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Raffinose Family Oligosaccharides: Crucial Regulators of Plant Development and Stress Responses

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ABSTRACT

Raffinose family oligosaccharides (RFOs), the α-galactosyl derivatives of sucrose, are nearly ubiquitous in Plantae, and they have been demonstrated to play pivotal roles in regulating plant responses to various abiotic stresses. RFOs accumulate to high levels in plant kernels/fruits or vegetative parts and are commonly associated with storability and desiccation or cold tolerance. Recent studies have also revealed the regulatory roles of RFOs in seed germination, plant development, and biotic stress resistance. Here, we provide an overview of the metabolism, transport, and evolution of RFOs as well as their physiological importance in plants. Recent research highlights the general importance of RFOs in plant development and stress response.

KEYWORDS

Development; evolution; metabolism; raffinose family oligosaccharides; stress response; transport

I. Introduction

As sessile organisms, plants encounter changing environmental conditions including stresses, which can significantly impair their growth, development, and productivity (El Sayed *et al.*, 2014; Sharma *et al.*, 2019). To survive, plants have evolved numerous mechanisms to cope with suboptimal environments; these include triggering a series of signal transduction responses and accumulating compatible metabolic substances. These substances include quaternary compounds, amino acids, soluble sugars, and raffinose family oligosaccharides (RFOs) (El Sayed *et al.*, 2014). RFOs, such as raffinose, stachyose, and verbascose, are derived from sucrose and activated galactose moieties, which are donated by galactinol. Galactinol and raffinose are ubiquitous in plants and have been demonstrated to play important roles in seed desiccation tolerance/seed storability (Sengupta *et al.*, 2015; Li *et al.*, 2017; Jing *et al.*, 2018). In recent years, more and more evidence has demonstrated the involvement of galactinol and RFOs in various abiotic stress responses in plants, and in general, the levels of RFOs (or galactinol) in different tissues can be used as an indicator of the degree of stress tolerance in plants (Sengupta *et al.*, 2015; Selvaraj *et al.*, 2017; Han *et al.*, 2020). The associations between RFOs (or galactinol) and plant development and biotic stresses have also been demonstrated by several recent studies (Unda *et al.*, 2017; Zhou *et al.*, 2017; La Mantia *et al.*, 2018; Hua *et al.*, 2021; Liu *et al.*, 2022). These findings have greatly broadened our knowledge of the intrinsic molecular mechanisms underlying plant stress tolerance and disease resistance. In this review, we summarize the transport, evolution, and physiological importance of RFO metabolism pathways in plants with an emphasis on the specific functions of RFOs in development and stress responses. Finally, future research directions including the potential use of RFOs for crop improvement are discussed.

II. RFO metabolism in plants: biosynthesis and catabolism

RFOs are synthesized through the sequential addition of galactosyl moieties to sucrose by the action of α-galactosyltransferases, resulting in a series of oligosaccharides

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with degrees of polymerization up to 15 (Peterbauer and Richter, 2007). The biosynthesis of RFOs has been thoroughly studied in several plant species including *Ajuga reptans*, *Medicago sativa*, and *Arabidopsis thaliana* (Bachmann *et al.*, 1994; Blöchl *et al.*, 2005; Iftime *et al.*, 2011; Sengupta *et al.*, 2015).

To date, two pathways have been identified for RFO biosynthesis. One is the galactinol-dependent pathway (Figure 1). The first key step of this pathway is the synthesis of galactinol from UDP-galactose and L-*myo*-inositol by galactinol synthase (GolS) (Peterbauer *et al.*, 2001; Sengupta *et al.*, 2015). Raffinose is then formed by the addition of galactose units from galactinol to sucrose in a reaction catalyzed by raffinose synthase (RS), whereas stachyose is synthesized by the addition of galactose units from galactinol to raffinose in a reaction catalyzed by stachyose synthase (STS) (Peterbauer *et al.*, 2001; Sengupta *et al.*, 2015). The second RFO biosynthetic pathway is the galactinol-independent pathway (Figure 1), which has been identified in *A. reptans* and *Coleus blumei*; this pathway includes galactan: galactan galactosyltransferase (GGT), which belongs to the acid α-galactosidase (AGAL) protein family (Bachmann *et al.*, 1994; Gilbert *et al.*, 1997; Haab and Keller, 2002; Tapernoux-Lüthi *et al.*, 2004; Elango *et al.*, 2022). GGT catalyzes the chain elongation of RFOs by transferring a terminal galactosyl moiety from one RFO molecule to one another. For instance, GGT can produce raffinose and verbascose when incubated with stachyose (Bachmann *et al.*, 1994; Bachmann and Keller, 1995). However, it should be noted that GGT enzymatic activity has been found in leaves but not in seeds, suggesting that the galactinol-independent RFO synthesis pathway may exist in plant leaves, but not in plant seeds.

RFO catabolism in plants has received relatively little attention even though this process is as important as the biosynthesis reaction. RFOs are hydrolyzed to sucrose and D-galactose by the action of AGAL and alkaline α-galactosidase (AGA) (Blöchl *et al.*, 2008; Sengupta *et al.*, 2015). The resulting sucrose and D-galactose may either be used as energy sources or reutilized to form RFOs (Blöchl *et al.*, 2008). To serve as an energy source, sucrose can be degraded into glucose and fructose by invertase or into UDP-glucose and fructose by sucrose synthase (Ruan, 2014). Subsequently, glucose, fructose, and D-galactose can readily enter other metabolic pathways. Free D-galactose can be rapidly converted into galactose-1-phosphate by GalK and further metabolized either by the conventional Leloir pathway or by a pyrophosphorylase-dependent pathway (Peterbauer *et al.*, 2001; Sengupta *et al.*, 2015). Intriguingly, all RFO biosynthesis and catabolism reactions are reversible.

III. The source-to-sink transport of RFOs in plants

In plants, <80% of carbohydrates produced by photosynthesis in leaves are exported to heterotrophic tissues and organs to enable their growth and development (Kalt-Torres *et al.*, 1987; Ainsworth and Bush, 2011; Kölling *et al.*, 2013; Brauner *et al.*, 2018). Sucrose is the major carbohydrate used for long-distance transport. The initial step of source-to-sink transport of sucrose is phloem transport, which is the transfer of sugars from mesophyll cells (MCs) to companion cells (CCs) and then the sieve elements (SEs) of minor veins (Knop *et al.*, 2001; Voitsekhovskaja *et al.*, 2006; McCaskill and Turgeon, 2007). As shown in Figure 2, the two main phloem loading strategies in most species are apoplasmic loading and passive symplasmic loading. The former is a transporter-mediated and energy-coupled process, while the latter is an osmotic driving force-dependent process with sucrose transport from MCs to CCs occurring via plasmodesma (Rennie and Turgeon, 2009; Slewinski and Braun, 2010; Ma *et al.*, 2019). In addition, some tree species use an active symplastic loading strategy in addition to the passive strategy, such as the gymnosperm *Gnetum gnemon* and the angiosperms *Quercus robur*, *Fraxinus excelsior*, *Fagus sylvatica*, and *Magnolia kobus* (Liesche *et al.* 2011; Öner-Sieben and Lohaus 2014; Fink *et al.*, 2018). Following long-distance transport through SEs and arrival at the sink tissues, the same strategies are used for phloem unloading, i.e., active apoplasmic unloading or passive symplasmic unloading (Oparka *et al.*, 1999; Stadler *et al.*, 2005a, 2005b; Ma *et al.*, 2019). Sucrose is then used directly to provide carbon and energy for growth and development or metabolized into RFOs or starch for storage in sink tissues (Ruan and Patrick, 1995; Ruan *et al.*, 2001).

A third phloem loading strategy, polymer-trapping loading, is used in a limited number of plant families, such as *Cucurbitaceae*, *Lamiaceae*, and *Oleaceae* (Figure 2; Rennie and Turgeon, 2009; Gil *et al.*, 2011; Ma *et al.*, 2019). In this strategy., raffinose and stachyose, rather than sucrose, are the predominant carbohydrates transported in phloem. In the polymer-trapping loading model, sucrose produced by photosynthesis in leaves diffuses into specialized CCs (intermediary cells, ICs) from MCs through specialized plasmodesmata, where it is then polymerized to

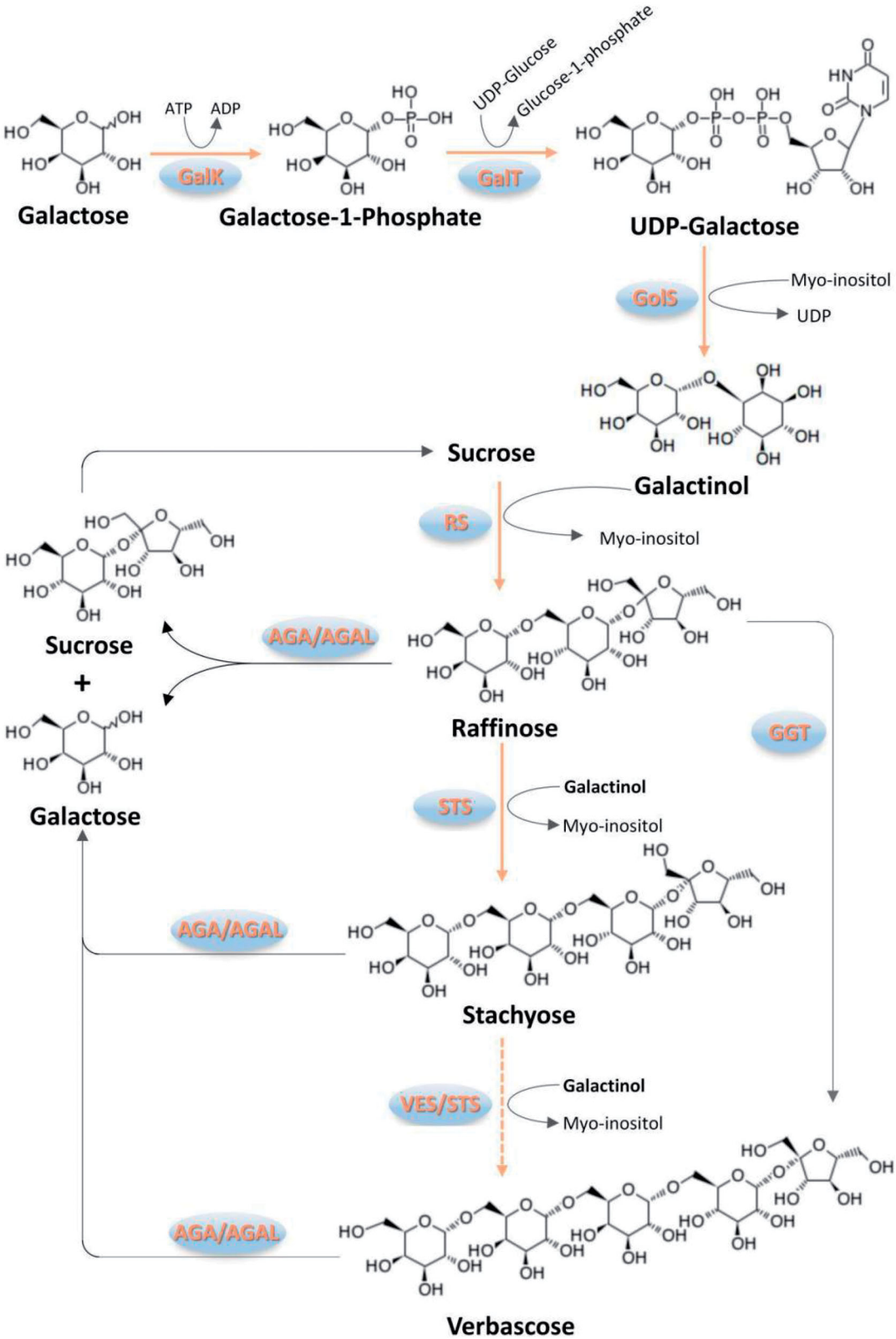

Figure 1. The proposed RFO metabolism pathway in plants. α-D-galactose is phosphorylated by galactokinase (GalK, EC 2.7.1.6) to form galactose-1-phosphate. Then, a uridine monophosphate (UMP) group is transferred from UDP-glucose to galactose-1-phosphate by galactose-1-phosphate uridylyltransferase (GalT), producing glucose-1-phosphate and UDP-galactose (Peterbauer *et al.*, 2001; Coelho *et al.*, 2015). GolS: galactinol synthase; RS: raffinose synthase; STS: stachyose synthase; VES: verbascose synthase; AGA: alkaline α-galactosidase; AGAL: acid α-galactosidase; GGT: galactan: galactosyl-transferase.

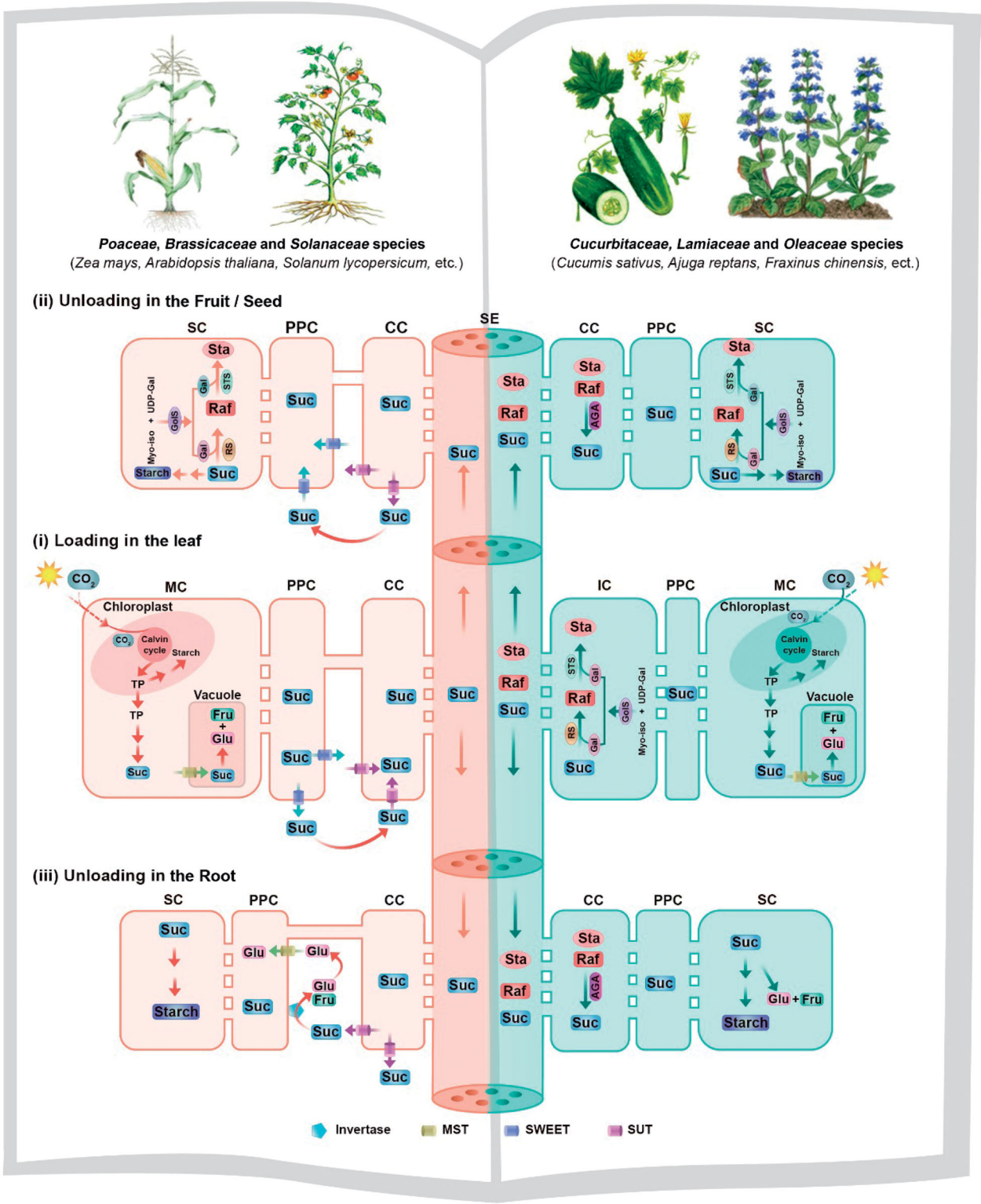

Figure 2. A proposed model of RFO transport in plants. SC: sink cell; PPC: phloem parenchymal cell; CC: companion cell; IC: intermediate cell (specialized CC); SE: sieve element; MC: mesophyll cell; TP: triose phosphate; Fru: fructose; Glu: glucose; Raf: raffinose; Sta: stachyose; Gal: galactinol; Myo-Iso: myo-inositol.

form RFOs, i.e., raffinose and stachyose (Yadav *et al.*, 2015). RFOs are thought to be unable to diffuse back to the MCs on account of their larger size, and they enter the SEs through wider plasmodesmata-pore units (Hannah *et al.*, 2006; Zhang and Turgeon, 2018). In sink tissues, RFOs are unloaded from the phloem, and AGAs hydrolyze the RFOs to sucrose and galactose, which can also be partitioned via the

apoplasmic pathway. The RFO transport pathway was recently demonstrated to function in sweet watermelon (*Citrullus lanatus*) and cucumber (*Cucumis sativus*). In these species, the alkaline AGA genes *ClAGA2* (Ren *et al.*, 2021) and *CsAGA2* (Liu *et al.*, 2022) were identified as the key factors controlling stachyose and raffinose hydrolysis, and they were found to be specifically expressed in the vascular bundle. Ren *et al.* (2021) showed that knocking out *AGA2*, Sugars Will Eventually Be Exported Transporter 3 (*SWEET3*), and Tonoplast Sugar Transporter 2 (*TST2*) affected fruit sugar accumulation in *C. lanatus*.

In *A. reptans* which is a frost-hardy, perennial labiate that contains high levels of RFOs, two different RFO pools are observed (Figure 2). One is a long-term storage pool of RFOs, which are synthesized in the MCs, and the other is a transport pool of RFOs, which are synthesized in the phloem. The storage RFOs serve as an energy source and support frost tolerance, while the transport RFOs enter sink tissues to maintain plant growth (Bachmann *et al.*, 1994; Bachmann and Keller, 1995; Kannan *et al.*, 2018). Interestingly, isoforms encoded by two allelic variants of *GolS* (*ArGolS1* and *ArGolS2*) from *A. reptans* display different roles in RFO biosynthesis as evidenced by their differential gene expression and enzyme activity (Sprenger and Keller, 2000). *ArGolS1* is primarily expressed in MCs and is involved in the synthesis of storage RFOs, whereas *ArGolS2* is predominantly expressed in ICs and is involved in the synthesis of transport RFOs. Further compartmentalization analysis of MCs revealed that the RFO transport pool, GGT, stachyose, and verbascose are all vacuolar localized, whereas the RFO storage pool, GolS, STS, and galactinol are located outside the vacuole, and raffinose is distributed in both the cytosol and the vacuole (Bachmann *et al.*, 1994; Bachmann and Keller, 1995; Kannan *et al.*, 2018). It was proposed that stachyose is synthesized outside the vacuole (probably in the cytosol) and then transported into the vacuole via a stachyose transporter in the tonoplast, although multi-omics evaluation of the tonoplast membrane did not identify this transport protein (Tohge *et al.*, 2011). A later report indicated that raffinose, which accumulates in the chloroplasts of cold-treated *A. reptans*, is originally synthesized outside the chloroplast and subsequently taken up into the chloroplast by a raffinose transporter on the chloroplast envelope (Schneider and Keller, 2009). A shift in carbohydrates (mainly RFOs) from the cytosol to the vacuole and chloroplast, and from winter leaves to summer leaves, has also been identified in *A. reptans*, suggesting that RFOs play important roles in frost tolerance in this evergreen plant (Findling *et al.*, 2015).

Several studies have shown that the metabolism and transport of RFOs can be affected by exogenous stress treatment or by overexpression of the key RFO metabolism enzymes in plants (Gilbert *et al.*, 1997; Ayre *et al.*, 2003; Hannah *et al.*, 2006; McCaskill and Turgeon, 2007; Obata and Fernie, 2012). For example, salt stress significantly induces the accumulation of RFOs (verbascose and raffinose) in both the source and white sink tissues of *Coleus blumei*, with stressed plants preferentially transporting sucrose over RFOs, as determined by phloem-sap analysis (Gilbert *et al.*, 1997). Galactinol is the second most abundant sugar synthesized in the ICs of *C. blumei*, but galactinol accumulation is also observed in non-phloem compartments, such as MCs, in transgenic tobacco plants with CC-specific overexpression of *CmGolS1* (Ayre *et al.*, 2003). Hence, these transgenic tobacco plants accumulate large amounts of galactinol in the leaves, but long-distance transport of galactinol is limited, with only a small amount being transported to sink tissues. Moreover, in transgenic potato plants displaying constitutive or CC-specific overexpression of GolS or GolS in combination with RS, both galactinol and raffinose were transported in the phloem. However, although significant amounts of galactinol were observed in the phloem, only a small amount of raffinose was able to be transported even when there was a high concentration of raffinose (Hannah *et al.*, 2006). In addition, simultaneous silencing of two GolS genes (*VpGolS1* and *VpGolS2*) in *Verbascum phoeniceum* resulted in the inhibition of both RFO biosynthesis and RFO transport in phloem (McCaskill and Turgeon, 2007).

IV. Evolution of RFO metabolism in plants

To investigate the evolution of RFO metabolic pathways in plants, we searched for genes encoding homologous proteins of GolS, RS/STS, AGAL, and AGA, which are involved in RFO catabolism, in 26 representative plant species from nine plant lineages, namely glaucophytes, rhodophytes, chlorophytes, charophytes, bryophytes, lycophytes, gymnosperms, monocots, and dicots (Figure 3A), and performed BLAST analyses using HMMER software. After removal of redundant and incomplete protein sequences which may be encoded by pseudogenes, a

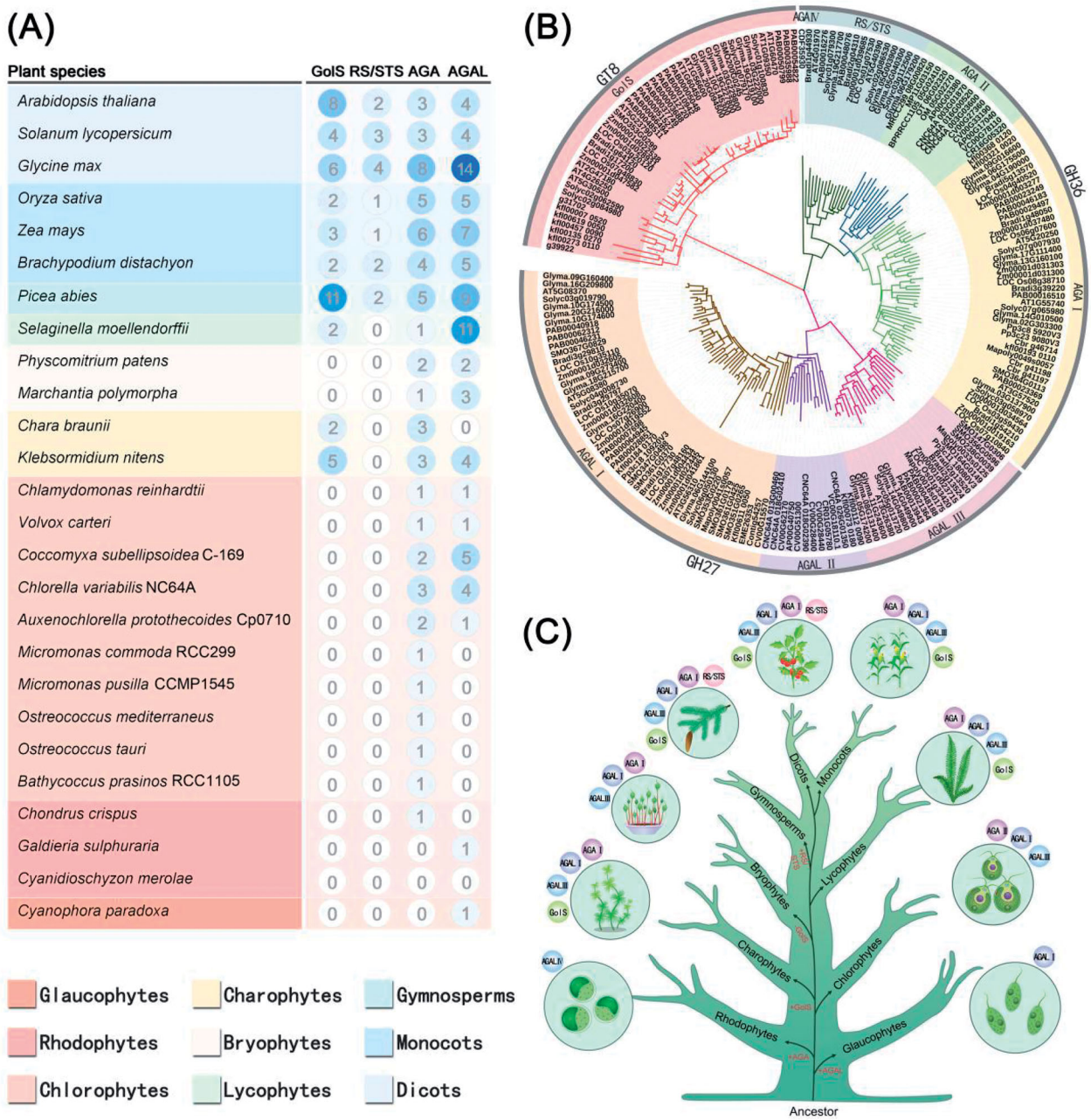

Figure 3. Phylogenetic relationships between key RFO metabolic enzymes in plants. (A) The distribution of key enzymes in plant lineages from glaucophytes to angiosperms. (B) The phylogenetic relationships between the key enzymes in 26 plant species. Each member of the GolS, RS/STS/AGA, and AGAL protein families were grouped into different branches based on their protein sequence homology. (C) A model for the evolution of RFO metabolic enzymes in the glaucophyte to angiosperm lineages based on phylogenetic data is shown in Figure 3B. In this model, key enzymes of the RFO metabolic pathway were gained and/or lost during the divergence of the aquatic algae and land plant lineages. One of the key rate-limiting enzymes in RFO synthesis, GolS, was gained in Charophytes but lost in Bryophytes. The other rate-limiting enzymes in RFO synthesis, i.e., RS and STS, first evolved from GH36 proteins in the most recent common ancestor of the gymnosperm, monocots, and dicots and were retained in all lineages.

total of 45 GolSs, 15 RS/STSs, 59 AGAs, and 82 AGALs were identified (Figure 3A and Table 1). The number and distribution of these key enzyme genes in the selected representative species were distinctly different. AGA and AGAL were found in most of the species examined including algae, with the number of paralogous genes ranging from 1 to 14. GolSs were found in all vascular plants and in charophyte algae with 2 to 11 paralogous genes, whereas RS/STS genes were only found in gymnosperms, monocots, and dicots with 1 to 4 paralogous genes (Figure 3A). These observations suggested that gene expansion

and gene loss occurred during the evolution of the RFO metabolic pathway in plants.

Phylogenetic analysis revealed that the clustering of GolSs, which belong to glycosyl transferase family 8 (GT8), is consistent with the evolutionary relationships of plant species (Figure 3B). RS/STS and AGA proteins belong to the glycosyl hydrolase family 36 (GH36), and the GH36 family was classified into four subfamilies, i.e. AGA I, AGAII, AGAIV, and RS/STS (Figure 3B). Unlike subfamily I, which contained sequences found in most of the plant lineages, from charophytes to monocots and dicots, subfamilies II, RS/STS, and IV only contained sequences from chlorophytes, vascular plants (gymnosperms, monocots, and dicots), and rhodophytes, respectively. These observations suggest that the functions of GH36 proteins have diverged during plant evolution, resulting in the specialized functions of the RS/STS subfamily members, which have been demonstrated to harbor multifunctional RFO synthase/galactosyl hydrolase activities. For example, the maize RS ZmRS (Zm00001d039685) displays both raffinose synthesis and galactinol hydrolysis activities (Li *et al.*, 2020), while *Arabidopsis* AtRS4 (also named AtSTS, AT4G01970) exhibits not only stachyose synthesis activity but also stachyose- and galactinol-specific hydrolysis activity (Gangl *et al.*, 2015). AGALs, which belong to glycosyl hydrolase family 27 (GH27), were classified into three subfamilies (I–III), with subfamily I present in most plant lineages from glaucophytes (*Cyanophora paradoxa*) to monocots and dicots. However, subfamilies II and III were only present in chlorophytes/charophytes and land plants (bryophytes, lycophytes, gymnosperms, monocots, and dicots), respectively (Figure 3B). The GGT enzyme in *A. reptans*, which belongs to AGAL subfamily I, can catalyze the chain elongation of RFOs and produce raffinose and verbascose (Bachmann *et al.*, 1994; Bachmann and Keller 1995), which suggests that AGALs may also harbor multifunctional RFO synthase/galactosyl hydrolase activities.

The key enzymes of the RFO metabolic pathway were gained and/or lost during the divergence of the aquatic algae and land plant lineages. AGAs and AGALs were present in the common ancestor but subsequently evolved into AGAL I proteins in glaucophytes and AGA IV proteins in rhodophytes (Figure 3C). Thereafter, specialized subfamilies, such as GAL II and III and AGA II and III also evolved. The key rate-limiting enzyme in RFO synthesis, GolS, was gained in Charophytes, but lost in Bryophytes, whereas RSs and STSs evolved from GH36 proteins in the most recent common ancestor of the gymnosperm, monocots, and dicots and were retained in all lineages (Figure 3C).

In plants, there are two pathways for RFO biosynthesis: a galactinol (α-galactosyl-myo-inositol)-dependent pathway and a galactinol-independent pathway. The former pathway, in which one or two α-galactosyltransferase steps (RS, EC 2.4.1.82, and STS, EC 2.4.1.67) result in the biosynthesis of short-chain RFOs, such as raffinose and stachyose, has been comprehensively studied. Strikingly, although there are no RS or STS homologs in chlorophytes, raffinose was detected in some algae, such as *Chlorella variabilis* and *Spirulina platensis* (de Medeiros *et al.*, 2021), suggesting that AGAs other than RS or STS may be involved in raffinose biosynthesis in chlorophytes. However, we should point out that the presence of a metabolite may be due to contamination of a culture with a species that genuinely synthesize it (Tohge *et al.*, 2013). The galactinol-independent pathway is regarded as being involved in the biosynthesis of long-chain RFOs, such as verbascose, and it catalyzes the transfer of an α-galactosyl residue from one RFO molecule to another, resulting in higher-order RFO oligomers. Indeed, GGT has been shown to catalyze the biosynthesis of long-chain RFOs in *A. reptans* (Bachmann *et al.*, 1994; Bachmann and Keller, 1995). This observation, combined with the phylogenetic relationship of RS/STS/AGAs and GALs, leads us to speculate that the galactinol-dependent pathway for short RFO biosynthesis may be present in all green lineages, while the galactinol-independent pathway may occur only in certain land plants, such as *A. reptans*, and may have evolved as an environmental adaptation. Indeed, the specialized RS/STS subfamily likely coevolved with vascular development in higher plants, which are also adapted to the land environment. In fact, the transition of plants from aquatic to terrestrial environments required plant adaptation to drought-stress environments. In addition to structural changes in the vascular sheath, the emergence of new metabolic pathways and/or metabolites is also a means of plant adaptation to drought-stress environments. RFOs, especially raffinose, is thought to play a role in plant drought stress tolerance because of the increased raffinose accumulation observed in leaves when plants encounter drought stress (Egert *et al.*, 2016; Li *et al.*, 2020).

V. The potential roles of RFOs in plant development

Several studies have demonstrated the regulatory roles of RFOs in plant growth and development. During

seed germination, RFOs cannot be used directly and need to be broken down into sucrose and galactose by the hydrolytic enzyme AGAL. A recent report demonstrated that AGAL activity gradually increases during seed maturation and early germination in *Cicer arietinum*, with the latter stage requiring more energy (Arunraj *et al.*, 2020). When the breakdown of RFOs in pea seeds is blocked by treatment with 1-deoxygalactono jirimycin (DGJ), a galactosidase-specific inhibitor, the treated seeds have a significantly lower germination rate, accompanied by depressed activities of GalK and UDP-galactose pyrophosphorylase, when compared with the control seeds (Blöchl *et al.*, 2007). The inhibition of germination could be relieved by the addition of exogenous galactose and partially relieved by the addition of exogenous sucrose (Blöchl *et al.*, 2007), suggesting that the content of galactose rather than sucrose was positively correlated with seed germination. Similarly, wild-type soybean seeds also show a delay in germination when treated with DGJ. However, soybean seeds with low RFO content (18% raffinose and 33% stachyose) show no significant differences in germination compared with wild-type seeds under normal conditions, and no remarkable delay in germination was observed when the low-RFO seeds were treated with DGJ (Dierking and Bilyeu, 2009). These results suggest that unlike in *C. arietinum* RFOs are not essential for soybean seed germination; however, sucrose levels in low-RFO seeds were significantly higher than those in the wild-type plants (Dierking and Bilyeu, 2009), which might at least partially explain the lack of effect of RFO content on the germination of soybean seeds. *RS4/5* double knock-out *Arabidopsis* seeds exhibited a five-day delay in germination in darkness and upregulated expression of a repressor of germination; these phenotypes were attributed to the absence of RFOs in germinated seeds (Gangl and Tenhaken, 2016). Taken together, previous studies indicate that the levels of RFOs and RFO pathway-derived metabolites are closely related to seed germination, but the exact function of RFOs varies among different seeds.

Three recent reports demonstrated that RFOs are also involved in the growth and development of hybrid poplar (*Populus alba* × *grandidentata*) and cucumber (Unda *et al.*, 2017; Hua *et al.*, 2021; Liu *et al.*, 2022). Transgenic poplar plants with the highest levels of *AtGolS3* transgene expression formed tension wood, as manifested by an increased number of vessels and the appearance of a G-layer in the fibers, whereas transgenic poplar plants with moderate *AtGolS3* expression showed only moderate alterations in secondary cell wall composition and ultrastructure, such as lower lignin and higher cellulose contents (Unda *et al.*, 2017). In cucumber, transcription of *CsAGA1* was found to gradually increase during fruit development, especially in the fruit vasculature. *CsAGA1*-overexpressing plants showed bigger fruits compared with wild type, whereas *CsAGA1*-RNAi plants exhibited delayed fruit development due to altered hexose production in the peduncle and main vascular bundle of the fruit (Hua *et al.*, 2021). Further analysis showed that manipulation of *CsAGA2* expression influences phloem loading, sugar production, and exportation from leaves and petioles, and thus affects cucumber fruit set and development (Liu *et al.*, 2022). These results collectively illustrate that ectopic expression of *GolS/AGA* influences RFO metabolic pathways and that RFO may function as a molecular signal to trigger a series of metabolic changes, ultimately impacting sugar transport, cell differentiation, and development in plants (Unda *et al.*, 2017).

VI. The roles of RFOs in plant abiotic stress tolerance

A. The roles of RFOs in seed desiccation tolerance, seed storability, and seed vigor

Desiccation tolerance, which is necessary for the maturation of orthodox seeds, refers to the ability of seeds to withstand dehydration, to reduce the deleterious effects of dehydration and slow their metabolic activity, and finally to maintain viability in a dry state for a long period of time (Wang *et al.*, 2015; Jing *et al.*, 2018). There is evidence that RFOs play a key role in desiccation tolerance. For example, the *GolS* enzymes are significantly upregulated in the alpine aeroterrestrial alga *Klebsormidium crenulatum* under strong desiccation-stress conditions (Holzinger *et al.*, 2014), and raffinose accumulation is associated with the acquisition of desiccation tolerance as well as the tolerance to high-temperature drying in cereal seeds (Chen and Burris, 1990; Brenac *et al.*, 1997). Conversely, loss of raffinose accumulation is accompanied by the loss of desiccation tolerance during the germination of maize seeds (Koster and Leopold, 1988). More recently, the positive correlation between RFOs and seed desiccation tolerance has been further validated by overexpressing *GolS1*, *GolS2*, and/or *RS5* in *Arabidopsis* (Jing *et al.*, 2018). The resultant transgenics display higher levels of RFOs and greater desiccation tolerance than the wild-type plants, whereas *gos1 gos2* double mutant plants and *rs5* single mutant plants, which have lower levels of RFOs, exhibit delayed

acquisition of desiccation tolerance compared with the wild-type plants (Jing *et al.*, 2018). High RFO levels might be required to maintain a steady state level of reducing monosaccharide sugars to confer desiccation tolerance to seeds, starting from dry seeds to all the way through the post-germination stage (Arunraj *et al.*, 2020).

Seed storability, defined as the longevity of seeds after storage, is partially correlated with seed desiccation tolerance, and seed vigor is also closely correlated with seed longevity (Bentsink *et al.*, 2000; Gurusinghe and Bradford, 2001). As a result, seeds with good desiccation tolerance frequently exhibit longer seed longevity and higher seed vigor. In addition to playing important roles in desiccation tolerance, high levels of RFOs are also required for maintaining seed vigor or longevity in plants (Bernal-Lugo and Leopold, 1992; Pukacka *et al.*, 2009; Vandecasteele *et al.*, 2011; de Souza Vidigal *et al.*, 2016; Salvi *et al.*, 2016; Li *et al.*, 2017; Han *et al.*, 2020). In hybrid rice seed, the level of raffinose is positively correlated with the seed germination rate under natural aging conditions (Yan *et al.*, 2018), but the galactose content is negatively correlated with the seed germination rate under both natural and artificial aging conditions (Chen *et al.*, 2022). In maize seeds, a low level of raffinose is associated with lower seed vigor (Bernal-Lugo and Leopold, 1992). Consistent with this, the maize Dehydration-Responsive Element-Binding 2 A mutant (*zmdreb2a*) exhibits decreased seed longevity due to reduced expression of a *ZmRS* gene and a decreased level of raffinose accumulation in the embryo (Han *et al.*, 2020). In *Arabidopsis*, overexpression of *ZmAGA1*, which decreases both RFO and galactinol contents in mature seeds, results in a higher seed germination percentage but decreased seed aging tolerance (Zhang *et al.*, 2021). Further analysis showed that RFO levels were lowest in imbibed *ZmAGA1* overexpressing seeds and rapidly increased post-imbibition. When seeds transitioned to the germination stage, the RFOs were rapidly hydrolyzed to monosaccharide sugars, which may be incorporated into either cell membranes or cell walls of the growing shoot and root tips, providing energy leading to increased germination vigor (Zhang *et al.*, 2021). Intriguingly, Li *et al.* (2017) found that monocot and dicot plants have different requirements for RFOs in modulating seed vigor. An important discovery was that raffinose is the only RFO that accumulates in seeds of the monocot maize, and the seeds of the *zmrs* mutant, which lacks raffinose, show remarkably reduced vigor even though they survive desiccation. By contrast, several RFOs (raffinose, stachyose, and verbascose) are detected in the seeds of *Arabidopsis*. It seems that seed vigor of *Arabidopsis* is positively correlated with either the total RFO content or the RFO/sucrose ratio, instead of the absolute amounts of individual RFOs, and moreover, that stachyose and verbascose contribute more than raffinose to seed vigor in *Arabidopsis* (Li *et al.*, 2017). Nevertheless, in some plant species, there is no direct association between RFOs and desiccation tolerance or seed vigor (Bentsink *et al.*, 2000; Gurusinghe and Bradford, 2001; Dierking and Bilyeu, 2009), suggesting that a broader analysis of this phenomenon is necessary. In conclusion, these observations demonstrate the pivotal roles of RFOs in controlling desiccation tolerance, storability, and vigor of plant seeds, and show that the specific roles of RFOs in these processes might be plant species-dependent to some extent.

B. The roles of RFOs in temperature stress tolerance

Extreme low (cold, chilling, or frost) and high temperatures, can have highly detrimental effects on plant growth and crop yield worldwide (Suzuki, 2019). Previous studies have indicated that cold treatment induces the accumulation of raffinose in both cold-tolerant and cold-sensitive Arabidopsis and rice accessions but that raffinose levels are remarkably higher in cold-tolerant accessions than in cold-sensitive accessions (Klotke *et al.*, 2004; Morsy *et al.*, 2007; Nägele and Heyer, 2013). Keller *et al.* (2021) demonstrated that accumulation of raffinose in the pith tissue is correlated with freezing tolerance of both freezing–sensitive and freezing-tolerant sugar beet. Increased transcription levels of the GolS and RS genes as well as the accumulation of raffinose were also observed in rice seedlings exposed to chilling stress, and in grapevine woody tissues subjected to cold stress (Saito and Yoshida, 2011). Moreover, excised *A. reptans* leaves accumulated more RFOs during frost treatment under temperatures ranging from −10.5 to −24.5 °C, with more severe damage being observed in photosystem II in the RS mutant than in the corresponding wild-type plants during freezing (Peters and Keller, 2009; Knaupp *et al.*, 2011). A recent report demonstrated that the maize *zmrs* mutant lines, in which raffinose is eliminated, show decreased tolerance to cold stress compared with control plants. Further analysis also verified that the maize ZmDREB1A protein can bind directly to the promoter of *ZmRS* to activate its expression, and consequently lead to the accumulation

of raffinose and increased cold tolerance in maize (Han *et al.*, 2020). Similarly, ethylene-responsive factor 108 (ERF108) has been observed to directly target an RS enzyme gene to modulate the cold stress response of trifoliate orange (*Poncirus trifoliata L.*) (Khan *et al.*, 2021). Moreover, calmodulin-like protein 42 (MtCML42) has been demonstrated to positively regulate the C-repeat binding factor (CBF) pathway, which in turn increases the transcription of *MtGolS1* and *MtGolS2*, resulting in raffinose accumulation and enhanced cold tolerance in *Medicago truncatula* (Sun *et al.*, 2021). These results together suggest a positive role of RFOs in plant cold stress response.

Plants acquire heat stress tolerance through priming, which enables them to build stress memory when subjected to heat stress. A recent report found that primed plants perform better than non-primed plants under heat stress, partially because of increased RFO contents, suggesting an important role of RFOs in plant heat stress tolerance (Serrano *et al.*, 2019). Indeed, researchers demonstrated that overexpression of chickpea (*Cicer arietinum*) *CaGolS1* and *CaGolS2*, which are strongly induced by heat and oxidative stress, results in increased galactinol and raffinose contents as well as enhanced heat and oxidative stress tolerance through a reduced accumulation of reactive oxygen species (ROS) and consequent lipid peroxidation in *Arabidopsis* transgenic plants (Salvi *et al.*, 2018). Furthermore, *Arabidopsis* plants overexpressing *ZmGolS2* showed increased heat stress tolerance, which was attributed to the enhanced galactinol and raffinose contents (Gu *et al.*, 2016). Recently, overexpression of the heat shock factor A2 (*ZmHSFA2*) in *Arabidopsis* plants was found to lead to increased transcription of *AtGolS1*, *AtGolS2*, and *AtRS5*, increased raffinose levels in leaves and enhanced heat tolerance, whereas overexpression of the maize heat shock binding protein 2 (*ZmHSBP2*) in *Arabidopsis* reduced the expression of *AtGolS1*, *AtGolS2*, and *AtRS5*, which prevented raffinose accumulation in leaves and decreased heat tolerance (Gu *et al.*, 2019). In summary, all these results indicate the positive regulatory roles of RFOs in temperature stress response in plants.

C. The roles of RFOs in tolerance to drought, salt, and oxidative stresses

In addition to their roles in temperature stress, RFOs are also associated with enhanced drought stress tolerance in plants; for example, there is a strong accumulation of RFOs (raffinose, stachnose, and verbascose) in the resurrection plant *Xerophytaviscosa* when it is subjected to drought stress treatment (Peters *et al.*, 2007) Overexpression of *AtGolS2* in *Brachypodium distachyon*, *Arabidopsis*, and rice also remarkably increases drought tolerance (Taji *et al.*, 2002; Himuro *et al.*, 2014; Selvaraj *et al.*, 2017). Transgenic rice plants overexpressing *AtGolS2* exhibit increased galactinol content, grain yield in terms of panicle number, and grain fertility compared with control plants. In addition, ectopic expression of *BhGolS1* leads to significant accumulation of galactinol and raffinose as well as elevated drought tolerance in tobacco, and ectopic expression of *CsGolS4* results in increased galactinol and stachyose contents and improved drought tolerance in cucumber (Ma *et al.*, 2021). Interestingly, the expression of *BhGolS1* was found to be directly activated by the *BhWRKY1* gene *via* the binding of BhWRKY1 to W-box elements in the *BhGolS1* promoter (Wang *et al.*, 2009). Modulating the expression of *RS* genes has also been found to result in altered drought stress responses in plants. The maize *Zmrs* mutant, which lacks raffinose, shows decreased drought tolerance, whereas *Arabidopsis ZmRS*-overexpressing plants show increased tolerance to drought stress (Li *et al.*, 2020). This enhanced drought tolerance conferred by overexpression of *ZmRS* was found to be due to increased *myo*-inositol levels following galactinol hydrolysis, with the increased ratio of *myo*-inositol to raffinose positively regulating plant drought stress responses.

Thellungiella salsuginea, which is an important model for studying abiotic stress responses, shows increased levels of galactinol and raffinose and a high ratio of raffinose to sucrose when subjected to salt, drought, or cold stress (Amtmann, 2009). *TsGolS2* is significantly induced by NaCl, polyethylene glycol, and abscisic acid (ABA) treatments. Overexpression of *TsGolS2* in *Arabidopsis* improves salt and osmotic stress tolerance as manifested by the higher rates of germination and seedling growth of *TsGolS2* overexpressors compared with those of the control plants (Sun *et al.*, 2013). As mentioned above, ZmHSFA2 can regulate the expression of *AtGolS1*, *AtGolS2*, and *AtRS5* in *Arabidopsis* plants. Analogously, *Arabidopsis* plants overexpressing AtHsfA2 also exhibit increased transcription of *AtGolS1*, *AtGolS2*, *AtGolS4*, and *AtRS2* and increased galactinol and raffinose contents compared with control plants. Exogenous methylviologen treatment, which can mimic the oxidative stress in *Arabidopsis*, significantly increases the expression of not only *AtHsfA2* but also the *AtGolS* and *AtRS* genes and the levels of galactinol and raffinose. Overexpression of *AtGolS1* and *AtGolS2* results in

Table 1. The biological functions of genes involved in RFO metabolic pathways.

Gene name	Species	Function	References
Galactinol synthase (GolS)			
ZmGolS1/2/3	*Zea mays*	Response to heat and drought stress	Zhao *et al.*, 2004a, 2004b; Gu *et al.*, 2016
OsGolS1/2	*Oryza sativa*	Response to chilling, cold, drought, salt stress, and N deficiency	Phan *et al.*, 2009) Saito and Yoshida, 2011; Mukherjee *et al.*, 2019; Cui *et al.*, 2020
AtGolS1	*Arabidopsis thaliana*	Response to drought, high-salinity stresses, and fungal pathogens; negatively regulates seed germination	Taji *et al.*, 2002; Jang *et al.*, 2018; Jing *et al.*, 2018
AtGolS2	*A. thaliana*	Response to oxidative stress, drought, salt, chilling, and high-light stress; seed longevity	Himuro *et al.*, 2014; de Souza Vidigal *et al.*, 2016; Selvaraj *et al.*, 2017; Jing *et al.*, 2018
AtGolS3	*A. thaliana*	Response to cold and oxidative stress and pathogens Regulates cell wall growth and development	Taji *et al.*, 2002; Jing *et al.*, 2018; La Mantia *et al.*, 2018
AtGolS4/5/6/7	*A. thaliana*	Drought and cold stress; oxidative damage	Taji *et al.*, 2002; Nishizawa *et al.*, 2008; Jing *et al.*, 2018
AtGolS10	*A. thaliana*	Protects plants from oxidative damage	Nishizawa *et al.*, 2008
CsGolS1	*Corynespora cassiicola*	Involved in induction of systemic resistance	Zhou *et al.*, 2017
CsGolS1/2/3	*Camellia sinensis*	Responses to water deficit, lower temperature, pest attack, and ABA.	Zhou *et al.*, 2017
CaGolS1/2	*Cicer arietinum*	Improves seed vigor and prolongs seed life; response to heat and oxidative stress	Salvi *et al.*, 2016, 2018
BnGolS1	*Brassica napus*	Response to desiccation during seed development	Li *et al.*, 2011
XvGolS	*Xerophyta viscosa*	Transcript level increases under water deficit	Peters *et al.*, 2007
BhGolS1	*Boea hygrometrica*	Dehydration- and ABA-inducible expression	Wang *et al.*, 2009
TaGolS1/2	*Triticum aestivum*	Response to cold stress	Shimosaka and Ozawa, 2015
AnGolS	*Ammopiptanthus nanus*	Response to cold, salt, and drought stresses	Liu *et al.*, 2016
PsGolS	*Pisum sativum*	Expression induced by dehydration in seedlings	Lahuta *et al.*, 2014
LeGolS1	*Lycopersicon esculentum*	Expression induced by dehydration and cold in seedlings	Downie *et al.*, 2003
MtGolS1/2	*Medicago truncatula*	Involved in cold stress tolerance	Sun *et al.*, 2021
CsGolS1	*Cucumis sativus*	Response to nematode infection	Wang *et al.*, 2022
Raffinose synthase (RS)			
ZmRS8 (*ZmRS*)	*Z. mays*	Response to drought stress; regulates seed vigor	Gu *et al.*, 2016; Han *et al.*, 2020; Li *et al.*, 2020
OsRS5	*O. sativa*	Expression induced by dehydration in seedlings	Saito and Yoshida, 2011
AtRS4 (*AtSTS*)	*A. thaliana*	Response to abiotic stress	Gangl *et al.*, 2015
AtRS5	*A. thaliana*	Promotes seed germination in darkness	Gangl and Tenhaken, 2016; Jing *et al.*, 2018
CsRS	*C. sativus*	Promotes seed germination; overexpression enhances ROS tolerance and represses the defense response to leaf rust disease; response to nematode infection	Sui *et al.*, 2012; La Mantia *et al.*, 2018; Wang *et al.*, 2022
AmSTS1	*Alonsoa meridionalis*	Involved in phloem loading	Voitsekhovskaja *et al.*, 2009
VaSTS	*Vigna angularis*	Response to cold stress	Iftime *et al.*, 2011
CsSTS	*C. sativus*	Involved in phloem loading; response to nematode infection	Lü *et al.*, 2017; Zhang *et al.*, 2020; Wang *et al.*, 2022
PsRS	*P. sativum*	Response to dehydration in seedlings	Lahuta *et al.*, 2014
PtrRS	*Poncirus trifoliata*	Enhances cold stress tolerance	Khan *et al.*, 2021
LcRS	*Lens culinaris*	Seed development	Kannan *et al.*, 2021
VviRS5	*Vitis vinifera*	Response to cold stress	Noronha *et al.*, 2021

(*Continued*)

Table 1. Continued.

Gene name	Species	Function	References
Alkaline α-galactosidase (AGA)/seed imbibition protein (SIP)			
ZmAGA1 (ZmRS10)	*Z. mays*	Response to cold, drought, heat, ABA, and SA; regulates seed germination and seed aging	Zhao *et al.*, 2006; Zhang *et al.*, 2021
OsAGA1 (Osh69)	*O. sativa*	Leaf senescence; response to darkness, JA, SA, H_2O_2, and wounding	Lee *et al.*, 2004
AtSIP2 (AtRS2)	*A. thaliana*	Involved in unloading raffinose from the phloem	Peters *et al.*, 2010
AtSIP3 (AtDIN10)	*A. thaliana*	Response to light; regulates seed germination	Gangl and Tenhaken, 2016
ClAGA2	*Citrullus lanatus*	Regulates seed germination	Ren *et al.*, 2021
CsAGA2	*C. sativus*	Phloem unloading	Liu *et al.*, 2022
CsAGA1, CsAGA2	*C. sativus*	Fruit development; response to nematode infection	Hua *et al.*, 2021; Wang *et al.*, 2022
Alpha-galactosidase (AGAL)			
AtAGAL2	*A. thaliana*	Leaf development	Chrost *et al.*, 2007
ArGGT1	*A. reptans*	Response to chilling	Peterbauer *et al.*, 2002; Tapernoux-Lüthi *et al.*, 2004
VvAGAL1	*V. vinifera*	Response to salt and drought stress	Daldoul *et al.*, 2012

increased levels of galactinol and raffinose, which are positively correlated with enhanced tolerance to oxidative, salinity, and chilling stresses in *Arabidopsis* (Nishizawa *et al.*, 2008). These results together indicate the positive roles of RFOs in regulating plant responses to drought, salt, and oxidative stresses.

So far, three regulatory mechanisms have been proposed to explain the roles of RFOs in mediating different abiotic stress responses in plants. First, raffinose could maintain the stability of the cell membrane during air-drying and prevent leakage of cellular contents and membrane fusion after rehydration (Cacela and Hincha, 2006). Second, galactinol and raffinose could function as osmoprotectants and ROS scavengers to mitigate oxidative damage generated by adverse conditions (Nishizawa *et al.*, 2008; Van den Ende and Valluru, 2009; Van den Ende, 2013). Third, raffinose could be transported into chloroplasts to protect thylakoids and stabilize photosystem II, maintaining plant photosynthesis under adverse conditions (Knaupp *et al.*, 2011).

D. The role of RFOs in biotic stress response

Induced systemic resistance (ISR) and systemic acquired resistance are two types of resistance against pathogenic attacks induced in plants upon appropriate stimulation before contact with pathogens (Kim *et al.*, 2008). Two previous studies have indicated the important roles of RFOs in regulating *Pseudomonas chlororaphis* O6-mediated ISR in plants (Kim *et al.*, 2008). Transcriptional induction of *C. sativus GolS1* (*CsGolS1*) and the resultant accumulation of galactinol are observed earlier in O6-treated cucumber plants than in control plants after *Corynespora cassiicola* challenge. *CsGolS1*-overexpressing transgenic tobacco plants, which show increased accumulation of galactinol, exhibit constitutive resistance against the pathogens *Botrytis cinerea* and *Erwinia carotovora* (Kim *et al.*, 2008). Exogenous galactinol treatment remarkably increases the resistance of wild-type tobacco plants against pathogen infection, at least partially through the activation of defense-related genes, such as pathogenesis-related protein 1a (PR1a) and PR1b.

In *Arabidopsis*, *AtGolS1*, the ortholog of cucumber *CsGolS1*, is specifically induced by the pathogen *B. cinerea* (Cho *et al.*, 2010). Simultaneous overexpression of melon *CmGolS1*, cucumber *CsRS*, and *Alonsoa meridionalis AmSTS* in *Arabidopsis* resulted in a strong accumulation of RFOs in the transgenic plants, which showed enhanced resistance to the green peach aphid. Contrary to the positive roles of *GolS* in

regulating disease and pest resistance, *GolS* genes in poplar seem to play negative roles in resistance; the expression levels of two endogenous *GolS* genes are significantly downregulated and the galactinol level is reduced in wild-type poplar leaves inoculated with leaf rust (*Melampsora aecidiodes*) (La Mantia *et al.*, 2018). In addition, poplar plants overexpressing *AtGolS3* and *CsRS* exhibit increased galactinol and raffinose contents and decreased leaf rust resistance, while *GolS*-silenced poplar plants, which have lower galactinol concentrations, show significantly higher leaf rust resistance than the *AtGolS3*-overexpressing plants. The decreased leaf rust resistance of *AtGolS3* and *CsRS* overexpressing plants may be at least in part due to the reduced expression of salicylic acid (SA) signaling genes as well as the reduced level of SA in these transgenics (La Mantia *et al.*, 2018). Intriguingly, antagonism between SA and myo-inositol, an essential substance for the synthesis of galactinol, was identified in *Arabidopsis* plants infected with *Pseudomonas syringae* (Chaouch and Noctor, 2010). Myo-inositol suppresses the accumulation of SA and abolishes the resistance to virulent bacteria in catalase-deficient plants. Taken together, these results suggest that RFOs play pivotal roles in plant biotic stress response and that their specific roles depend on the pathogen the plant encounters.

VII. Concluding remarks and future perspectives

Compelling evidence is accumulating that induction of the expression of RFO biosynthetic genes (especially *GolS* and *RS*) and the accumulation of RFOs (mainly galactinol and raffinose) are general responses of plants to various abiotic and biotic stresses and that high levels of RFOs lead to enhanced tolerance of plants to different stresses. More strikingly, the roles of RFOs in seed vigor, plant growth, and development have also been reported by recent studies, supporting the existential importance of RFOs in the plant kingdom.

Although great progress has been made, our knowledge of the specific roles of RFOs in plant development and the responses of RFOs to different plant stresses are still very limited. Systematic studies on the origin and evolution of RFO metabolic pathways will help us to understand the relationship between RFO functions and adaptive evolution in plants. In addition, comparative analysis of anatomical structure in more species, together with spatially resolved metabolomics studies will help reveal the mechanism of source-to-sink transport of RFOs in plants. Moreover, systemic studies on the molecular mechanisms by which RFOs modulate plant development, stress tolerance, and seed vigor are still largely lacking. With the application of cutting-edge technologies, we can expect that more specific roles of RFO metabolic genes will be validated and their regulatory mechanisms fully understood in the next few years. Crucially, the development of genome editing technology means that in the near future key RFO metabolism enzymes could serve as promising targets for improving crop yield or quality by achieving the right balance of RFOs. This is especially important for legumes, where reduction of the RFO content in seeds could make them more suitable for consumption by humans and monogastric animals.

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