most GRAS genes downregulated

under GA treatment (Zhang et al., 2019). In the present study, expression analysis of GRAS genes under GA treatment suggested the involvement of RcGRAS in GA regulatory pathways. Expression of GRAS genes of four sub-families, including DELLA, LISCL, HAM, and SCL32, declined to zero. This emphasized the important role of these GRAS genes in GA signaling and regulatory pathways. Some RcGRAS (*RcGRAS1* and *RcGRAS6*) showed differential expression patterns in different tissue suggested the specific role of *GRAS* genes in different tissues. Expression of the rose specific *GRAS* gene (*RcGRAS3*) did not alter in any of the tissues, indicating that RcGRAS had some other role in plant growth than GA signaling.

Further, we have also observed a significant difference in RcGRAS genes' expression under drought stress, which suggested a promising role of GRAS genes in drought stress signaling in rose. Earlier studies also directed GRAS genes' involvement in various abiotic stresses (Liu et al., 2017; Mayrose et al., 2006; L. Wang et al., 2020; Xu et al., 2015). In Soybean, the majority of GRAS genes were up-regulated under drought stress; however, few (18 out of the 52) *GRAS* genes were repressed under drought stress suggested a promising and dynamic role of GRAS genes in providing stress tolerance. During the present study, surprisingly, none of the five gene showed higher expression under drought stress. One possible reason might be that consideration of very few genes during the present study for expression profiling under drought stress and we might lose the information of some potent genes which actually upregulate. Second, down-regulation of these four genes might play an important role in the activation of downstream genes for providing tolerance to plant under drought stress conditions.

5. Conclusions

In total, we systematically identified 59 GRAS genes in rose using the recently available rose genome information. These *RcGRAS* genes were randomly distributed on seven rose chromosomes. The identified *RcGRAS* genes were further categorized into 17 subfamilies based on their phylogenetic grouping with *Arabidopsis* and rice. Gene structure and motif pattern analyses indicated the GRAS members belong to the same subfamily exhibited similarities, which may specify their parallel gene functions. It was also observed that most *RcGRAS* genes lack introns, suggesting that the *RcGRAS* gene structures were prominently conserved. Moreover, CARE and expression analyses suggested their prevalent role in regulations of plant growth and development, GA and drought stress signaling. This study could assist the future function validation of *GRAS* genes and be utilized in rose improvement programs.

Declarations

CRedit authorship contribution statement

Priya Kumari: Data curation, Formal analysis, Writing - original draft

Vijay Gahlaut: Conceptualization, Investigation, Formal analysis, Methodology, Writing-review & editing

Ekjot Kaur: Investigation, Formal analysis

Sanatsujat Singh: Resources, Formal analysis

Sanjay Kumar: Supervision, Writing - review & editing, Funding acquisition.

Vandana Jaiswal: Supervision, Conceptualization,. Writing-review & editing, Funding acquisition, Project administration.

Declaration of competing interest

The authors report no declarations of interest.

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Table

Table 1. Details of various features of the *RcGRAS* genes reported in the present study.

Gene	Gene ID	Sub-family	Chromosome ^a (Mb)	CDS length (bp)	No. of exon	Protein		
						Length (aa)	pI	MW (KDa)
RcGRAS1	RchiOBHm_Chr1g0319641	SCL3	07.28	1440	1	479	6.04	54.23
RcGRAS2	RchiOBHm_Chr1g0334751	Rc2	26.86	1299	1	432	6.34	48.37
RcGRAS3	RchiOBHm_Chr1g0334761	Rc2	26.87	1764	1	587	5.00	66.11
RcGRAS4	RchiOBHm_Chr1g0349141	HAM	42.18	2106	2	701	5.57	77.21
RcGRAS5	RchiOBHm_Chr1g0367811	LISCL	58.05	2202	1	733	5.94	82.04
RcGRAS6	RchiOBHm_Chr1g0378081	PAT1	64.70	2001	1	666	6.59	73.28
RcGRAS7	RchiOBHm_Chr2g0089571	DELLA	03.72	1623	1	540	5.64	59.10
RcGRAS8	RchiOBHm_Chr2g0101761	SCL4/7	13.22	1854	1	617	5.34	67.61
RcGRAS9	RchiOBHm_Chr2g0102171	HAM	13.62	1677	1	558	5.85	62.53
RcGRAS10	RchiOBHm_Chr2g0107531	SCL32	18.80	1392	1	463	5.65	51.82
RcGRAS11	RchiOBHm_Chr2g0108031	SHR	19.55	1533	1	510	5.26	57.34
RcGRAS12	RchiOBHm_Chr2g0118791	NSP2	30.98	624	2	207	8.48	24.03
RcGRAS13	RchiOBHm_Chr2g0118801	NSP2	30.98	1071	1	356	4.98	40.17
RcGRAS14	RchiOBHm_Chr2g0145631	NSP1	63.36	1659	1	552	5.65	60.86
RcGRAS15	RchiOBHm_Chr2g0153661	SCR	70.98	1314	1	437	5.68	47.57
RcGRAS16	RchiOBHm_Chr2g0156761	PAT1	73.33	1677	1	558	6.21	62.59
RcGRAS17	RchiOBHm_Chr2g0160531	SCL32	76.35	1344	1	447	5.04	49.24
RcGRAS18	RchiOBHm_Chr2g0176081	RAM1	88.01	2124	2	707	5.83	78.10
RcGRAS19	RchiOBHm_Chr3g0459291	SCR	07.74	2376	2	791	5.89	86.27
RcGRAS20	RchiOBHm_Chr3g0462211	NSP2	9.959	1608	1	535	4.63	60.37
RcGRAS21	RchiOBHm_Chr3g0462231	NSP2	9.956	1608	1	535	4.69	60.60
RcGRAS22	RchiOBHm_Chr3g0466391	LISCL	12.84	2304	1	767	6.17	86.19
RcGRAS23	RchiOBHm_Chr3g0493011	DELLA	40.10	1695	1	564	5.18	62.02
RcGRAS24	RchiOBHm_Chr4g0431901	RAD1	56.03	1533	1	510	5.33	57.14
RcGRAS25	RchiOBHm_Chr4g0443621	DELLA	64.68	1860	1	619	5.26	68.31
RcGRAS26	RchiOBHm_Chr5g0000891	PAT1	00.48	1638	1	545	5.54	61.27
RcGRAS27	RchiOBHm_Chr5g0024171	RAD1	18.35	1620	1	539	4.89	60.54
RcGRAS28	RchiOBHm_Chr5g0037031	PAT1	31.36	1722	1	573	5.91	63.92
RcGRAS29	RchiOBHm_Chr5g0042621	PAT1	37.46	1584	2	527	5.57	58.34
RcGRAS30	RchiOBHm_Chr5g0050921	SCL3	51.70	1353	1	450	5.82	50.81
RcGRAS31	RchiOBHm_Chr5g0065461	LISCL	71.31	1998	1	665	5.69	75.01
RcGRAS32	RchiOBHm_Chr5g0065481	LISCL	71.34	1992	1	663	5.96	74.54
RcGRAS33	RchiOBHm_Chr5g0065491	LISCL	71.36	1986	1	661	5.89	74.49
RcGRAS34	RchiOBHm_Chr5g0065511	LISCL	71.46	1647	3	548	8.32	61.76
RcGRAS35	RchiOBHm_Chr5g0065531	LISCL	71.48	1809	2	602	6.10	68.15
RcGRAS36	RchiOBHm_Chr5g0065541	LISCL	71.49	2124	3	707	5.41	79.43
RcGRAS37	RchiOBHm_Chr5g0065561	LISCL	71.53	2190	1	729	5.43	81.50
RcGRAS38	RchiOBHm_Chr5g0065571	LISCL	71.54	2499	1	832	5.92	93.79
RcGRAS39	RchiOBHm_Chr5g0065581	LISCL	71.56	2442	1	813	5.37	91.59
RcGRAS40	RchiOBHm_Chr5g0065591	LISCL	71.57	2097	1	698	5.70	79.45
RcGRAS41	RchiOBHm_Chr5g0065601	LISCL	71.59	2073	1	690	5.67	78.26
RcGRAS42	RchiOBHm_Chr5g0065621	LISCL	71.60	1755	1	584	8.67	67.54
RcGRAS43	RchiOBHm_Chr5g0065631	LISCL	71.61	831	1	276	9.15	32.45
RcGRAS44	RchiOBHm_Chr5g0065651	LISCL	71.63	1035	2	344	9.13	39.82
RcGRAS45	RchiOBHm_Chr5g0065671	LISCL	71.67	2061	1	686	5.31	77.89
RcGRAS46	RchiOBHm_Chr5g0065721	LISCL	71.68	705	2	234	9.33	27.95
RcGRAS47	RchiOBHm_Chr5g0065751	LISCL	71.70	483	1	160	9.62	19.08
RcGRAS48	RchiOBHm_Chr5g0065791	LISCL	71.72	1989	1	662	6.09	76.09
RcGRAS49	RchiOBHm_Chr5g0065811	LISCL	71.76	2010	1	669	5.9	76.90
RcGRAS50	RchiOBHm_Chr5g0074111	LAS	80.14	1362	1	453	6.15	51.40
RcGRAS51	RchiOBHm_Chr6g0272211	SCLA	32.37	1458	1	485	6.24	55.12
RcGRAS52	RchiOBHm_Chr6g0277581	PAT1	40.08	1749	1	582	4.9	64.77
RcGRAS53	RchiOBHm_Chr6g0287671	SCR	51.01	1509	2	502	6.17	56.43
RcGRAS54	RchiOBHm_Chr6g0295411	NSP2	57.42	1548	1	515	5.25	56.64

RcGRAS55	RchiOBHm_Chr7g0190881	RAD1	09.79	1590	1	529	5.93	58.82
RcGRAS56	RchiOBHm_Chr7g0199131	DLT	17.17	1983	1	660	5.54	72.80
RcGRAS57	RchiOBHm_Chr7g0202931	HAM	20.57	2337	1	778	5.92	85.33
RcGRAS58	RchiOBHm_Chr7g0241071	Rc2	67.03	1968	1	655	5.63	74.40
RcGRAS59	RchiOBHm_Chr7g0241091	Rc2	67.07	1962	1	653	5.63	74.36

a - starting position

Figures

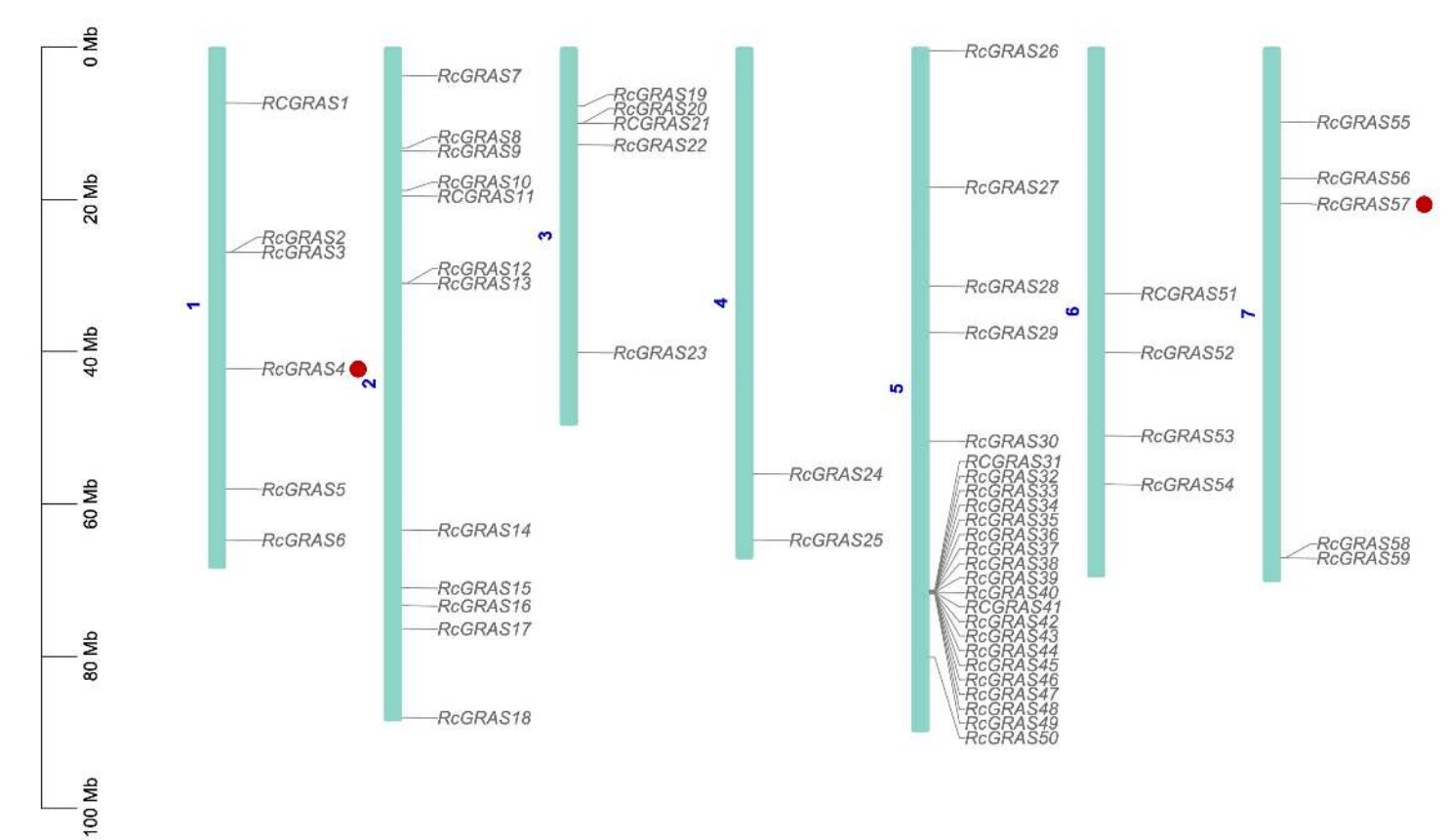


Figure 1

Localization of 59 RcGRAS genes on the seven rose chromosomes. The chromosome number is denoted to the left of each chromosome. A red circle indicates the genes targeted by miR171. The scale is in the megabases (Mb).

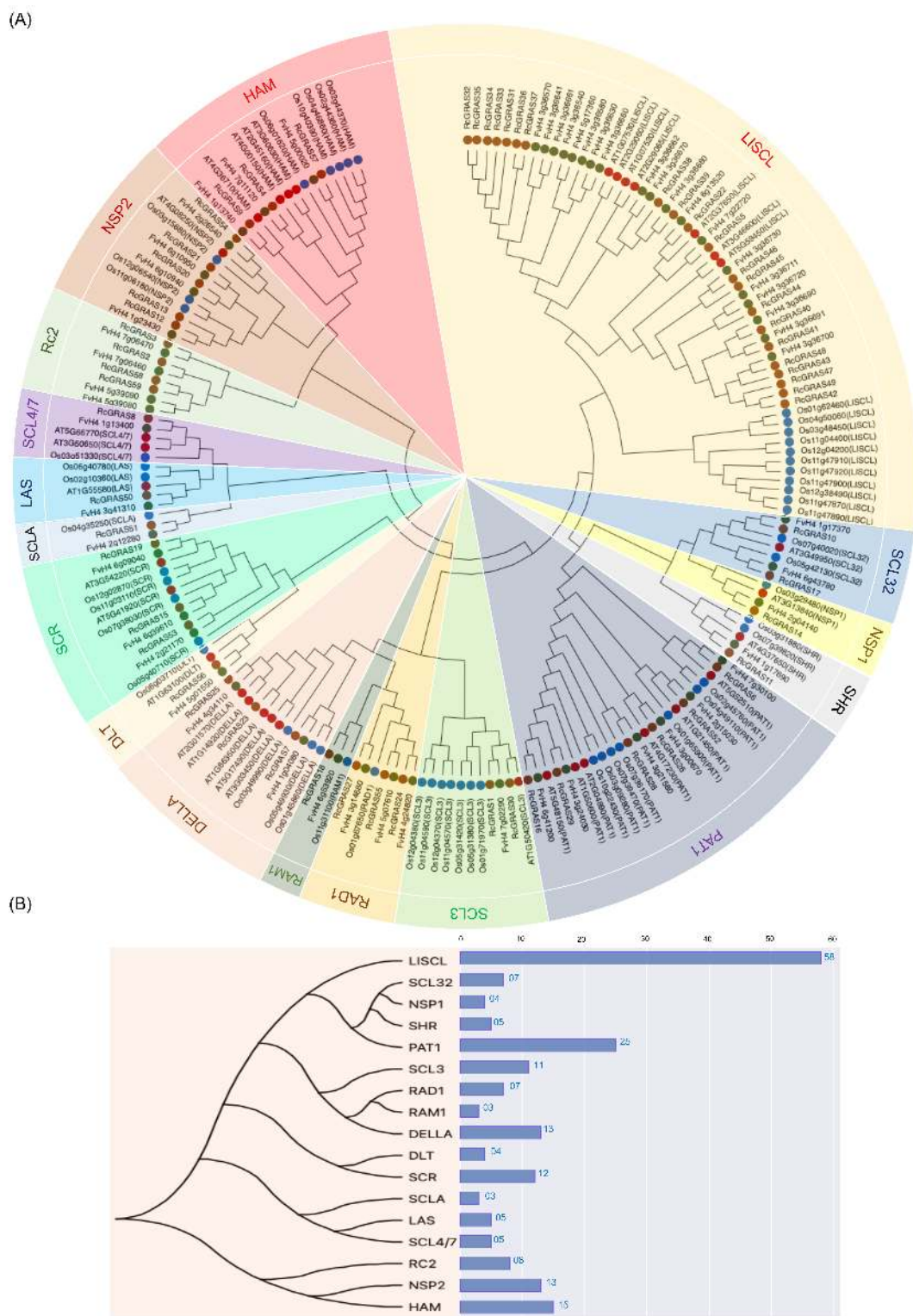


Figure 2

Localization of 59 RcGRAS genes on the seven rose chromosomes. The chromosome number is denoted to the left of each chromosome. A red circle indicates the genes targeted by miR171. The scale is in the megabases (Mb).

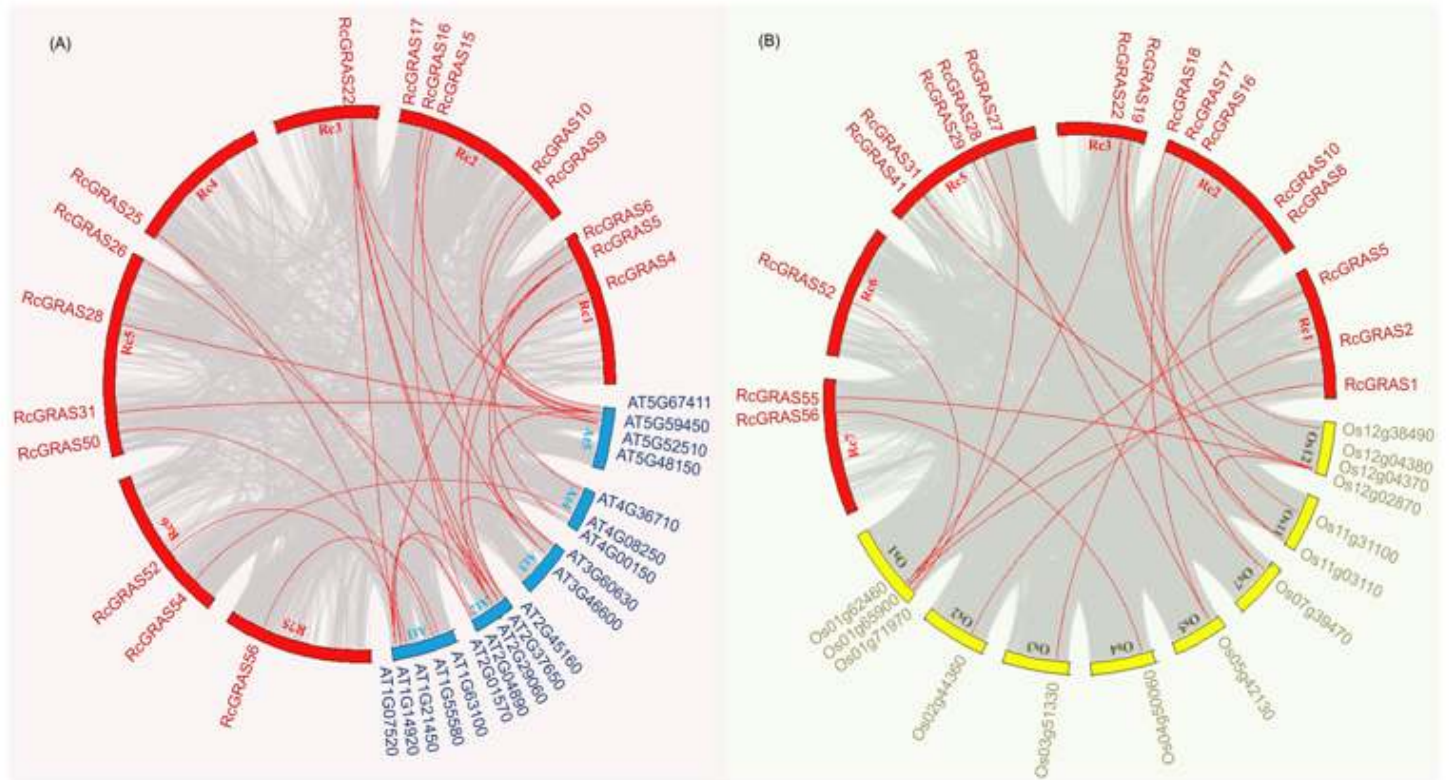


Figure 3

Synteny analysis of GRAS genes between rose and Arabidopsis (A), and rose and rice (B). Red curve lines denote syntenic GRAS gene pairs between two species (rose vs Arabidopsis and rose vs rice). Gray curves lines in the background denote the collinear blocks within pineapple and other plant genomes.

Figure 4

Phylogenetic clustering, gene structures and motif patterns of the RcGRAS members. (A) Phylogenetic clustering showed that the RcGRAS members were grouped into 17 different subfamilies. Phylogenetic analysis was performed using MEGA-X with the neighbor-joining (NJ) method. (B) Gene structures of the 59 RcGRAS genes. CDS and introns are indicated by pink boxes and black lines, respectively. Light blue boxes indicate untranslated 5' and 3' regions. (C) The schematic represent the motif patterns of the RcGRAS proteins. Different colored boxes indicated the ten different MEME-motifs. The sequence information for each MEME-motif in the form of sequence LOGO was also provided.

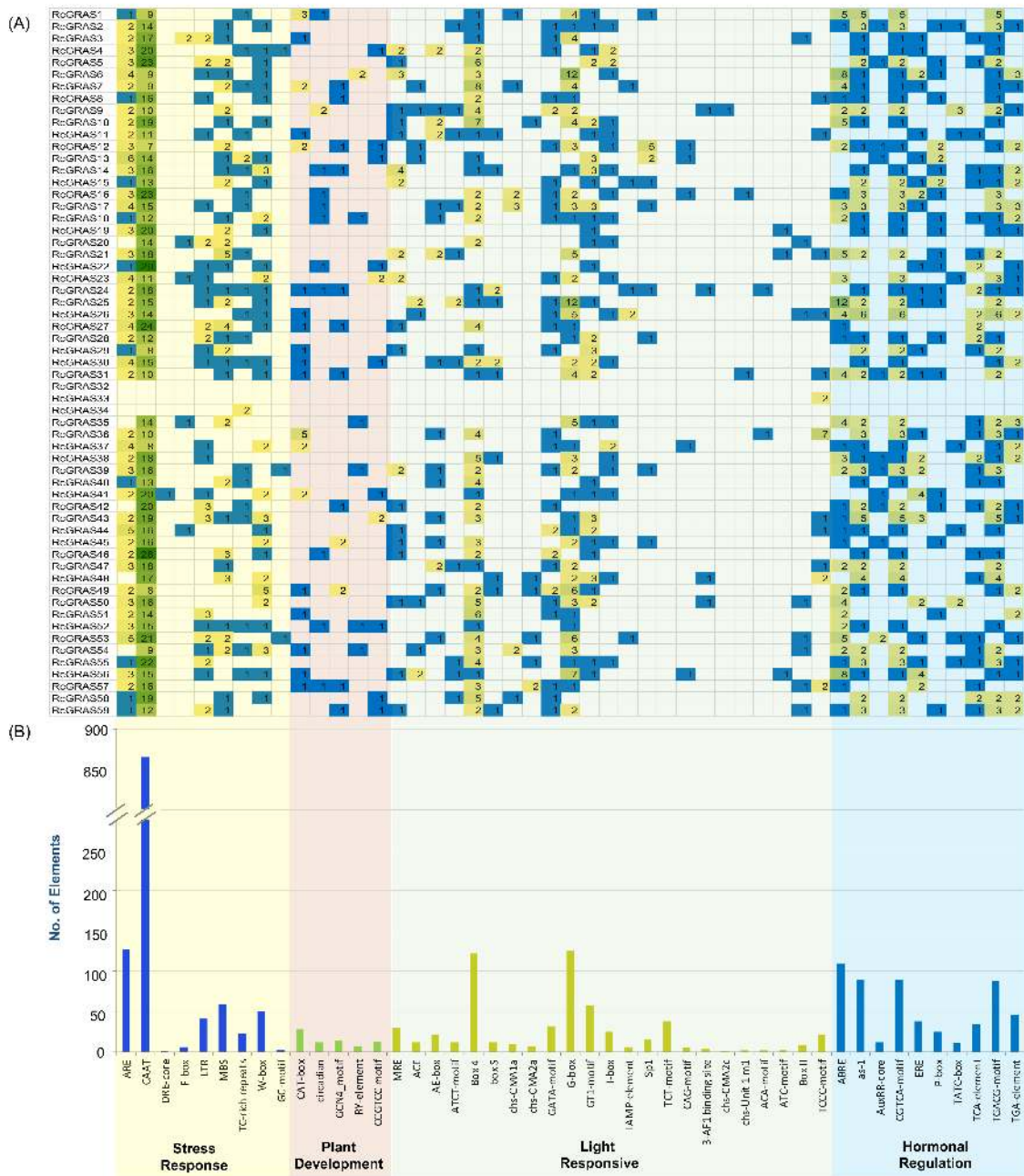


Figure 5

Predicted cis-acting regulatory elements (CAREs) in the promoter regions of RcGRAS genes. (A) Illustrating the number of different CAREs in the promoters of RcGRAS genes. (B) The sum of each CARE belongs to four different biological categories are represented by different coloured histograms.

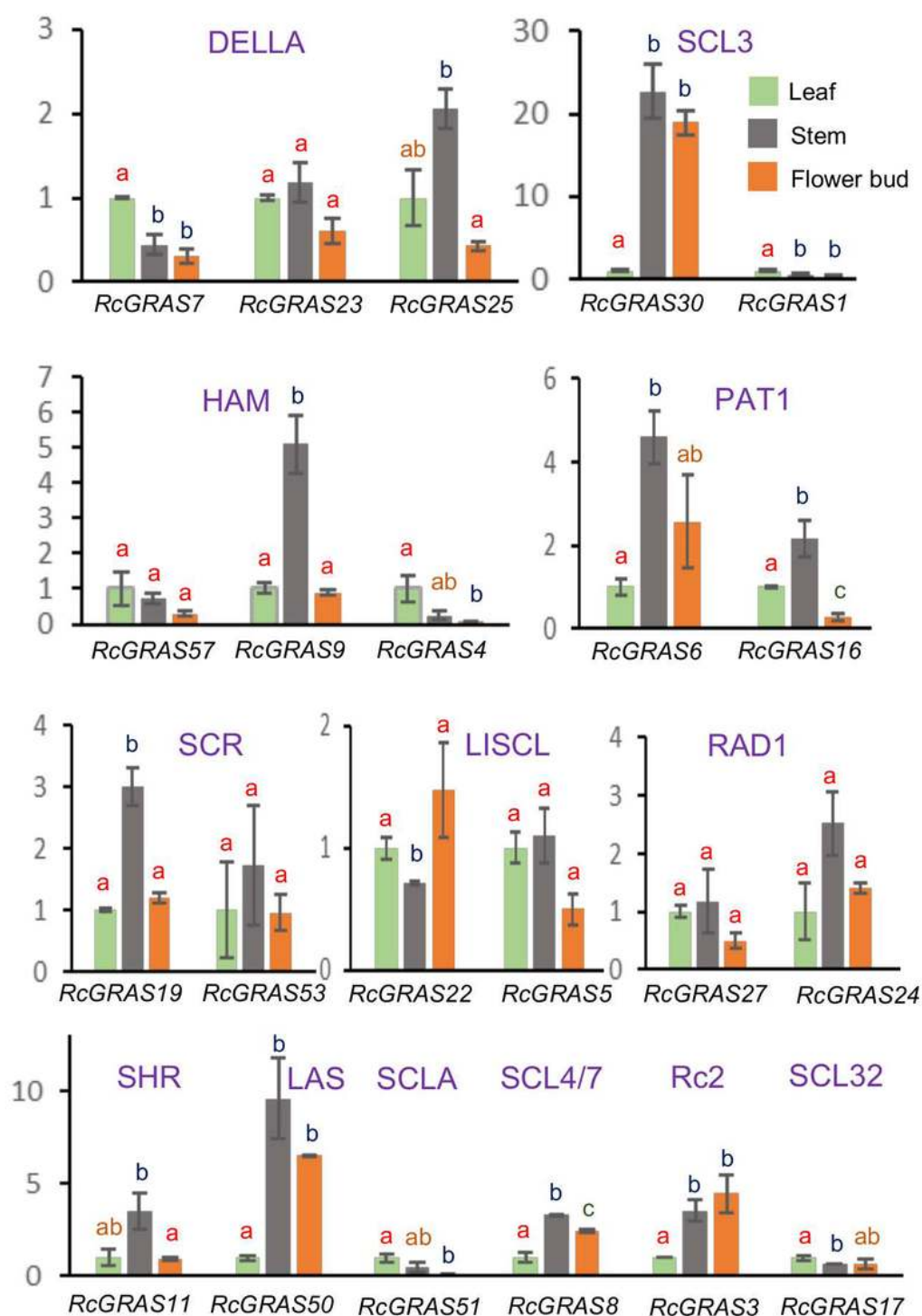


Figure 6

Tissue-specific expression profiles of 22 selected RcGRAS genes in leaf, stem and flower bud tissues. Y-axis represents the relative expression value. Bars represent the mean values of two biological and technical replicates $\pm$ SE. Lowercase alphabets represent significant difference ($p < 0.05$, Student's t-test).

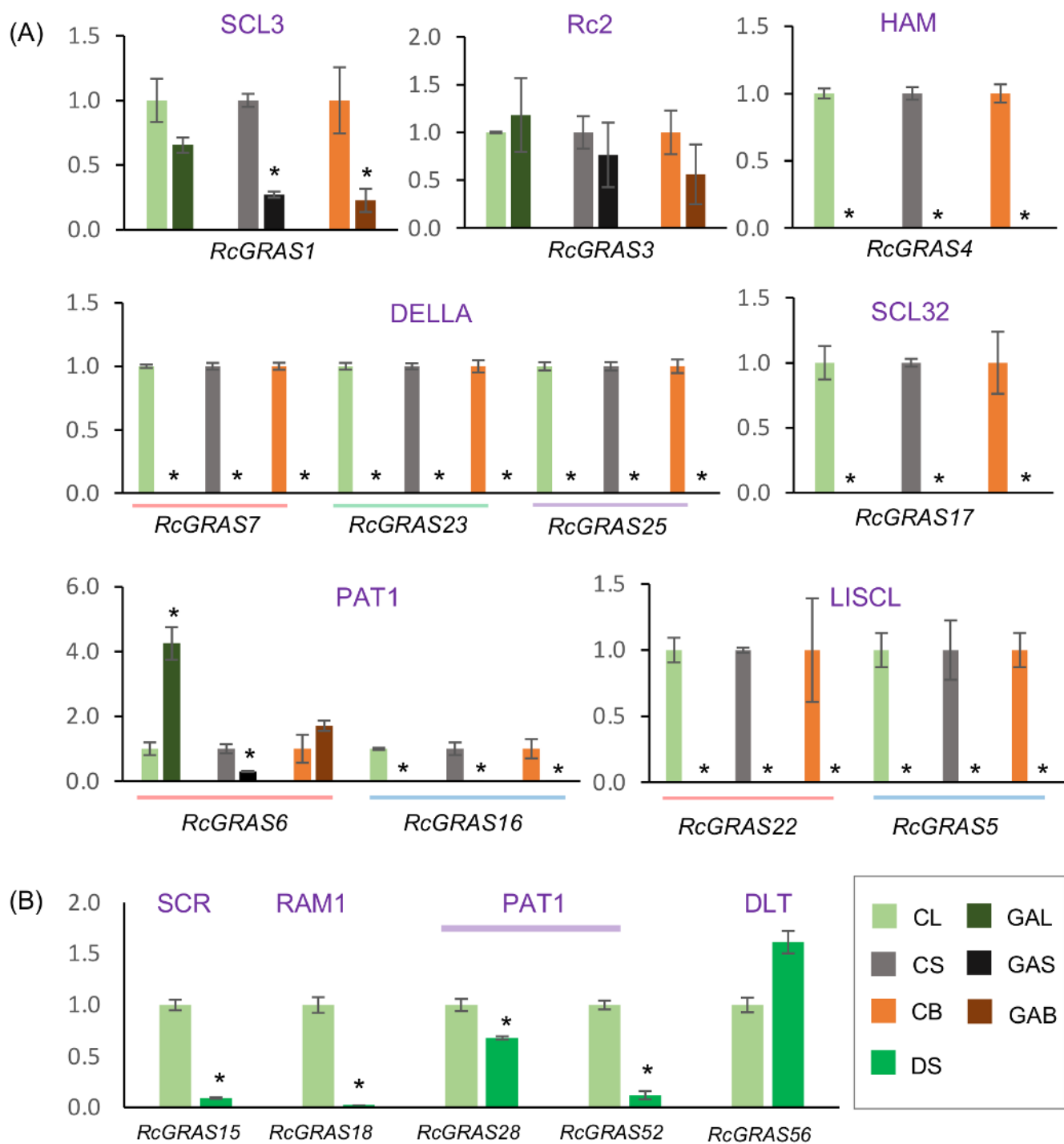


Figure 7

Differential expression detected for RcGRAS genes in response to GA treatment (A) and drought stress treatment (B) in different rose plant tissues. Untreated tissue was considered as control (C) to determine relative fold change. Y-axis represents the relative expression value. Bars represent the mean values of two biological and technical replicates $\pm$ SE. Asterisks (*) marks on the bars represent significantly up-or down-regulation as compared to control (P < 0.05, Student's t-test). CL, leaf tissue of control sample; CS,

stem tissue of control sample; CB, flower bud tissue of control sample; GAL, leaf tissue of GA treated sample; GAS, stem tissue of GA treated sample; GAB, flower bud tissue of GA treated sample; DS, leaf tissue of drought stress treated sample.

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