Multifarious Roles of GRAS Transcription Factors in Plants

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Abstract

The GAI-RGA - and -SCR (GRAS) proteins belong to the plant-specific transcription factor gene family and involved in several developmental processes, phytohormone and phytochrome signaling, symbiosis, stress responses etc. GRAS proteins have a conserved GRAS domain at C-terminal and hypervariable N-terminal. The C-terminal conserved domain directly affects the function of the GRAS proteins. For instance, in Arabidopsis, mutations in this domain in Slender rice 1 (SLR1) and Repressor of GA (RGA) proteins cause significant phenotypic changes. GRAS proteins have been reported in more than 30 plant species and till now it has been divided into 17 subfamilies. This review highlighted GRAS protein's importance during several biological processes in plants, structural features of GRAS proteins, their expansion and diversification in the plants, GRAS-interacting proteins complexes and their role in biological processes. We also summarized available recent research that utilized CRISPR-Cas9 technology to manipulate GRAS genes in a plant for different traits. Further, the exploitation of GRAS genes in crop improvement programs has also been discussed.

Keywords: GRAS protein, DELLA, Intrinsically Disordered Proteins, Arbuscular Mycorrhizal association, abiotic stress

Introduction

The GRAS transcription factors (TFs) are important plant-specific TF family and play multiple biological functions in plants including growth, development, cell signaling, phytochrome signaling, symbiosis, biotic and abiotic stress tolerance etc. (Di Laurenzio *et al.*, 1996; Greb *et al.*, 2003; Bolle, 2004; Fode *et al.*, 2008; Zhang *et al.*, 2020). The abbreviation for GRAS was given based on the first three identified members: GIBBERELLIN-ACID INSENSITIVE (GAI), REPRESSOR of GA1 (RGA) and SCARECROW (SCR) (Pysh *et al.*, 1999). The GRAS proteins

have conserved C-terminal GRAS domain and highly variable N-termini with homopolymeric stretches of certain amino acids (Hakoshima, 2018). The GRAS TFs family found throughout the plant kingdom and already reported in more than 30 species of plants including important species like Arabidopsis (Tian *et al.*, 2004), rice (Tian *et al.*, 2004), soybean (Wang *et al.*, 2020*a*), cassava (Shan *et al.*, 2020), barley (To *et al.*, 2020), cabbage (Song *et al.*, 2014), tomato (Huang *et al.*, 2015), tobacco (Chen *et al.*, 2015), and tea plant (Wang *et al.*, 2018). Based on conserved protein domains and functions, the GRAS TF family further divided into SCR, SCARECROW-LIKE3 (SCL3), SHORT-ROOT (SHR), DELLA, LATERAL SUPPRESSOR (LS), LIGHT SIGNALING through INTERACTIONS LIGHT-RESPONSIVE TRANSCRIPTION FACTOR PIFs (LISCL), HAIRY MERISTEM (HAM), NODULATION SIGNALING PATHWAY 2 (NSP2) and PHYTOCHROME A SIGNAL TRANSDUCTION1 (PAT1) subfamilies (Tian *et al.*, 2004). Functional characterization of many GRAS genes emphasized their involvement during different stages and pathways of plant development, including gibberellic acid (GA) signaling (Peng *et al.*, 1997; Ikeda *et al.*, 2001), brassinosteroid (BR) signaling (Ma *et al.*, 2010; Davière and Achard, 2013), shoot and axillary meristem maintenance (Stuurman *et al.*, 2002; Greb *et al.*, 2003), radial organization of the root and shoot (Helariutta *et al.*, 2000), phytochrome signal transduction (Bolle *et al.*, 2000), nodulation and arbuscular mycorrhiza (AM) symbiosis (Kaló *et al.*, 2005; Heckmann *et al.*, 2006; Shtark *et al.*, 2016), abiotic stress responses (Ma *et al.*, 2010; Guo *et al.*, 2019). The GRAS genes were also involved in the 1960s green revolution, the *Reduced height1* (*Rht1*) and *Rht2 genes* encode mutant DELLA proteins (belongs to GRAS TFs), which have reduced sensitivity for the GA (Hedden, 2003). It was resulting in the development of semi-dwarf plant stature with improved yield and lodging resistance. Further, SHR and SCR (GRAS proteins) were found to form a complex to regulate asymmetric cell division resulted in radial roots (Cui *et al.*, 2007). GRAS protein AtSCL14 interacts with TGACG motif-binding factor (TGA) TFs and regulates stress-response in Arabidopsis (Fode *et al.*, 2008). Another GRAS gene *PeSCL7* was reported to provide tolerance to high salinity, osmotic, and drought stresses (Ma *et al.*, 2010).

GRAS TFs are excellent candidate genes that can be manipulated or engineered for plant improvement due to their dynamic functions. In the following review, we detailed the GRAS protein structure, GRAS-interacting proteins, and their involvement in various biological processes, i.e. plant growth and development, phytochrome and phytochrome signal, AM

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association, biotic and abiotic stresses. Finally, we summarized recent studies that utilized clustered regularly interspaced short palindromic repeats-CRISPR-associated (CRISPR-Cas) based genome editing to manipulate GRAS genes in plants.

Structural features of the GRAS proteins

GRAS proteins are usually 400–770 amino acids long, and their carboxyl (C-) terminal have conserved GRAS domain and amino (N-) terminal have a variable region. The GRAS domain comprises ~390 amino acids and contains five distinct motifs in a specific order i.e. leucine heptad repeat I (LHR I), VHIID, LHR II, PFYRE and the SAW motif (Lee *et al.*, 2008; Hakoshima, 2018). Among these motifs, LHRI is conserved across all GRAS proteins and has nuclear localization signals (NLSs) (Sun *et al.*, 2012). The LHRI-VHIID-LHRII motif complex has been involved in GRAS protein-protein or-DNA partner interactions (Pysh *et al.*, 1999).

The fourth motif PFYRE contains the following three distinct sequence signatures; (i) proline (P) residue, (ii) phenylalanine and tyrosine (FY) residues, and (iii) arginine and glutamic acid (RE) residues. The SAW motif contains the sequence signatures WX7G, LXW, and SAW and is well conserved in GRAS proteins. Exact function of these two motifs PFYRE and SAW are not well defined in plants; however, their conserved nature may suggest that these two motifs are essential for the structural or functional integrity of the GRAS proteins. For instance, PFYRE and SAW motifs are shown to important for normal phenotypic growth, as the mutations in these motifs have resulted in phenotypic changes. SLR1 (SLENDER RICE1), RGA, and NSP1 are important examples of mutation in PFYRE and SAW motifs (Itoh *et al.*, 2002; Heckmann *et al.*, 2006). Unlike the GRAS domain, N- terminal of GRAS proteins are highly variable (Pysh *et al.*, 1999), where the variable length and unique stretch sequence comprised of intrinsically disordered domains (IDDs) intricate in specific molecular recognitions (Sun *et al.*, 2012). These features showed its involvement in protein-protein interactions and gene-specific functions, therefore, imparting the functional specificity to the GRAS proteins so they could act as activators during different regulatory processes (Fig. 1). For instance, GRAS protein's DELLA subfamily contains three motifs (DELLA, VHYNP and LR/KXI motifs) with repeated hydrophobic or aromatic residues (Fig. 1A). It has been reported that these motifs interact with the GA-bound receptor GA-

INSENSITIVE DWARF 1 (GID1) and hence play a crucial role in GA signaling and ultimately plant development (Murase *et al.*, 2008). Similarly, in the case of other GRAS subfamilies, the repeated hydrophobic/aromatic residues (SHR; poly-Q, poly-T, Poly-S/H and SCR; poly-S/P, poly-S, polyQ/P) play an analogous role in the binding of GRAS proteins with their interacting partners (Fig. 1B, C) (Tian *et al.*, 2004; Sun *et al.*, 2012).

GRAS proteins: Functions as transcription factors (TFs)

Based on several structural features of GRAS proteins, including homopolymeric sequences of amino acid residues and the existence of LHR domains, they have been supposed to work as TFs in plants. Additionally, several GRAS proteins also had NLSs and localized in nuclear (Tian *et al.*, 2004) further strengthen its function as TF. In the case of *M. trancatula* and *Populus euphratica*, *MtNSP1* and *PeSCL7* were localized in the nucleus (Smit *et al.*, 2005; Ma *et al.*, 2010). Similarly, a LiSCL protein from lily has two highly basic regions, which are essential for nuclear localization (Morohashi *et al.*, 2003). There are certain exceptions too, highlighting the reasons for the diverse function of GRAS proteins. *MtNSP2* is found to be associated with the endoplasmic reticulum/nuclear envelope, and after the addition of the Nod factor, it relapses into the nucleus to execute its function (Kaló *et al.*, 2005). In Bottle gourd, 10 genes were found to be located in the chloroplast, one each in mitochondria, endomembrane system, and the extracellular space (Sidhu *et al.*, 2020). In case of Cassava, four MeDELLAs proteins were found to localize in cytoplasm and also in nuclei (Li *et al.*, 2018). The protein-protein interaction and gene expression analysis revealed that these genes have tissue-specific localization and their interaction with various other genes probably regulates the interacting partners in specific plant tissues.

GRAS gene family: Expansion and diversification in the plants

The GRAS genes have been reported in more than 30 plant species, and number of genes ranged from 33 (Arabidopsis) to 150 (cotton) (Table 1). However, their classification into different subfamilies is still not standardized. Initially, the GRAS genes were clustered into eight subfamilies, including- (i) DELLA, (ii) HAM, (iii) LISCL, (iv) PAT1, (v) LS, (vi) SCR, (vii) SHR and (viii) SCL9 in case of rice and Arabidopsis (Tian *et al.*, 2004; Lee *et al.*, 2008). Similar to Arabidopsis and rice, eight subfamilies of GRAS genes were also reported in apple (Fan *et al.*,

2017), walnut (Quan *et al.*, 2019) and clover (Zhang *et al.*, 2017). However, in other plant species, the number of subfamilies is higher, 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) and 16 subfamilies were identified in bottle gourd (Sidhu *et al.*, 2020). While combined phylogenetic analysis using eight different angiosperm species, regroup GRAS TF into 17 subfamilies (Cenci and Rouard, 2017). To further understand the evolutionary relationship, we also performed the phylogenetic analysis using 581 GRAS proteins from 12 plant species and found that GRAS proteins were grouped into 17 subfamilies (For detail, see Fig. 2). The studies mentioned above showed considerable divergence among the GRAS family genes in flowering plants, and in the future, more studies on GRAS genes in the plant kingdom may identify more subfamilies.

GRAS proteins are not present in algae, but were first identified in bryophytes, *Marchantia Polymorpha* and *Sphagnum fallax* (Song *et al.*, 2017; Guo *et al.*, 2019). GRAS protein sequences across different plant species, including lower plants and higher plant species, showed 12 subtle clades in a phylogenetic tree, and in flowering plants, the GRAS family showed maximum divergence. There is enormous variability in the number of subfamilies of GRAS TFs among different plant species (Table 1), suggest that there is some species or lineage specific retention of GRAS subfamilies during evolution. Further, the less conservation of the GRAS domains across different subfamilies, but more sequence similarities (up to 98%) within subfamilies highlights similar functions of GRAS genes (Sun *et al.*, 2012). For example; most of the genes characterized yet from the DELLA subfamily are involved in GA signaling pathway. It has also been confirmed with GRAS proteins functional characterization in different plants, which has indicated a conserved function/pattern among putative orthologues of each subfamily and/or subclade in different plants. Another good example is the NSP1 gene, which is conserved in leguminous and non-leguminous crops and associated with nodule formation (Smit *et al.*, 2005; Heckmann *et al.*, 2006).

In the case of bryophytes, ESTs with a similar GRAS gene sequence suggest that this protein family has aroused before the rise of the land plants (Nishiyama *et al.*, 2003). In plant, GRAS genes are mostly intronless including grapevine (88.46%), barley (88.2%), tomato (77.4%),

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capsicum (84%), *Brassica rapa* (83.3%), *Prunus* (82.2%), Arabidopsis (67.6%), rice (55%) and populus (54.7%) (Abarca *et al.*, 2014; Liu and Widmer, 2014; Song *et al.*, 2014; Huang *et al.*, 2015; Lu *et al.*, 2015; Grimplet *et al.*, 2016; Liu *et al.*, 2018; To *et al.*, 2020). The high proportion of intronless GRAS genes unveils that the GRAS proteins are closely related during evolution. The probable causes for the intronless genes in eukaryotic genomes are duplication events and reteroposition of intron-containing genes (Zou *et al.*, 2011). Additionally, intronless genes might also result from the horizontal gene transfer (HGT) from the prokaryotic genome, since intronless genes are a typical prokaryote feature. GRAS-like proteins were initially reported in bacteria and found to involve in methylase activity (Zhang *et al.*, 2012) and later developed in the sister lineage (streptophyte/charophyte) to the land plants (Wilhelmsson *et al.*, 2017).

Intrinsically disordered proteins (IDPs) and functional versatility in GRASs

In eukaryotes, 25-30% of proteins are intrinsically disordered proteins (IDPs) and play a significant role in various molecular and cellular functions. More than 70% of signaling proteins and 82–94% of TFs have intrinsically disordered regions (IDR) (Iakoucheva *et al.*, 2002; Oldfield *et al.*, 2005; Liu *et al.*, 2006). IDR, present within IDP, enables proteins to undergo disorder-to-order transitions to recognize and bind at specific binding interfaces among different partners (Sun *et al.*, 2011, 2012). Computational and bioinformatics analysis revealed that the GRAS proteins also fall under the IDP category (Sun *et al.*, 2011). The GRAS protein's N-terminus contains molecular recognition features (MoRFs), short interaction-prone sites within IDR that upon interaction and recognition of partner's proteins allows specific disorder-to-order transitions to form functional complexes (Sun *et al.*, 2011, 2012). The functional diversification of GRAS proteins is attributed to the intrinsically disordered N-domain, which is reflected by; a) its expansion into different subfamilies, b) role in transcriptional regulation along with signaling pathways, and, c) some proteins can show cross-talk in different signal pathways forming homo- or heterodimers. For example, BdSLR1 and BdSLRL1 can form homodimers but cannot form heterodimers (Niu *et al.*, 2019).

Intrinsic disorder characteristic in the GRAS proteins has provided them binding plasticity, which is directly correlated to the functional polymorphism to these proteins (Dunker *et al.*, 2005;

Haynes *et al.*, 2006; Sun *et al.*, 2011). In the DELLA subfamily, the N-terminal has been characterized as intrinsically disordered, whereas the C-terminal possesses only basic structural protein folding with a series of highly conserved motifs (Sun *et al.*, 2011). This highly variable N-terminal domain with intrinsically disordered characteristics enables this gene family with dynamic functions. The intrinsically disordered N-domain allows GRAS proteins to act as the key regulators in several signaling pathways.

Additionally, phosphorylation and dephosphorylation have widely been observed for the functionality of GRAS proteins. The phosphorylation and dephosphorylation events are at high-probability in GRAS proteins' N-terminal domains because they have a homopolymeric stretch of serine, threonine and tyrosine amino acids. In the functioning of these disordered protein complexes, phosphorylation plays an essential role in adjusting the interactions of disordered protein and ultimately signal transduction events (Iakoucheva *et al.*, 2004; Mittag *et al.*, 2010). For instance, in rice, GID2 interacts specifically with phosphorylated SLR1 protein (a DELLA protein), not with unphosphorylated SLR1 protein; and strongly suggested that only phosphorylated state of SLR1 protein can undergo GA-dependent degradation(Gomi *et al.*, 2004). Moreover, a rice kinase (EL1) may also directly phosphorylate SLR1 proteins (Dai and Xue, 2010). In SLR1 proteins, Ser residues present within DELLA/TVHYNP and polyS/T/V domain (at N-terminal domain) undergo phosphorylation process (Itoh *et al.*, 2005). The experimental demonstrations have confirmed that rice SLR1 and *Brachypodium* BdSLR1 show that the DELLA domain and TVHYNP motif are the key regulators for transcriptional activation activity and GID1 interaction (Hirano *et al.*, 2012; Niu *et al.*, 2019). Further, two nodulation specific GRAS proteins, NSP1 and NSP2, are phosphorylated by CCaMK (calcium- and calmodulin-dependent protein kinase) to facilitate nodulation signaling (Mitra *et al.*, 2004; Gleason *et al.*, 2006). Reversible phosphorylation is also playing a significant role in regulating the stress-induced response of some GRAS proteins. For instance, NtGRAS1 in *Nicotiana tobacum* (Czikkel and Maxwell, 2007), and CIGR1 (chitin-inducible gibberellin-responsive) and CIGR2 in the case of rice (Day *et al.*, 2004) are good examples. Therefore, the phosphorylation and dephosphorylation states of DELLA proteins are critical to govern the stability or degradation of proteins and regulate intracellular signal transduction under different stress conditions (Hall *et al.*, 2002; Day *et al.*, 2004; Czikkel and Maxwell, 2007). Although the knowledge about GRAS proteins' phosphorylation and

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dephosphorylation is still elusive, but disorder-assisted phosphorylation may provide a different dimension to study GRAS proteins and their recognition patterns during signaling mechanisms.

The IDPs also provided a functional advantage over structurally ordered proteins for molecular recognition *via* protein-protein interactions. To facilitate specific recognition, they undergo binding-induced conformational changes and display different binding sites to recognize different partners during protein interactions, giving binding plasticity to these proteins. The MoRFs are responsible for this disorder-to-order transition to recognize their interacting partners. The N-domain is highly variable between subfamilies, but it is found to be highly conserved within the subfamily (Sun *et al.*, 2011). For example; in the DELLA subfamily, repeated hydrophobic/aromatic residues are the restricted motifs in the N domain, and are crucial for GID1 interaction and GA signaling and ultimately plant growth (Murase *et al.*, 2008). The GRAS TFs exhibit a higher level of divergence due to the presence of MoRFs at their N-terminal region, which allows them to function as an activator in different signaling and molecular pathways. Whereas, C-terminal region is highly conserved in GRAS family and interacts with downstream proteins (Pysh *et al.*, 1999; Bolle, 2004). For instance, Leu heptad repeats in the conserved LHRI-VHIID-LHRII domain involved in protein-protein interactions, which has also been proved experimentally by following interacting pairs, where these interactions are conferred by either an individual motif or the entire region: DELLA–PIFs, DELLA–JAZ1, DELLA–GID2, AtSHR– AtSCR, NSP1–NSP2 and BnSCL1–AtHDA19 (Cui *et al.*, 2007; Fode *et al.*, 2008; Hirsch *et al.*, 2009; Hou *et al.*, 2010). It has been observed that the GRAS proteins are mostly the transcriptional coactivators, which may block or enhance the activity of interacting partners (Sun *et al.*, 2012). Another conserved C-terminus, VHIID domain is involved in protein-DNA interactions (Pysh *et al.*, 1999). Therefore, this transition in protein structure for molecular recognition based upon intrinsic disorder-to-order transition due to the presence of MoRFs provides a hypothetical framework that can help to explore more probable functioning of GRAS TFs in signaling and regulation pathways which can future be confirmed by experimental elucidations.

GRAS -interacting proteins: Regulates the function of GRAS TFs

GRAS proteins mostly function via interaction with an interacting protein partner such as transcription factors (for e.g., IDD, TGA, MYB), transcriptional regulators (BOIs, SPL), chromatin remodeling complexes (Pickle; PKL), and co-chaperones (PFDS) (Zhang *et al.*, 2012,

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2014). In *Arabidopsis*, Indeterminate Domain (AtIDD) proteins (plant-specific TF) have been reported to regulate the expression of SCR by interacting with SHR and SCR, which are further involved in root tissue formation by accommodating their zinc finger motifs into the groove of the SHR GRAS domain (Colasanti *et al.*, 2006; Hakoshima, 2018; Aoyanagi *et al.*, 2020). Further, it was shown that IDD proteins also act as transcriptional coactivators with DELLA proteins. The DELLA/IDD protein complex induces the expression of the *SCL3* gene and regulates the GA signaling pathway in plants (Yoshida *et al.*, 2014). The TGA TFs belong to group D bZIP TFs, which are found ubiquitously in eukaryotes (Johnson *et al.*, 2003). Arabidopsis *LlSCL/SCL9* branch member, *SCL14 (SCARECROW-LIKE 14)* can be recruited to its target promoter region only in the presence of TGA factors. The TGA/SCL14 protein complex regulates the activation of detoxification mechanism upon the challenge of plants with xenobiotics, i.e. isonicotinic acid 2,4,6-triiodobenzoic acid (Fode *et al.*, 2008).

In eukaryotes, chromatin remodeling is essential for gene expression, and chromatin-remodeling factor PKL/Enhanced Photomorphogenic1(EPP1) was found to suppress phytochrome signaling in plants (Jing *et al.*, 2013). Interaction of PKLs with GRAS protein was also reported in the plant (Zhang *et al.*, 2014). PKL interacts with BZR1 and PIF3 to stimulate hypocotyl growth by inhibiting the histone methylation of genes involved in cell elongation (i.e. IAA19, PRE1, TCH4). DELLA proteins interacted with PKL and negatively regulated its activity by constricting its binding ability. Therefore, the PIF-BZR1-PKL-DELLA module regulates H3K27me3 modification status and ensures seed etiolation in plants (Fig. 3A).

Several GRAS TFs are involved in AM symbiotic association mechanism. However, the mechanism of their regulation is a big question. *RAM1*, a major gene involved in the induction of hyphopodia formation in AM fungal association, whose transcription is regulated explicitly by CYCLOPS and DELLA (Pimprikar *et al.*, 2016). The CCaMK, calcium, and calmodulin-dependent kinase, interacts with CYCLOPS to phosphorylate CYCLOPS, which directly binds to a conserved palindromic region cis-element (GGCGCC box/AM-CYC box) located on *RAM1* promoter (Pimprikar *et al.*, 2016). CYCLOPS also interacts with the DELLA, which further boosts its capability to transactivate the *RAM1* promoter along with promoters of other nodulation genes such as, *NIN (NODULE INCEPTION)* and *ERN1(ERF REQUIRED FOR NODULATION1)* (Jin *et al.*, 2016; Pimprikar *et al.*, 2016). Another GRAS interacting protein BOTRYTIS SUSCEPTIBLE1 INTERACTOR (BOI) that acts as a transcriptional regulator of GRAS protein

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has also been reported (Park *et al.*, 2013). It was shown that BOI and DELLA interact and target the promoters of a subset of GA-responsive genes in plants and inhibit their expression that eventually affects plant flowering.

Self-Regulatory module of GRAS TFs

The proteins of two different subfamilies of the GRAS family; SCR and SHR play a critical role in maintaining each other levels during radial root organization. *Arabidopsis*, *AtSCR* is expressed in the quiescent centre and is responsible for generating ground tissue by regulating asymmetric cell division of the initial cells (Sabatini *et al.*, 2003). Whereas, *AtSHR* is expressed in stele and known to show outward movement into the adjacent ground tissue. Its nuclear movement is critical for the quiescent centre and endodermis formation (Nakajima *et al.*, 2001). SCR levels are restricted to the ground tissue and are positively regulated by SHR. As SHR enters into the root endodermis, SCR and SHR interact to form the SCR-SHR protein complex, and SCR translocates SHR into the nucleus. As the SHR is sequestered in the nucleus, the SCR-SHR complex stimulates more SCR production to guarantee that SCR is present in ample amount to trap more and more SHR in the endodermal cell layer forming a functional feedback loop (Cui *et al.*, 2007). Comparable homologues of *AtSCR* are found in maize (*ZmSCR*) and rice (*OsSCR*), which showed almost similar expression patterns with slight variability in *OsSCR* expression (already mentioned above in functions), suggesting similar, but not identical, regulatory mechanism during radial root patterning in dicots and monocots (Kamiya *et al.*, 2003; Lim *et al.*, 2005). Also, NSP1 and NSP2 can form homo and heterodimers to direct the nodulation process (Hirsch *et al.*, 2009). Also, NSP2 can interact with RAM1 protein (Gobbato *et al.*, 2012). Additionally, RAM1 also interacts with RAD1 for transcriptional regulation of several downstream genes (Xue *et al.*, 2015; Park *et al.*, 2015*a*).

MicroRNA based regulation of GRAS TFs

MicroRNAs are 21 to 24 nucleotide long highly conserved noncoding RNA sequences that regulate plant growth and developmental processes by directing cleavage of mRNA sequences or translational inhibition. The role of microRNAs is already known in regulating transcript levels in different developmental processes like auxin signaling, organ development and others (Mallory *et al.*, 2004; Lanet *et al.*, 2009; Song *et al.*, 2019). The variants of *Arabidopsis AtSCL6* gene have

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been predicted to be targeted by miR171 (Llave *et al.*, 2002; Rhoades *et al.*, 2002). The miR171 targets three *SCL6* transcripts, which are critical for differentiation of axillary meristem and promoting shoot elongation (Schulze *et al.*, 2010; Wang *et al.*, 2010). Similarly, in rice and barley, the function of miR171 has been identified phase transitions and floral meristem determinacy, thereby highlighting the conserved regulation of GRAS genes in monocots and dicots (Curaba *et al.*, 2013; Fan *et al.*, 2015). Additionally, SCL6, SCL22, and SCL27 transcripts are regulated by miR171 to control chlorophyll biosynthesis (Ma *et al.*, 2014). In *M. truncatula* and *Lotus japonicus*, it has been observed that that miR171 targets NSP2 transcripts, which are essential regulators of nodule formation and mycorrhiza association (Hofferek *et al.*, 2014; Hossain *et al.*, 2019). It was shown that a regulatory loop regulating NSP2 cleavage by miR171h during AM symbiotic association (Lauressergues *et al.*, 2012). In Soybean, GmSCL-6 and GmNSP2 mRNAs are cleaved by miR1710 and miR171q to regulate the nodulation process. In tomato, of the six SIG genes; *SlGRAS24* and *SlGRAS40* transcripts are known targets of miR171, whereas, *SlGRAS8* is assumed to be targeted by miR171 via translational inhibition mechanism (Huang *et al.*, 2015, 2017). These studies emphasized the crucial role of miRNAs in regulating GRAS genes during different developmental stages of plants.

Biological functions regulated by GRAS TFs

As mentioned earlier, the GRAS TF family members play an essential role in the regulation of various biological processes, such as plant growth and development (GA signal transduction, shoot/root formation, male gametogenesis) phytochrome signal, AM association, biotic and abiotic stress responses. Some of the important examples are discussed individually below. The recent data presented here summarized the function of most GRAS TFs in regulating the transcriptional reprogramming associated with various biological processes in different plant species (Fig. 3). These multifarious roles played by GRAS TFs make them potential candidates for improving plants.

Shoot apical and axillary meristem development and maintenance

As plants differ from animals by the fact that there is a continuous growth to form new organs during post-embryonic shoot development, which depends on shoot apical meristem (SAM). In

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tomato, the *Lateral suppressor (LAS)*, a GRAS gene, was involved in axillary meristem initiation and lateral shoot formation (Schumacher *et al.*, 1999). Similarly, in Arabidopsis, an *AtLAS* (ortholog of *LeLs*) gene was involved in axillary shoot formation (Greb *et al.*, 2003), thus signifying a conserved mechanism for lateral shoot formation in tomato and *Arabidopsis*. However, the ortholog in rice i.e. *MOC1* is involved in regulating tiller number, signifying the differences between monocotyledonous tillering pathway and dicotyledonous branching patterns. A GRAS gene *Hairy Meristem* (*HAM*) has been identified in *Petunia* involved in the lateral organogenesis and meristem maintenance. The *ham* mutants of *Petunia* showed non-maintenance of the meristem. Further, it was also shown that *HAM and WUSCHEL* (*WUS*) worked in parallel during specification and maintenance of meristem, and HAM is essential for prolonged response to *TERMINATOR* and *SHOOTMERISTEMLESS* (*PhSTM*) in *Petunia* (Stuurman *et al.*, 2002). Therefore, this pathway serves as a novel mechanism to signal the meristem cell in differentiating tissues at the shoot meristem.

Plant tillering

MONOCULM 1 (*MOC1*), encodes a GRAS protein in rice, expressed in the axillary buds and regulates axillary bud initiation and therefore, executes the tiller formation in rice. The *moc1* null mutant exhibited single culm only (without tillering), where overexpression lines showed enhanced tillering. The degradation of MOC1 is regulated by a co-activation of *ANAPHASE-PROMOTING COMPLEX* (*APC/C*); *TILLER AND DWARF 1* (*TAD1*) in a cell-cycle-dependent manner (Lin *et al.*, 2012; Xu *et al.*, 2012). Gibberellin (GA) has long been known to inhibit tillering in plants (Li *et al.*, 2003). Another gene dwarf and low- tillering (*DLT*), which encodes a GRAS protein, is also characterized in rice in the regulation of plant morphology via BR signaling. The mutant of the *DLT* gene showed dwarf phenotype and low-tillering in rice (Tong *et al.*, 2009).

Microsporogenesis and fruit ripening

The involvement of GRAS protein in fruit ripening and their transcriptional regulatory mechanisms are also reported. *LiSCL* is a nuclear-localized microsporocytes gene which expressed explicitly during premeiotic anther developmental in *Lilium longiflorum* anthers. It is involved in meiosis and activates the pollen mother cell as shown by transient expression assay (Morohashi *et*

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al., 2003). The *Lels* mutants in tomato showed the blocked axillary meristem initiation (Schumacher *et al.*, 1999). Tomato GRAS1 is also differentially expressed in breaker and mature fruits emphasizing its role in fruit development (Huang *et al.*, 2015). Recently, it is demonstrated that a GRAS TF SlGRAS4 was involved in the tomato ripening by regulating ethylene biosynthesis genes and MADS TFs (Liu *et al.*, 2021).

Radial patterning of root and shoot

In plants, the regulation of asymmetric cell divisions is of great importance in organ development as it is continued throughout the life of the organism. Almost 1.5 decade ago, At*SCR*, a GRAS TF has been identified to express in the cortex and endodermis (Di Laurenzio *et al.*, 1996), shoot apical meristem (Fukaki *et al.*, 1998), bundle sheath (Wysocka-Diller *et al.*, 2000) and quiescent center (Sabatini *et al.*, 2003). The *scr* mutant in Arabidopsis showed aberrant plant growth with defective roots, hypocotyl stem, and inflorescence, and suggested that *AtSCR* is essential in radial patterning of root and shoot formation. *SHORT-ROOT* (*SHR*), a GRAS TFs, is also required for quiescent center identity along with its role in radial root patterning (Sabatini *et al.*, 2003). *AtSHR* gene is exclusively expressed in provascular tissue, whereas its protein was found in the root stele, cortex/endodermis initial, quiescent center, cortex/endodermis initial daughter and endodermis (Nakajima *et al.*, 2001). *shr* mutants were found to possess only one layer of cortex similar to that in *scr* mutants (Helariutta *et al.*, 2000), signifying that presence of SHR and SCR is critical for periclinal division and endodermis formation (Gallagher *et al.*, 2004; Cui *et al.*, 2007; Cruz-Ramírez *et al.*, 2012). In *shr* mutants, *SCR* expression levels were greatly reduced, suggesting that transcription of SCR is directly or indirectly controlled by SHR gene (Nakajima *et al.*, 2001). The SHR gene transcript is initially present in the stele, from where it moves to the adjacent cell layers and interacts with its target SCR. SHR functions downstream to SCR and is primarily responsible for asymmetric cell division in *Arabidopsis* (Di Laurenzio *et al.*, 1996; Heidstra *et al.*, 2004). However, SCR restricts SHR movement to endodermis (Cui *et al.*, 2007). The SCL23, is another GRAS TF, which is found to be operative in the same pathway. No alterations in the root pattern have been observed in the single mutant under normal growth conditions, which suggests that it functions redundantly with closely related members and under some other regulatory control. However, the double mutant *scrscl23* mimicked the *shr* phenotypes and resulted in smaller roots and plants. Since it can spread bi-directionally, SCL23 protein shows

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zonation-dependent movement. SCL23 interacts with SCR and SHR to restrict their movement. Therefore, SCL23, SCR and SHR genes play a significant role in endodermis formation in the root meristem (Cui *et al.*, 2014; Long *et al.*, 2015; Kim *et al.*, 2017) (Fig. 3A). Another GRAS gene SCL3 found to be involved in maintaining GA pathway and helps in the development of root endodermis (Heo *et al.*, 2011; Yoshida *et al.*, 2014). The *scl3* null mutant showed decreased GA responses and enhanced GA biosynthesis genes expression specifying that *SCL3* functions as a positive regulator of the GA signaling pathway (Heo *et al.*, 2011; Zhang *et al.*, 2011). However, the tissue-specific maintenance of the GA pathway in endodermis is regulated by *SCL3* combined with the *SHR/SCR* genes, highlighting a regulatory network of the GRAS TFs in the root endodermis. In the case of *Zea mays*, *SCARECROW* (*ZmSCR*), an ortholog of *AtSCR*, found to be localized to the endodermal cell layer, proposing conserved pathways to be involved in radial patterning, despite variation in the size and configuration of the quiescent center in two species i.e. *Arabidopsis* and maize (Lim *et al.*, 2000).

An ortholog of Arabidopsis SCR, in rice, SCR (*OsSCR*) was found to express in a similar pattern; *OsSCR* was found upregulated in endodermis while downregulated in the daughter cortex. *OsSCR* transcripts were also found in leaf primordium at the P3 stage, during stomatal development, as they showed polarized expression in guard mother cells (GMCs) before cytokinesis. A higher transcript level of *OsSCR* was also found in the ligule initiation tissues. It suggests that *OsSCR* has dynamic functions and is involved in the development of cortex/endodermis, stomata and ligule (Kamiya *et al.*, 2003). Since the two putative rice orthologs to *Arabidopsis SHR* have been identified in rice, of which *OsSHR1* was found to express in leaves and roots. *OsSHR1* expresses during the stomatal development, and its transcript was observed in P3 leaf primordia at Stage 1. The *OsSCR*, was predominately expressed in stomata, whereas the OsSHR1 transcript was almost ubiquitously found in the L1- layer cells and not restricted in the stomatal row. Somehow, this co-expression of *OsSHR1* and *OsSCR* during different developmental tissues suggested that their co-operative networking is important for forming root endodermis, stomata and subsidiary cells. Though the expression of *OsSHR1* is found in root similar to *Arabidopsis* SHR, but the exact location within the root is not identical in both species. For instance, *AtSHR* is expressed exclusively expressed in the stele (Helariutta *et al.*, 2000); however, *OsSHR1* is expressed in the endodermis and cortex along with stele. The variability in rice and *Arabidopsis SHR* localization suggests that *OsSHR* might not share similar functions, and

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rice prevail different mechanism of radial pattering of the root. Therefore, it can be concluded that SHR acts as a key player and activates *SCL23* and *SCR* genes in the endodermis. SHR TF enters into endodermis and forms protein complexes of SHR-SCR, SHR-SCL23, or SHR-SCR- SCL23 to specifically regulate the *SCR* and *SCL2*, respectively and negative feedback loop functions to control each other levels in maintaining the cell specificity in both roots (Yoon *et al.*, 2016).

Seed germination

The germination of seeds governed by the external environment i.e. temperature, moisture and light, and internally by plant growth regulators i.e. ABA and GA. Basically, ABA regulates seed dormancy and GAs regulate germination induction (Koornneef *et al.*, 2002). DELLA protein belongs to the GRAS family is also involved in regulating the seed germination process. The DELLA protein (which is itself regulated by GA levels) make a complex with ABSCISIC ACID INSENSITIVE 3 (ABI3) and ABI5 and binds to the promoter of *SOMNUS* (*SOM*), that involved in the negative regulation of the seed germination process (Fig. 3A) (Lim *et al.*, 2013). It was demonstrated that during unfavorable situations (for example heat stress, darkness), the ABA levels were increased and GA levels decreased, leading to the binding of DELLA/ABI3/ABI5 protein complex on *SOM* promoter. It causes transcription activation of *SOM* genes and inhibits seed germination during unfavorable environmental/external conditions (Lim *et al.*, 2013).

Arbuscular mycorrhiza (AM) symbiosis

A symbiosis between most of the land plants (mostly legumes) and fungi that occur to exchange nutrients between the two partners is AM association. A large transcriptomic level reprogramming occurs in the root, which itself undergoes morphological changes to establish this mutualism (Gutjahr and Paszkowski, 2013; Pimprikar and Gutjahr, 2018). During this AM Symbiotic association, a bidirectional exchange occurs, where the release of lipo-chito-oligosaccharides and myc factors by the nearby fungus to provoke pre-symbiosis responses in the host (Maillet *et al.*, 2011; Genre *et al.*, 2013). However, the fungus detects the plant host, primarily through strigolactones being secreted by the host roots (Akiyama *et al.*, 2005; Besserer *et al.*, 2006). In this highly conserved signaling cascade that functions for a symbiosis-specific association, the GRAS TF has come into the picture after the calcium signaling, which activates the calcium and

calmodulin-dependent kinase (CCaMK), and further regulates CYCLOPS TF (Singh *et al.*, 2014; Pimprikar *et al.*, 2016). The two GRAS genes, *NSP1* and *NSP2* are well characterized in *M. truncatula*, are shown to be involved in the early events of signal transduction during rhizobial and mycorrhizal symbiotic association (Kaló *et al.*, 2005; Smit *et al.*, 2005). Further, in the lotus, LjNSP1 and LjNSP2 function as the Nod factor-activated transcription regulators (Heckmann *et al.*, 2006). A GRAS TF, Reduced Arbuscular Mycorrhization 1 (RAM1) from *M. truncatula* has also been reported to be involved in arbuscule development (Gobbato *et al.*, 2012). In rice, DELLA INTERACTING PROTEIN (DIP1) is found as a crucial gene for mycorrhizal colonization. Mutations in *DIP1*, *RAM1* and *DELLA (SLENDER RICE1)* showed a strong reduction of AM formation although an exact picture of arbuscular branching has not been investigated yet (Yu *et al.*, 2014). MYCORRHIZA-INDUCED GRAS (MIG1), a novel GRAS-domain TF found in *M. truncatula*, which expresses in arbuscule-containing cells, suggesting a specific function during fungal association. It was also reported that MIG1 also interacts with DELLA and this complex regulates radial cell expansion in roots to adjust fungal infection structures during the process of AM symbiosis (Heck *et al.*, 2016) (Fig. 3B). Recently, MtGRAS7 has been identified during plant growth and development before and after *Rhizobium* bacterium inoculation in *M. truncatula* based upon its expression, whereas its exact role needs to be elucidated (Revalska *et al.*, 2019). *NSP1* and *NSP2* are significantly required in early steps for Nod-factor signaling and nodule formation in the roots during rhizobial and mycorrhizal symbiosis (Hirsch *et al.*, 2009). *RAM1* acts as a major gene in the transcriptional activation of the late arbuscule-related genes that are essential during the nutrient exchange by targeting carbohydrate and lipid metabolism genes(Park *et al.*, 2015*b*; Luginbuehl and Oldroyd, 2017; Bravo *et al.*, 2017; Jiang *et al.*, 2018). Moreover, in *Petunia hybrida*, the genes involved in the early stages, are not affected by the *ram1/ata* mutation. However, the genes involved in late arbuscule functioning, for example *PT4* and *STR*, are specifically activated by *RAM1* (Rich *et al.*, 2017). In *M. truncatula*, *RAM1* and *NSP2* are required to form cutin monomers during fungal entry (Murray *et al.*, 2013). Another important feature is that these GRAS proteins can form homo- or heteropolymers, such as NSP1-NSP2 (Hirsch *et al.*, 2009), RAM1-NSP2 (Gobbato *et al.*, 2012) and RAD1-LjNSP2 (Xue *et al.*, 2015) and RAM1-RAD1 (Xue *et al.*, 2015; Park *et al.*, 2015*a*), DIP1-RAM1, which interact to direct the nodulation process. NSP1 and NSP2 can form a heterodimer, which binds to promoter of the *Early Nodulin 11* (*ENOD11*) gene, and a single mutation in the LHR I domain of *NSP2* is affected its binding

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ability and causes to nodule formation and nitrogen fixation (Itoh *et al.*, 2002; Hirsch *et al.*, 2009). *NSP2* is under the regulatory control of microRNA miR171h in angiosperms (Lauressergues *et al.*, 2012; Hofferek *et al.*, 2014). Further, the expression of *RAM1* was found to be regulated by CCaMK/CYCLOPS and DELLA (Pimprikar *et al.*, 2016). CCaMK interacts with CYCLOPS to activate *RAM1* by directly binding at a conserved palindromic region (GGCGCC box) of the *RAM1* promoter (Pimprikar *et al.*, 2016). *DIP1* is shown to interact with mycorrhizal GRAS protein, RAM1 to directly regulate mycorrhizal associated gene expression (Yu *et al.*, 2014). Further GRAS proteins are also involved in AM degeneration regulation, MYB1 TF interacts with DELLA and NSP1 and this interaction assay regulates the Cysteine protease 3 (CP3) gene that was known to involve in AM degeneration (Floss *et al.*, 2017). The above studies suggested that Arbuscule formation involves calcium and hormone signaling. The RAM1 promoter act as a central integration node of this calcium (CCaMK/CYCLOPS) and hormonal (DELLA/GA) signaling (See Fig. 3B) and plays a vital role in the regulation of AM development in plants.

Stress responses

GRAS genes were found to play a significant role in providing tolerance against different abiotic and biotic stresses. For instance, the functional characterization of the *PeSCL7* gene of populous emphasizes its involvement in salinity, osmotic and water stresses (Ma *et al.*, 2010). Further, overexpression transgenics of *Brassica napus* gene *BnLAS* in Arabidopsis showed increased chlorophyll contents, increased stomatal density on leaves, enhanced tolerance to drought and salt stress, reduced water loss rates and better recovery after dehydration treatment (Yang *et al.*, 2011). The epidermal wax deposition has also been observed in transgenic leaves and the expression of many wax synthesis and regulatory genes like *CER1, CER2, KCS1* and *KCS2* were also enhanced. It suggested that the overexpression of *BnLAS* results in enhanced cuticle formation to reduce transpiration (Yang *et al.*, 2011). *TaSCL14* is a wheat LISCL subfamily GRAS gene that showed enhanced expression under high-light stress. *TaSCL14* acts as a multifunctional regulator as the silencing of *TaSCL14* produced various defects including (i) lowered tolerance to photooxidative stress; (ii) increases injury to biological membranes, therefore critical for the survival of plants in extreme environmental stresses; (iii) increased rate of leaf senescence; (iv) decreased photosynthetic capacity; (v) inhibited plant growth (Chen *et al.*, 2015). PAT1 proteins that regulate phytochrome signaling are also known to regulate abiotic stress responses. Overexpression lines

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of the *VaPAT1* gene showed increased tolerance against cold, drought and salt stresses (Yuan *et al.*, 2016). OsGRAS23, a nuclear localized novel stress-responsive GRAS TF, belongs to the LISCL subfamily of GRAS proteins, has been identified in rice. It has strong transcriptional activation activity and could bind to the upstream regulatory region of its potential target genes. *OsGRAS23* overexpression rice transgenics exhibited enhanced tolerance against drought and oxidative stresses, and decreased accumulation of H_2O_2 (Xu *et al.*, 2015).

Four MeDELLAs proteins have been identified in Cassava. The overexpression of these genes in *Nicotiana benthamiana* leaves showed lesser bacterial growth, and silenced plants showed decreased resistance against bacteria, highlighting *MeDELLAs* confer improved resistance against cassava bacterial blight (Li *et al.*, 2018). SCL14, a member of LlSCL/SCL9 subfamily of GRAS protein in the *Arabidopsis* is important for xenobiotic stress tolerance. Ectopic expression of SCL14 in the plants showed enhanced tolerance to toxin like, isonicotinic acid and 2,4,6-triiodobenzoic acid, 2,4-D, *p*-chlorophenoxyisobutyric acid, whereas the mutants were found to be more susceptible. It suggests that *SCL14* is essential for the induction of genes functioning in the detoxification or xenobiotic stress in *Arabidopsis* (Fode *et al.*, 2008). SCL28 is a nuclear-localized transcriptional activator found in the meristem zone of root in *Arabidopsis* and suggested that the *SCL28* function orchestrates between cell division and elongation pattern in regulating the root growth during various stress condition (Choe *et al.*, 2017). Certain GRAS homologs found in *Nicotiana tabacum* can be induced by hydrogen peroxide (H_2O_2). For example; *NtGRAS1* expression is strongly enhanced under various stresses such as; antimycin A, H_2O_2, salicylic acid and L-cysteine suggested its role under stress condition (Vandenabeele *et al.*, 2003; Czikkel and Maxwell, 2007). The above studies suggested that GRAS proteins are also involved in managing various biotic and abiotic stresses in plants in addition to their role in growth and development.

Phytohormones and growth regulation

Phytohormones are the master regulators of various biological processes in plants and play a central role in various stress signaling pathways. For instance, GAs are involved in several plant developmental processes, including leaf expansion, stem elongation, hypocotyl elongation, seed germination, pollen maturation, trichome development, and flowering. Most of the GA signaling pathway components have been identified in plants and among them the DELLA (a major GRAS

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TFs subfamily) was found as an important component of growth inhibition (Achard and Genschik, 2009; Davière and Achard, 2013; Fukazawa *et al.*, 2014, 2017). In earlier reports, it has been shown that the GAI and RGA (DELLA proteins) degradation occurs via the E3 SCF^{SLY} complex. A conformational change occurs by the interaction between GA-GID1-DELLA complex in the presence of GA, which is subsequently recognized by $SCF^{SLY1/GID2}$ and causes the degradation of DELLA protein via E3 ubiquitin ligase complex. The degradation of DELLA proteins subsequently activates the downstream genes (Dill and Sun, 2001; Fu *et al.*, 2002). DELLA gain-of-function mutants showed dwarf phenotype and insensitivity against GA; however, loss-of-function mutants showed enhanced GA sensitivity resulted in slender or tall phenotypes (Cheng *et al.*, 2004; Wild *et al.*, 2012). Degradation of RGA proteins can be delayed by *Xanthomonas* effector protein, which helps plants provide tolerances under different stress conditions. The ERF-associated amphiphilic repression (EAR) motif of *Xanthomonas* effector protein interacts with the DELLA motif of RGA proteins and regulates the GA-induced binding of GID1 (Tan *et al.*, 2014). In contrast to *Arabidopsis*, only one but highly conserved DELLA was found in the case of tomato, grapevine, and cereals. In *Solanum lycopersicum*, a DELLA gene called *PROCERA* (*PRO*) has been characterized by a missense mutation in the GRAS domain region (Bassel *et al.*, 2008; Carrera *et al.*, 2012). This mutant showed partial responsiveness to GA; however, enhanced GA responsive mutant phenotype suggested the retention of its partial activity (Van Tuinen *et al.*, 1999), which confers that tomato has both DELLA independent and dependent pathways for GA-signaling (Livne *et al.*, 2015). During the "green revolution," dwarf cultivars (*RHT-B1* and *RHT-D1*, an ortholog of *GAI*) have been selected from wheat, which lacks GA response. These dwarf mutants (*Rht-B1* and *Rht-D1*) have showed reduced response to GA because of truncation near the DELLA domain (Peng *et al.*, 1999). Similar truncation has been found in D8 genes of maize. Maize contains two duplicated DELLA genes, *DWARF PLANT 8* (*D8*) and *DWARF PLANT 9* (*D9*), found on different chromosomes. Both D8 and D9 genes are gibberellin-insensitive and produced dwarfing phenotype. The *Arabidopsis* transgenics carrying the dominant Maize *D8* and *D9* alleles also showed a reduced length of rosette leaves, siliques, inflorescence, stems, root structures, shortened filaments produced dwarf floral structures with a few seeds. In maize, *D8* and *D9* mutants also exhibited male sterility (Winkler and Freeling, 1994; Lawit *et al.*, 2010). The *dwarf plan t8* (*d8*) is a homolog of *Reduced height* (*Rht1d*) from wheat, *Slender1* from barley and *Slender rice1* from rice (Winkler and Freeling, 1994; Lawit *et al.*, 2010). The *Arabidopsis*

582 transgenic carrying the grapevine *Gibberellin Insensitive1 (*VvGAI1) mutant alleles with three
583 different promoters of wheat (*Rht-B1b*), barley (*Sln1d*) and *Brassica* (*Brrga1-d*) (Chandler *et al.*,
584 2002; Muangprom *et al.*, 2005) showed very short internodes and dwarf phenotype regardless of
585 which promoter was used (Zhong and Yang, 2012). Two DELLA genes in *Brachypodium*
586 *distachyon* (*BdSLR1* and *BdSLRL1*) have been characterized in *Arabidopsis* and found to be
587 involved in GA-mediated signaling and plant development. A similar function was also observed
588 for *Arabidopsis*, rice, maize, and wheat orthologs (Niu *et al.*, 2019). Single base deletion leading
589 to frameshift mutation was observed in rice *slender* mutant, resulting in mutants exhibiting
590 constitutive GA response phenotype (Ikeda *et al.*, 2001) and reduced JA sensitivity (Yang *et al.*,
591 2012), along with enhanced disease susceptibility (Yang *et al.*, 2013). Unlike rice, in Arabidopsis,
592 DELLA proteins are also found to promote susceptibility and resistance to the virulent biotrophs
593 and necrotrophs, respectively. This differential function of DELLA proteins in *Arabidopsis* and
594 rice might result from JA and SA signaling (Navarro *et al.*, 2008). It also throws light on the
595 diverged functions of DELLA in rice and *Arabidopsis*, which might be due to evolutionary or
596 signaling divergence between monocots and dicots. In barley (*Hordeum vulgare*), the *SLN* (for
597 *Slender*, rice *SLR1* ortholog) gene has been identified (Chandler *et al.*, 2002) and found to be
598 degraded in response to GA (Fu *et al.*, 2002), highlighting conservation in functional mechanisms.

599 BRs, one of the major groups of steroid phytohormones, regulates the range of biological processes
600 including agronomic traits in crop plants. OsDLT (DWARF AND LOW-TILLERING), a GRAS
601 TF, found to be involved in BR signaling in rice and *dlt* mutant showed phenotypic deformities
602 including the reduced height, less tillering, late-flowering etc similar to BR-mutants (Tong *et al.*,
603 2009, 2012). Gene *OsDLT* down-regulated in the presence of BR; OsBZR1 can bind to its
604 promoter to repress its expression, as experimentally proved by in vitro assays, which suggests
605 that OsDLT and OsBZR1 regulate each other in opposite ways. In rice, upregulation of *DWF4* (BR
606 biosynthesis gene) enhances the tillering number, suggesting a positive role of BR in tillering (Wu
607 *et al.*, 2008), whereas the BR negatively controls the *OsDLT* resulting in a reduction in tiller
608 number and grain size in the mutants. Therefore, two complex mechanisms dwarf phenotype and
609 fewer tillers are controlled by *DLT* expression in rice. Members of the GRAS family are also found
610 to be involved in auxin signaling. For instance, an ortholog of *AtSCL15* (Pysh *et al.*, 1999) from
611 *Arabidopsis*, BnSCL1 from *Brassica napus* SCARECROW-like genes highly expressed root

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tissue and its expression are influenced by auxin; highlighting the involvement of GRAS proteins during auxin response and signaling (Gao *et al.*, 2004). *SCL1* genes in *Pinus radiate (PrSCL1)* and *Castanea sativa (CsSCL1)* are the two GRAS genes predominately expressed in roots and influenced under exogenous auxin. *Pinus radiata* and *Castanea sativa* share sequence similarity and auxin-induced expression in roots and highlight a conserved auxin signaling pathway among distant relatives during evolution (Sánchez *et al.*, 2007).

Phytochrome signaling and growth regulation

In addition to regulate various developmental pathways, GRAS proteins also play a crucial role in light signaling. For instance, *SCL21*, *SCL5* and *PAT1* involved in phytochrome A (PhyA) signal transduction. Proteins encoded by these three genes contain conserve EAISRRDL motif which is absent in other family members, and the mutants of these three genes showed common Phy-A related deformities (Bolle *et al.*, 2000; Torres-Galea *et al.*, 2006, 2013). Further, *SCL13* (*Scarecrow-like 13*), another member of PAT1 subfamily involved in the phytochrome B (PhyB) pathway. SCL13 is both cytoplasmic (mostly) and nuclear-localized in Arabidopsis. It is particularly involved in red light signaling and is also known to modulate phyA responses in a phyB-independent manner. The SCL3 antisense lines showed lower sensitivity towards red light and execute its role in hypocotyl elongation in plants (Torres-Galea *et al.*, 2006). Two more cytoplasm and nuclear-localized proteins belonging to the PAT1 subfamily i.e. AtPAT1 and AtSCL21 have also been characterized as positive regulators of phyA signal transduction in *Arabidopsis*. Moreover, the biochemical evidence showed that AtPAT1 and AtSCL21 physically interact with each other. The double mutant showed an elongated hypocotyl under FR light, suggested that both SCL21 and PAT1 are essential for FR light-mediated signaling. These results suggested that the heterodimeric complex of SCL21 and PAT1 acts as a positive regulator of the phyA signaling pathway, which is further confirmed by their similar subcellular localization both before and after the interaction (Bolle *et al.*, 2000; Torres-Galea *et al.*, 2013).

GRAS proteins (especially DELLA) are also involved in regulation shade avoidance responses via phytochrome signaling in plants. In Arabidopsis, it was demonstrated that low red/far-red light ratio sense by the phyB photoreceptor and the phyB inactivates the PHYTOCHROME INTERACTING FACTOR 4 (PIF4) TFs through phosphorylation. PIF4 TFs is also a target by

GA signaling pathway, phyB promotes GA biosynthesis through regulating the expression of these two genes *GA20ox* and *GA3ox*, this leads to degradation of DELLA proteins, which otherwise prevent PIF4 function (Hisamatsu *et al.*, 2005; Colebrook *et al.*, 2014) (Fig. 3C).

Genome editing of GRAS genes for plant improvement

With recent advances in genome editing technologies [i.e. zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), RNA-guided CRISPR-Cas nuclease system], several new avenues have been opened up to improve plants for almost any important trait. These technologies have made more precisely target any gene of interest. Here we summarize studies that utilized CRISPR-Cas9 based genome editing to manipulate GRAS genes in the plant for different traits. The first study using CRISPR-Cas technology in plant genome editing was reported in 2013 (Feng *et al.*, 2013), where parallel editing was done in some endogenous loci including GAI in model plant system. In Arabidopsis, the DELLA domain in GAI locus was targeted and one amino acid was deleted. Due to structural deviation, DELLA proteins became insensitive to GA-induced degradation and resulted in repressed growth and dwarf phenotype (Feng *et al.*, 2013). Similarly, in tomato, the mutant was also developed using the CRISPR-Cas nuclease system where one amino acid deleted from DELLA protein resulted in dwarf phenotype. The mutation was found semi-dominant at the seeding stage where heterozygous plants showed intermediate height than homozygous mutant (dwarf) and wild type. However, this mutation was found dominant at the adult stage, as heterozygous plants were as dwarf as a homozygous mutant. Homozygous mutants were also found to only partially responsive against GA treatment (Tomlinson *et al.*, 2019). In rice, SLR genes (which encode DELLA) were edited by targeting the DELLA domain and 16 lines were developed involving six types of mutation in SLR genes (Jung *et al.*, 2020). Out of these, two mutants were insensitive towards GA, and were dwarf with shrunken leaf and short internode. Further, genes belonging to SHR subfamily (involved in root radial patterning) have also been edited through the CRISPR-Cas nuclease system. In *Glycine max*, mutants were developed with anomalous root radial patterning by editing some endogenous genes involving SHR genes (Cai *et al.*, 2015). GRAS genes have also been edited in polyploidy plant species where multiple paralogous genes are present. For instance, in *Brassica napus* (allotetraploid) four genes (*BnaA9.RGA, BnaC9.RGA, BnaA6.RGA* and *BnaC7.RGA*) belonging to the RGA subfamily of GRAS proteins were edited through CRISPR-Cas, and good mutation

673 frequency was observed (Yang *et al.*, 2017). Quadruple mutant involving four RGA genes was
674 found significantly taller than wild type. This information prominences that GRAS genes can be
675 precisely edited in diploid and polyploidy plant species, and since GRAS genes come under the
676 endogenous category, editing of these genes may result in severe phenotypic changes.
677

678 ## Conclusions and future outlook

679 Numerous studies highlight a conserved biological role of GRAS proteins in regulating organ
680 growth and development, phytohormones and phytochrome signaling, symbiosis, biotic and
681 abiotic stress regulation in the plant, prevalently by interaction with other proteins in the plants.
682 The identification and functional annotation of GRAS family proteins in model plants, crop plants,
683 and agronomically important plants have been carried out, which has opened up new avenues of
684 possibilities to understand the mechanisms underlying similarities and differences among these
685 plants species. Though certain mechanisms are conserved among monocotyledonous and
686 dicotyledonous plants, there are still several variations, suggesting unique developmental
687 pathways have emerged during the evolution. Several GRAS family genes are precariously
688 involved in regulating many agronomic traits in cereals, like, *Rht1* and *Rht2* in wheat and *MOC1*
689 in rice. The identification of dwarf mutants of *Rht-1* and *Rht-2* in wheat has created history by
690 contributing to the "green revolution." The usage of such paradigms of genetic variability has
691 enormous implications in crop improvement. The existence of conserved mechanisms of GRAS
692 TFs in regulating cell division and differentiation in angiosperms provides an insight to explore
693 more agronomy traits. More functional elucidation and characterization of GRAS TFs may provide
694 valuable information about their functions in the several plant processes and exploitation of useful
695 traits can build up new scope for crop improvement. The conserved functional mechanisms with
696 slight differences of some of the GRAS family genes among different species suggested
697 diversification among different plant species. The LS, LAS and MOC1 from in tomato,
698 *Arabidopsis*, and rice, respectively, share only about 50% sequence similarity and show conserved
699 function in promoting shoot branching or tillering. LAS genes in *Arabidopsis* and tomato are
700 functionally similar and a conserved control mechanism exists between two distantly related
701 species. However, the differences in the mutant phenotypes in both species may be attributed to
702 redundant gene activities during flower development in *Arabidopsis* leading to variability in

phenotype (Greb *et al.*, 2003). Many GRAS TFs are involved in combating different stress conditions, including environmental and biotic stress responses, which provides acumen into generating transgenic lines for agriculture and agronomic importance.

The protein interaction and transactivation activities have also been demonstrated in different plant species, such as BdSLR1 and BdSLRL1 shows similar interaction and transactivation activity to the homologs in *Arabidopsis*, rice and maize, demonstrating that orthologs have conserved functions. Therefore, it opens the possibility of predicting putative functions of unknown genes in different plant species. Henceforth, the GRAS gene family may be targeted for genetic engineering (overexpression or silencing lines) and genome editing (ZFNs, TALENs, CRISPR/Cas9) approaches improve several agronomic traits that lead to the development of higher-yielding crop varieties.

However, it will be more motivating if we further characterize the GRAS gene family members for their involvement in transcriptional regulation and distinct biological processes. It will provide a clearer view of the biological process or additional GRAS genes and GRAS interactions interacting protein complexes.

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Figure legends

Figure 1. Schematic depicting domains of the GRAS proteins. The conserved C- terminal GRAS domain comprises five distinct motifs, LHRI, VHIID, LHRII, PYRE, and SAW. The LHRI, VHIID, LHRII, PFYRE motifs are the part of the α-helical cap, while LHRII, PFYRE and SAW are the components of the α/β core subdomain. The N-terminus comprises variable motifs and regions (A) DELLA subfamily consists of two motifs DELLA and VHYNP and a poly-S/T/V region (B) Short-root (SHR) subfamily comprises of three poly-Q, poly-T and poly-S/H regions and, (C) SCARECROW (SCR) subfamily comprises of three poly-S/P, poly-S and poly-Q/P region on their N-terminal.

Figure 2. Phylogenetic tree of GRAS proteins in *Arabidopsis thaliana* (Lee *et al.*, 2008), *Amborella trichopoda* (Albert *et al.*, 2013), *Capsicum annum* (Liu *et al.*, 2018), *Coffea canephora* (Denoeud *et al.*, 2014), *Fragaria vesca* (Chen *et al.*, 2019*a*), *Gossypium hirsutum* (Zhang *et al.*, 2018), *Lagenaria siceraria* (Sidhu *et al.*, 2020), *Musa acuminate* (D'Hont *et al.*, 2012), *Oryza sativa* (Tian *et al.*, 2004), *Phoenix dactylifera* (Al-Mssallem *et al.*, 2013), *Theobroma cacao* (Argout *et al.*, 2011) and *Vitis vinifera* (Grimplet *et al.*, 2016). MEGA X aligned the GRAS protein sequences (581) of the 12 plant species with the MUSCLE method. The tree was built with the neighbor-joining (NJ) method and the bootstrap test carried out with 1000 iterations (Kumar *et al.*, 2018). Phylogenetic tree was visualized with iTOL v4 (Letunic and Bork, 2019). The GRAS proteins are clustered into 17 subfamilies, marked by various colours.

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Figure 3. Regulatory circuits mediated by GRAS transcription factors (TFs) during plant growth and development, arbuscular mycorrhizal development and phytochrome regulation.
(A) GRAS encoding protein DELLA acts as a positive regulator for the GA signaling pathway. DELLA and GA signaling interplay regulate MOC1 via modulating SLR1 protein levels to regulate tiller number and plant height. Moreover, DELLA proteins also regulate tiller number and hypocotyl development by interacting with chromatin remodeling complexes and brassinosteroid (BR) pathway. The DELLA interacts with chromatin remodeling complex, Pickle (PKL), which further interacts with PIF3 and BZR to regulate hypocotyl growth by inhibiting the histone methylation of genes involved in cell elongation. The SCL3 controls its functionality during ground tissue division and root elongation, where SCL3 regulates its level and is also being regulated by DELLA proteins and attenuates DELLA repressors positive regulators for the functional GA signaling pathway. The DELLA protein also makes a complex with ABSCISIC ACID INSENSITIVE 3 (ABI3) and ABI5. This complex binds to the promoter of the *SOMNUS* (*SOM*) gene, involved in the negative regulation of the seed germination process. The GRAS TFs are also involved in endodermis development; SHR acts as the master regulator and directly activates the expression of both SCR and SCL23 in the endodermis of root and equivalent tissues. The mobile SHR moves from stele to endodermis/starch sheath and interacts with either or both SCR and SCL23 to form SCR-SHR, SCR-SCL23, SCL23-SCR-SHR complexes to upregulate the SCR and SCL23 levels. SCL23, which can either alone upon interaction with SCR, negatively regulates the transcription of SHR such that any of these interactions are sufficient to prevent SHR from moving beyond endodermis. B) GA-signaling pathway regulates GRAS TFs that control gene expression during arbuscular mycorrhizal (AM) development. The GA-signaling via DELLA inhibits the activity of two GRAS genes, i.e., NSP1 and NSP2. Further, NSP1, combined with NSP2 and calcium (Ca^{2+}) levels, regulates the transcription of *ENDO1* genes that are involved in nodulation during rhizobial infection in plants. The fluctuations in Ca^{2+} levels activate CCaMK, which phosphorylates CYCLOPS TF, which is complex with DELLA protein regulates the transcript level of RAM1 (a GRAS TF). RAM1 interacts with RAD1 (another GRAS-domain protein); these interactions regulate the cascade of genes (*AMT2.2, STR, RAM2, PT4, EXO70I, FatM, SbtM1, ABCGs*) involved in arbuscular formation and function. The DELLA interacts GRAS-domain protein MIG1 and regulates genes involved in radial cell expansion. DELLA and NSP1 also interact with MYB1 and regulates transcriptional regulation of the CP3 gene involved in arbuscule degeneration. C) GA- signaling pathway indirectly also contributes to phytochrome regulation, where SCL13 under continuous FR (far-red) and R (red) light inhibits hypocotyl elongation through phyA and phyB modulation. GRAS proteins also regulate shade avoidance responses via phytochrome signaling in plants. The R/F-R light ratio sense by the phyB photoreceptor inactivates the PHYTOCHROME INTERACTING FACTOR 4 (PIF4) TFs through phosphorylation. PIF4 TFs are also a target by the GA signaling pathway. Black arrows indicate transcriptional controls, protein-protein interaction denoted by red arrows, green denote the movement of proteins, bars denote negative regulation, and the cross represents the confined movement.

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Table 1. Details of GRAS genes reported in different plant species.

Species (common name)	Family	Monocot/ Eudicot	No. of GRAS	No. of subfamilies	References
Arabidopsis thaliana (Arabidopsis)	Brassicaceae	Eudicot	33	08	(Lee *et al.*, 2008)
Brassica juncea (brown mustard)	Brassicaceae	Eudicot	88	09	(Li *et al.*, 2019)
Brachypodium distachyon (stiff brome)	Poaceae	Monocot	48	10	(Niu *et al.*, 2019)
Brassica napus (rapeseed)	Brassicaceae	Eudicot	92	09	(Guo *et al.*, 2019)
Brassica oleracea (cabbage)	Brassicaceae	Eudicot	35	09	(Li *et al.*, 2019)
Brassica rapa (mustard)	Brassicaceae	Eudicot	48	08	(Song *et al.*, 2014)
Camellia sinensis (tea plant)	Theaceae	Eudicot	52	13	(Wang *et al.*, 2018)
Capsicum annuum (pepper)	Solanaceae	Eudicot	50	10	(Liu *et al.*, 2018)
Citrus sinensis (sweet orange)	Rutaceae	Eudicot	50	11	(Zhang *et al.*, 2019)
Dendrobium catenatum (Chained Dendrobium)	Orchidaceae	Monocot	47	11	(Zeng *et al.*, 2019)
Fagopyrum tataricum (buckwheat)	Polygonaceae	Eudicot	47	10	(Liu *et al.*, 2019)
Fragaria vesca (woodland strawberry)	Rosaceae	Eudicot	54	14	(Chen *et al.*, 2019*a*)
Glycine max (soybean)	Fabaceae	Eudicot	117	09	(Wang *et al.*, 2020*a*)
Gossypium hirsutum (cotton)	Malvaceae	Eudicot	150	14	(Zhang *et al.*, 2018)
Hordeum vulgare (barley)	Poaceae	Monocot	62	12	(To *et al.*, 2020)
Ipomoea trifida (sweet potato)	Convolvulaceae	Eudicot	70	11	(Chen *et al.*, 2019*b*)
Jatropha curcas (physic nut)	Euphorbiaceae	Eudicot	48	12	(Wu *et al.*, 2015)
Juglans regia (english walnut)	Juglandaceae	Eudicot	52	08	(Quan *et al.*, 2019)
Lagenaria siceraria (bottle gourd)	Cucurbitaceae	Eudicot	37	17	(Sidhu *et al.*, 2020)
Malus domestica (apple)	Rosaceae	Eudicot	127	08	(Fan *et al.*, 2017)
Manihot esculenta (casava)	Euphorbiaceae	Eudicot	77	14	(Shan *et al.*, 2020)
Medicago truncatula (barrel clover)	Fabaceae	Eudicot	59	08	(Zhang *et al.*, 2017)
Musa acuminata (banana)	Musaceae	Monocot	73	16	(Cenci and Rouard, 2017)
Nelumbo nucifera (sacred lotus)	Nelumbonaceae	Eudicot	38	09	(Wang *et al.*, 2016)

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Nicotiana tabacum (tobacco)	Solanaceae	Eudicot	21	08	(Chen *et al.*, 2015)
Oryza sativa (rice)	Poaceae	Monocot	57	08	(Tian *et al.*, 2004)
Panax ginseng (ginseng)	Araliaceae	Eudicot	59	13	(Wang *et al.*, 2020*b*)
Physcomitrella patens (moss)	Funariaceae	Monocot	40	13	(Wu *et al.*, 2014)
Populus tremula (populus)	Salicaceae	Eudicot	106	13	(Liu and Widmer, 2014)
Prunus mume (Japanese apricot)	Rosaceae	Eudicot	46	11	(Lu *et al.*, 2015)
Ricinus communis (Castor beans)	Euphorbiaceae	Eudicot	48	13	(Xu *et al.*, 2016)
Selaginella moellendorffii (lycophyte)	Selaginellaceae	Eudicot	45	15	(Engstrom, 2011)
Solanum lycopersicum (tomato)	Solanaceae	Eudicot	54	10	(Niu *et al.*, 2017)
Vitis vinifera (grapevine)	Vitaceae	Eudicot	52	13	(Grimplet *et al.*, 2016)
Zea mays (maize)	Gramineae	Monocot	86	08	(Guo *et al.*, 2017)

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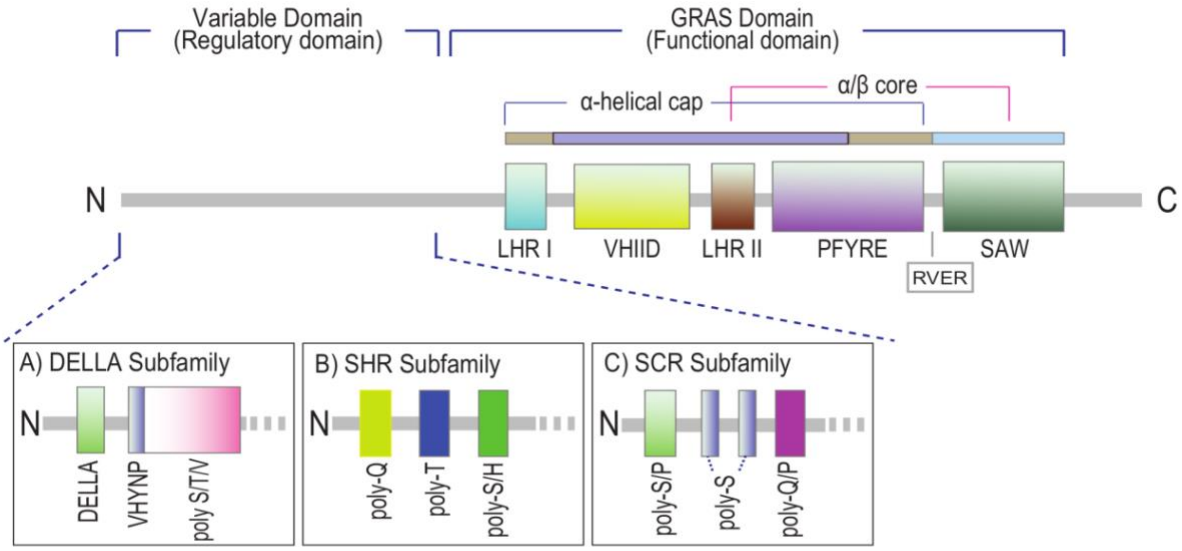

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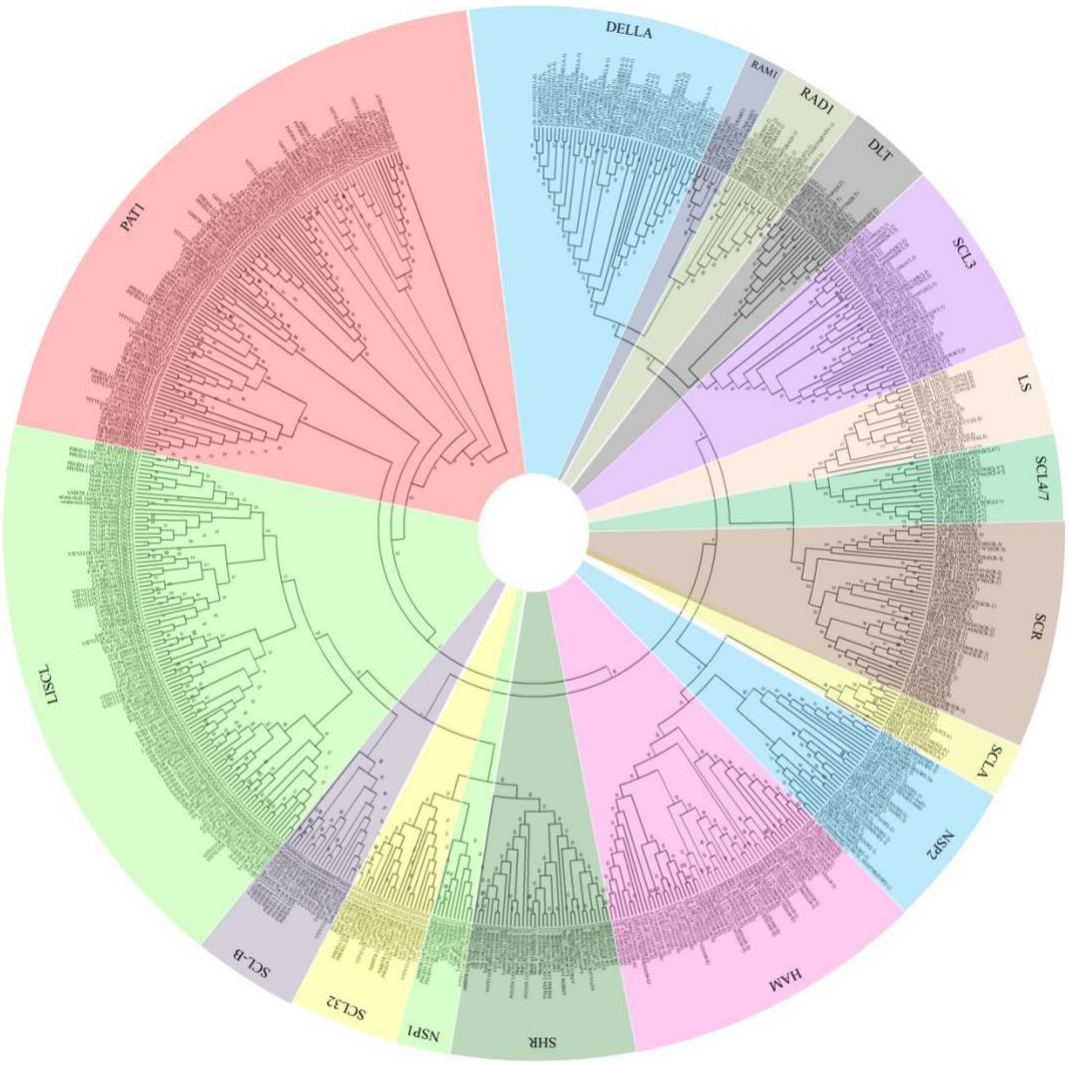

Figure 2. Phylogenetic tree of GRAS proteins in *Arabidopsis thaliana* (Lee *et al.*, 2008), *Amborella trichopoda* (Albert *et al.*, 2013), *Capsicum annum* (Liu *et al.*, 2018), *Coffea canephora* (Denoeud *et al.*, 2014), *Fragaria vesca* (Chen *et al.*, 2019*a*), *Gossypium hirsutum* (Zhang *et al.*, 2018), *Lagenaria siceraria* (Sidhu *et al.*, 2020), *Musa acuminate* (D'Hont *et al.*, 2012), *Oryza sativa* (Tian *et al.*, 2004), *Phoenix dactylifera* (Al-Mssallem *et al.*, 2013), *Theobroma cacao* (Argout *et al.*, 2011) and *Vitis vinifera* (Grimplet *et al.*, 2016). MEGA X aligned the GRAS protein sequences (581) of the 12 plant species with the MUSCLE method. The tree was built with the neighbor-joining (NJ) method and the bootstrap test carried out with 1000 iterations (Kumar *et al.*, 2018). Phylogenetic tree was visualized with iTOL v4 (Letunic and Bork, 2019). The GRAS proteins are clustered into 17 subfamilies, marked by various colours.

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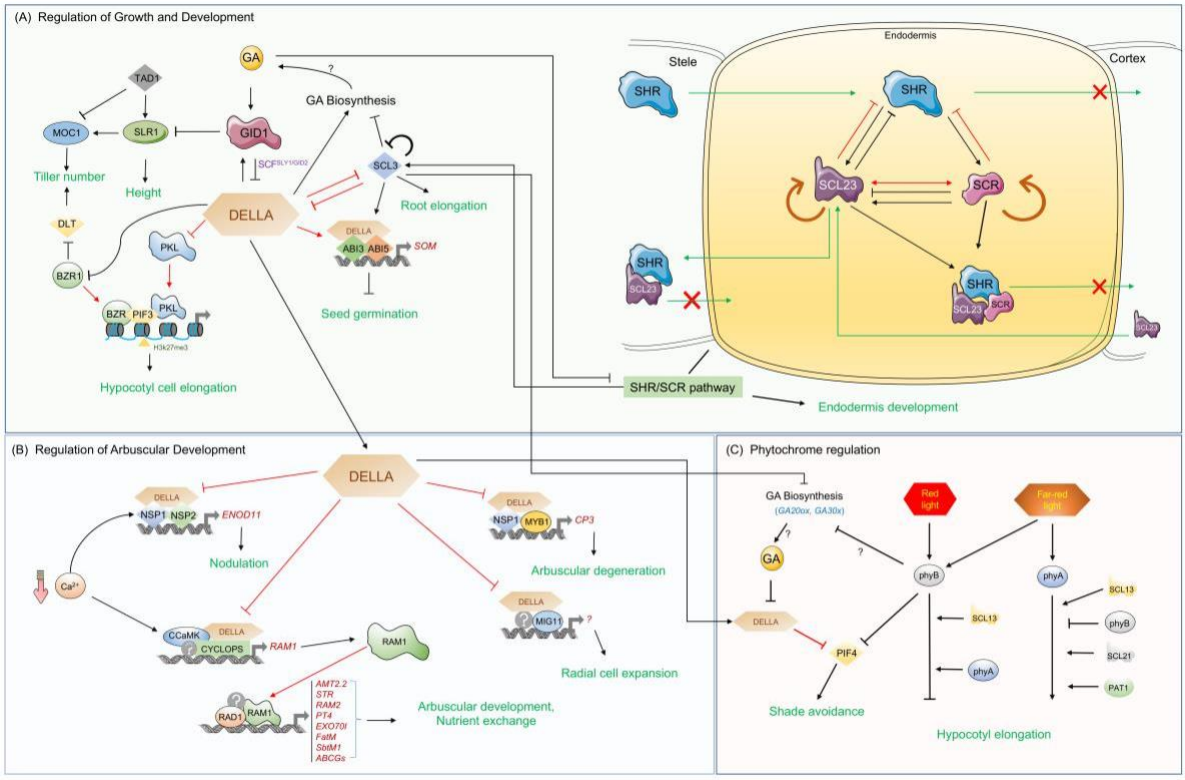

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Figure 3. Regulatory circuits mediated by GRAS transcription factors (TFs) during plant growth and development, arbuscular mycorrhizal development and phytochrome regulation.

(A) GRAS encoding protein DELLA acts as a positive regulator for the GA signaling pathway. DELLA and GA signaling interplay regulate MOC1 via modulating SLR1 protein levels to regulate tiller number and plant height. Moreover, DELLA proteins also regulate tiller number and hypocotyl development by interacting with chromatin remodeling complexes and brassinosteroid (BR) pathway. The DELLA interacts with chromatin remodeling complex, Pickle (PKL), which further interacts with PIF3 and BZR to regulate hypocotyl growth by inhibiting the histone methylation of genes involved in cell elongation. The SCL3 controls its functionality during ground tissue division and root elongation, where SCL3 regulates its level and is also being regulated by DELLA proteins and attenuates DELLA repressors positive regulators for the functional GA signaling pathway. The DELLA protein also makes a complex with ABSCISIC ACID INSENSITIVE 3 (ABI3) and ABI5. This complex binds to the promoter of the *SOMNUS* (*SOM*) gene, involved in the negative regulation of the seed germination process. The GRAS TFs are also involved in endodermis development; SHR acts as the master regulator and directly activates the expression of both SCR and SCL23 in the endodermis of root and equivalent tissues. The mobile SHR moves from stele to endodermis/starch sheath and interacts with either or both SCR and SCL23 to form SCR-SHR, SCR-SCL23, SCL23-SCR-SHR complexes to upregulate the SCR and SCL23 levels. SCL23, which can either alone upon interaction with SCR, negatively regulates the transcription of SHR such that any of these interactions are sufficient to prevent SHR from moving beyond endodermis. B) GA-signaling pathway regulates GRAS TFs that control gene expression during arbuscular mycorrhizal (AM) development. The GA-signaling via DELLA inhibits the activity of two GRAS genes, i.e., NSP1 and NSP2. Further, NSP1, combined with NSP2 and calcium (Ca^{2+}) levels, regulates the transcription of *ENDO1* genes that are involved in nodulation during rhizobial infection in plants. The fluctuations in Ca^{2+} levels activate CCaMK, which phosphorylates CYCLOPS TF, which is complex with DELLA protein regulates the transcript level of RAM1 (a GRAS TF). RAM1 interacts with RAD1 (another GRAS-domain protein); these interactions regulate the cascade of genes (*AMT2.2, STR, RAM2, PT4, EXO70I, FatM, SbtM1, ABCGs*) involved in arbuscular formation and function. The DELLA interacts GRAS-domain protein MIG1 and regulates genes involved in radial cell expansion. DELLA and NSP1 also interact with MYB1 and regulates transcriptional regulation of the CP3 gene involved in arbuscule degeneration. C) GA- signaling pathway indirectly also contributes to phytochrome regulation, where SCL13 under continuous FR (far-red) and R (red) light inhibits hypocotyl elongation through phyA and phyB modulation. GRAS proteins also regulate shade avoidance responses via phytochrome signaling in plants. The R/F-R light ratio sense by the phyB photoreceptor inactivates the PHYTOCHROME INTERACTING FACTOR 4 (PIF4) TFs through phosphorylation. PIF4 TFs are also a target by the GA signaling pathway. Black arrows indicate transcriptional controls, protein-protein interaction denoted by red arrows, green denote the movement of proteins, bars denote negative regulation, and the cross represents the confined movement.