Raffinose synthase enhances drought tolerance through raffinose synthesis or galactinol hydrolysis in maize and *Arabidopsis* plants

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ABSTRACT

Raffinose and its precursor galactinol accumulate in plant leaves during abiotic stress. RAFFINOSE SYNTHASE (RAFS) catalyzes raffinose formation by transferring a galactosyl group of galactinol to sucrose. However, whether RAFS contributes to plant drought tolerance and, if so, by what mechanism remains unclear. In this study, we report that expression of *RAFS* from maize (or corn, *Zea mays*) (*ZmRAFS*) is induced by drought, heat, cold, and salinity stresses. We found that *zmrafs* mutant maize plants completely lack raffinose and hyper-accumulate galactinol and are more sensitive to drought stress than the corresponding null-segregant (NS)

plants. This indicated that ZmRAFS and its product raffinose contribute to plant drought tolerance. *ZmRAFS* overexpression in *Arabidopsis* enhanced drought stress tolerance by increasing *myo*-inositol levels via ZmRAFS-mediated galactinol hydrolysis in the leaves due to sucrose insufficiency in leaf cells and also enhanced raffinose synthesis in the seeds. Supplementation of sucrose to detached leaves converted ZmRAFS from hydrolyzing galactinol to synthesizing raffinose. Taken together, we demonstrate that ZmRAFS enhances plant drought tolerance through either raffinose synthesis or galactinol hydrolysis, depending on sucrose availability in plant cells. These results provide new avenues to improve plant drought stress tolerance through manipulation of the raffinose anabolic pathway.

Raffinose is thought to play a role in plant drought stress-tolerance based on positive correlations between raffinose accumulation in leaves when plants encounter drought stress (1-3). GALACTINOL SYNTHASE (GOLS) and RAFFINOSE SYNTHASE (RAFS) are two key enzymes responsible for raffinose biosynthesis (4,5). Using *myo*-inositol and UDP-galactose, GOLS (EC 2.4.1.123) catalyzes the production of galactinol (6); whereas RAFS (EC 2.4.1.82) uses sucrose and galactinol to synthesize raffinose (4).

The expression and function of *GOLS* in response to plant abiotic stress have been well elucidated in many plant species. Expression of some *GOLS* genes is induced by abiotic stress, such as drought, heat shock, salinity and osmotic shock (7,8) while others are induced by pathogen attack (9). Overexpression of *GOLS* increased the galactinol and raffinose content while concurrently enhancing the abiotic stress tolerance of transgenic plants (7,10-12). Since both galactinol and raffinose are increased by overexpression of *GOLS* genes, it is unclear whether galactinol, raffinose, or both directly enhance abiotic stress tolerance. Further complicating this situation, GALACTINOL SYNTHASE has, as one of its substrates, *myo*-inositol, a sugar alcohol with abiotic stress alleviating properties of its own (13) and whose utilization to make galactinol, necessarily draws down its concentration in the cell.

Unlike GOLS, very few RAFS have been characterized *in vitro* or *in vivo* (4,5,14-17). *RAFS* genes are induced by dehydration stress (17), and their transcripts accumulate in the late stages of seed maturation and desiccation, coincident with increased raffinose content (18,19). There are six putative *RAFFINOSE SYNTHASE* genes (*AtRS1-6*) in *Arabidopsis thaliana* (Arabidopsis) (20). AtRS5 is the only genuine raffinose synthase that has been shown to have RAFS activity (16). The mutation of *AtRS5* abolishes raffinose in the leaves and reduces the raffinose content in seeds. AtRS4, identified as a STACHYOSE SYNTHASE (AtSTS), has some raffinose synthetic capacity and the *atrs4* mutant is devoid of stachyose in the

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seeds (5). In addition to *AtRS5* and *AtRS4* in Arabidopsis, *AtRS6* is predicted to be a drought-induced (through alternative splicing) *RAFS* (21). Knockout mutants in genes involved in raffinose biosynthesis in Arabidopsis have failed to show any plant-level phenotypic changes during normal growth other than perturbations in raffinose family oligosaccharides amounts and a repression of seed germination in darkness (5,16). Fewer *RAFS* genes have been predicted in the maize (*Zea mays*) genome based on bioinformatics studies (22), and some of these, such as GRMZM2G340656, have already been experimentally identified and annotated as encoding ALKALINE ALPHA GALACTOSIDASE (23). We recently characterized the unique maize *raffinose synthase* gene and found that it plays an important role in maize seed vigor and longevity (19). Currently, no evidence has shown that over-expression of *RAFS* can enhance the raffinose content in plant vegetative tissues. Despite extensive studies, the function of raffinose in regulation of, or response to, vegetative abiotic stress has not been distinguished from that of galactinol or *myo*-inositol and, thus, the role raffinose plays in plant drought stress tolerance remains unclear. There are three hypothetical mechanisms addressing how raffinose performs its function when plants are in, or are recovering from, adverse conditions: 1. As a protective agent, raffinose could stabilize membranes during dehydration and prevent leakage of cellular contents and membrane fusion after

rehydration (24). 2. As a ROS scavenger, raffinose (and galactinol) may mitigate oxidative damage under abiotic stress conditions (20). 3. Raffinose could be transported into chloroplasts, protecting thylakoids and stabilizing PSII (25,26).

In this study, we demonstrated that maize *zmrafs* mutants lacking raffinose and hyper-accumulating galactinol but with WT amounts of sucrose and *myo*-inositol, were more sensitive to drought stress than null segregant lines in two maize inbred lines. Similar to the *ZmGOLS2*-overexpressing Arabidopsis plants, *ZmRAFS*-overexpressing Arabidopsis plants displayed a significantly increased tolerance to drought stress. However, while the galactinol and raffinose amounts in *ZmGOLS2*-overexpressing plants were greater, both were significantly decreased in *ZmRAFS*-overexpressing Arabidopsis leaves. The ZmRAFS in *ZmRAFS*-overexpressing Arabidopsis leaves did not seemingly contribute to net raffinose accumulation but rather hydrolyzed galactinol without transfer of the galactose to sucrose, thereby generating large amounts of *myo*-inositol under drought stress. This study demonstrates that ZmRAFS enhances plant drought tolerance through either synthesis of more raffinose or by generation of more *myo*-inositol *via* hydrolysis of galactinol.

RESULTS

ZmRAFS is induced by abiotic stress

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ZmRAFS transcription was rapidly and dramatically up-regulated in leaves when the V3 stage (three leaves stage) seedlings were treated with heat shock, dehydration or subjected to increased salt within 2-3 h, but returned to background levels with prolonged treatment (Fig. 1A). Under cold stress, *ZmRAFS* exhibited weakly increased level of transcript in V3 stage seedlings treated for 8 h (Fig. 1A). ZmRAFS protein was undetectable in untreated leaves of V3 stage seedlings, but accumulated nearly concomitantly with transcript under heat shock and salt stress, but showed a gradual increase under dehydration treatment (Fig. 1B). No ZmRAFS protein could be detected in leaves under cold stress (Fig. 1B). The ZmRAFS mRNA abundance and its protein accumulation are imperfectly correlated in maize seedlings under abiotic stress. These data suggest that *ZmRAFS* expression might be regulated at both transcriptional and translational levels. Sub-cellular localization of ZmRAFS protein was determined by transient expression of a fusion protein comprising ZmRAFS and YFP in maize leaf protoplasts. Fluorescence signal from YFP was detected throughout the cytoplasm and chloroplasts but was not in the vacuole, while the fluorescence of the ZmRAFS-YFP fusion protein was only detected in the cytoplasm (Fig. 1C). Western blot analysis of ZmRAFS-YFP fusion protein expression showed that the fusion protein was present and intact in transformed maize leaf protoplasts as was endogenous RAFS (Fig. 1D).

zmrafs maize mutants, containing no raffinose but accumulating galactinol, are more sensitive to drought stress than NS

We next investigated the drought stress tolerance of the two *zmrafs* mutant lines in which *ZmRAFS* expression was interrupted by *mutator* insertion (19) (Fig. S1). The 7th leaf of maize plants was repeatedly photographed every other day during water withholding (Fig. 2A and S2A). The *zmrafs-1* mutant plants exhibited more severe injury compared to NS plants (Fig. 2A). *zmrafs-2* leaves showed greater longitudinal folding than NS when the rolled leaves were spread open (Fig. S2A). Electrolyte leakage, an indicator of cellular injury (27), of the mutant leaves was significantly greater than that of NS leaves (Fig. 2B) on the 16th, 18th and 20th day of the drought stress, but not during normal watering. A similar electrolyte leakage phenotype was observed in *zmrafs-2* mutant plants relative to their NS, occurring even earlier at day 14 of the stress (Fig. S2B). Sucrose and *myo*-inositol amounts in leaves prior to and during the drought were similar between NS and mutant plants except for *myo*-inositol at 10 days after the start of the drought stress (Fig. 2C-D). Galactinol accumulated to greater amounts in mutant leaves compare to those of the NS; while the reverse was true of raffinose which, as expected, was undetected in the mutant (Fig. 2E-2F).

Overexpression of *ZmRAFS* in Arabidopsis enhanced galactinol hydrolysis rather than raffinose synthesis in leaves yet still enhanced

drought stress tolerance

To further investigate the consequences of altered raffinose amounts on plant drought stress tolerance, three Arabidopsis lines overexpressing the *ZmRAFS* gene were characterized for drought stress tolerance. In addition to wild type control, the *GFP-* and *ZmGOLS2*-overexpressing *Arabidopsis* lines (12) were also included as controls. The ZmRAFS and ZmGOLS2 protein accumulation was determined by western blot analysis (Fig. S3A). The Arabidopsis *raffinose synthase* (*atrs5*) mutant was characterized by PCR (Fig. S3B-C) and also used as a drought-susceptible control. After drought stress and re-watering, the 3 *ZmRAFS*-overexpressing lines were obviously more resistant to drought stress than any of the control lines except for the *ZmGOLS2*-overexpressing plants known to possess superior drought tolerance (12) (Fig. 3A). The survival percentage of *ZmRAFS-* and *ZmGOLS2*-overexpressing lines after drought stress was similar and both were significantly greater than that of the wild type (WT), *GFP* expressing (OEGFP) or *atrs5* plants (Fig. 3B). For unstressed seedlings, there was no difference in leaf ion leakage percentages regardless of genotype (Fig. 3C). However, upon drought stress, those percentages for all *ZmRAFS-* and *ZmGOLS2*-overexpressing plants were similarly low, and both are significantly lower than that of the WT, GFP expressing, or *atrs5* plants (Fig. 3C).

Drought stress enhanced the sucrose content in the leaves of all plant genotypes; increased the *myo*-inositol content in the 3 *ZmRAFS*-overexpressing plants and in the *atrs5* mutant; increased the galactinol content in all leaves apart from those of the 3 *ZmRAFS* plants; and increased the raffinose content in all leaves excluding those of 2 of the 3 *ZmRAFS* plants (Fig. 3D). Overexpression of *ZmGOLS2* enhanced both galactinol and raffinose contents, regardless of stress, compared with control lines (WT and OEGFP) (Fig. 3D). The raffinose content was decreased while the galactinol content was increased in *atrs5* mutant leaves as compared to WT (Fig. 3D). Unexpectedly, the raffinose and the galactinol content in leaves of the 3 *ZmRAFS* lines were significantly decreased compared to WT and OEGFP whether the plants were well-watered or under drought conditions (Fig. 3D). Surprisingly, given what was observed in the leaves, the raffinose content in seeds of *ZmRAFS-* overexpressing Arabidopsis lines was significantly greater than that of other lines (19). To investigate whether ZmRAFS protein expressed in OEZmRAFS leaves was able to synthesize raffinose using sucrose and galactinol, enzyme activities of leaf extracts from all Arabidopsis lines were tested *in vitro* for raffinose-synthetic (transferase) and non-raffinose producing, galactinol-hydrolytic activities. Under the conditions tested, the leaf extracts of all three of the *ZmRAFS*-expressing Arabidopsis lines had both dramatically greater transferase (Fig. 3E) as well as hydrolytic (Fig.

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3F) activities relative to the leaf extracts of the other genotypes.

Characterization of the substrate binding site of ZmRAFS

RAFFINOSE SYNTHASE binds two substrates (galactinol and sucrose) and it has both raffinose synthetic and galactinol hydrolytic activities. To determine the substrate binding sites of the ZmRAFS, enzyme activity assay of mutated ZmRAFS was performed (Fig. 4). According to its closely related templates, the most probable ligand binding sites of ZmRAFS were predicted using the I-TASSER online resource (28) (Supplementary Table S1). To determine the putative substrate binding residues of ZmRAFS, those predicted amino acids (AA) were mutated to alanine to minimize potential structural alterations of the mutated protein (Fig. 4A). The solubility of the mutant ZmRAFS-His6 recombinant proteins were demonstrated using western blot analysis of an aliquot of the supernatant for each mutant (Fig. 4B). The bacterial-expressed, wild type ZmRAFS protein showed both raffinose synthetic- and unproductive galactinol hydrolytic-activities as determined by *in vitro* assay (Fig. 4C). Although mutation of many residues decreased or abolished either raffinose synthetic-activity or both raffinose synthetic- and unproductive galactinol hydrolytic-activity, none enhanced raffinose synthetic activity (Fig. 4C). The aspartate-264-alanine mutant had greatly increased (~2-fold) unproductive galactinol

hydrolytic activity but had no detectable raffinose synthetic activity. These results are suggestive that the galactinol hydrolytic activity of ZmRAFS is independent of forming raffinose, supportive of the previous characterization of the catalytic mechanism as a ping-pong, bi-bi reaction (4).

The relative amount of galactinol and sucrose to ZmRAFS, determines whether ZmRAFS completes the transferase reaction, forming raffinose, after hydrolyzing galactinol

To investigate the effects of galactinol or sucrose on the ZmRAFS enzyme, the activity of *E. coli*-expressed ZmRAFS-His6 recombinant protein was characterized in substrate concentration control systems (Fig. 5A-B). *Myo*-inositol is generated by both raffinose synthesis and galactinol hydrolysis; whereas raffinose is only generated when the enzyme completes its transferase function. By this logic, the molar ratio of *myo*-inositol to raffinose is expected to reflect whether the ZmRAFS performs the entire synthesis reaction or only hydrolyzes galactinol without transfer of galactose to sucrose. When sucrose was saturating (29.2 mM) and the galactinol concentration was low (1.1-2.6 mM), similar amounts of *myo*-inositol and raffinose were generated (Fig. 5A; ratio [*myo*-inositol/raffinose] was approximately one). As the galactinol concentration increased (≥5.3 mM compared with the apparent Km=2.93 mM; Supplementary Table S2), the amount of *myo*-inositol that was generated was significantly

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greater than that of raffinose and so the ratio became statistically significantly greater than one (Fig. 5A). When galactinol was saturating (15.9 mM), regardless of the sucrose concentration, the amount of *myo*-inositol that was generated was always greater than that of raffinose produced; however, the ratio of *myo*-inositol to raffinose gradually decreased as the sucrose concentration was increased (Fig. 5B). These results show that the relative amount of the two substrates, sucrose and galactinol determines whether ZmRAFS predominantly hydrolyzed galactinol unproductively or predominantly completed the synthesis of raffinose, suggesting that greater raffinose production in Arabidopsis leaves expressing *ZmRAFS* may be possible by boosting substrate amounts. The kinetic assays determined that ZmRAFS exhibited apparent lower affinities of the substrates than that of AtRS5 (Supplementary Table S2) (29). Under the conditions used, ZmRAFS has a higher affinity for galactinol (apparent Km=2.93) than sucrose (apparent Km=7.40) when both substrates were present; and exhibited higher apparent affinity for galactinol when sucrose was absent (Supplementary Table S2).

To attempt to boost raffinose amounts and to further investigate why ZmRAFS predominantly hydrolyzed galactinol without completing the transferase reaction to produce raffinose in *ZmRAFS*-expressing Arabidopsis leaves, *ZmGOLS2/ZmRAFS*-double expressing plants were generated to test whether ZmRAFS would synthesize raffinose if the galactinol content was

increased in leaves. The paradigm for this experiment was the *ZmGOLS2* overexpressing plants which produce greater amounts of both galactinol and raffinose (Fig. 3D). As characterized by western blot analysis, the accumulation of ZmRAFS protein in leaves of *ZmRAFS*- and *ZmGOLS2/ZmRAFS*-double expressing plants were, as expected, greater than that of WT, GFP or the *ZmGOLS2* line (Fig. S4). Similarly, the ZmGOLS protein accumulation in leaves of *ZmGOLS2*- and *ZmGOLS2/ZmRAFS*-double-expressing plants was greater than that of WT, *GFP* or the *ZmRAFS* line (Fig. S4). Comparison of the plant morphology, percentage survival, and the ion leakage of these different Arabidopsis genotypes after drought stress, showed that the *ZmGOLS2/ZmRAFS*-, *ZmRAFS*-, and *ZmGOLS2*-expressing lines were more tolerant of drought stress compared to WT plants (Fig. 6A-C).

Drought stress increased sucrose accumulation in leaves of all lines; increased *myo*-inositol in OEZmRAFS-25 and two double over-expressing lines (OEZmGOLS2/ZmRAFS-1 and -3) (Fig. 6D). Expression of *ZmGOLS2* in Arabidopsis significantly increased the galactinol and raffinose content in leaves, whether the plants were grown under normal or drought stress conditions, as compared to WT (Fig. 6D). The galactinol content in the leaves of *ZmGOLS2/ZmRAFS*-expressing plants was significantly less than that of the *ZmGOLS2* expressing plants, whether the plants were grown under either normal or drought conditions (Fig.

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6D). The raffinose content in the leaves of *ZmGOLS2/ZmRAFS* expressing plants was similar to that of the *ZmGOLS2* expressing line when the plants were grown under well-watered conditions, but was less than that of *ZmGOLS2* line when the plants were grown under drought conditions, although it was still superior to WT or GFP control plants (Fig. 6D). These data indicate that ZmRAFS still unproductively hydrolyzed galactinol in *ZmGOLS2/ZmRAFS* expressing plants. Infiltration of sucrose into the detached leaves of all the Arabidopsis genotypes, compared with that of infiltration of detached leaves with water, increased the sucrose content in the leaf (Fig. 6E). Of the water-treated group, galactinol was only detected in *ZmGOLS2* plants (Fig. 6F). The galactinol content was significantly increased in detached leaves of both the *ZmGOLS2*- and 1 of 3 *ZmGOLS2/ZmRAFS-1* expressing plants upon sucrose treatment compared with water treatment, but remained constant in *ZmGOLS2/ZmRAFS*-2 and -3 lines (Fig. 6F). Even with water infiltration, the raffinose content in *ZmGOLS2* plants was significantly greater than that of other plants (Fig. 6G). Interestingly, the raffinose content in the *ZmGOLS2/ZmRAFS-1* and -2 plants was significantly greater than that, not only of the control plants, but also that of *ZmGOLS2* plants when sucrose treated (Fig. 6G) despite the seemingly different ratios of protein of the two enzymes accumulated in those plants (Fig. S4). These results are consistent with the greater accumulation of raffinose in the seeds of the *ZmRAFS* plants (19), these data suggested that infiltration of sucrose to the detached leaves of *ZmGOLS2/ZmRAFS*-expressing Arabidopsis plants provided sufficient sucrose substrate for ZmRAFS to complete the transferase reaction after hydrolyzing galactinol and subsequently, to accumulate raffinose.

Overexpression of *ZmMIPS* increased *myo*-inositol accumulation in leaves and enhanced plant drought tolerance.

Over-expression of *ZmRAFS* in Arabidopsis decreased galactinol and raffinose content while it increased *myo*-inositol content in leaves and enhanced the plant drought tolerance (Fig. 3A-D and Fig. 6A-D). To further demonstrate that it is *myo*-inositol that functions in this scenario to protect the plants from drought stress, two maize homologues of *AtMIPS* (*myo*-inositol-1-phosphate synthase, the key enzyme for *myo*-inositol synthesis in Arabidopsis) were cloned and named *ZmMIPS1* (*GRMZM2G155242*) and *ZmMIPS2* (*GRMZM2G004528*). The expression of ZmMIPS1 and ZmMIPS2 protein were induced in bacteria and purified (Fig. S5A). Enzyme activity assay of the purified N-terminal His-tag fusion proteins of ZmMIPS1 or ZmMIPS2 showed that both ZmMIPS fusion proteins were able to generate *myo*-inositol-1-phosphate (Fig. S5B). Plant expression vectors for *ZmMIPS1* or *ZmMIPS2* were constructed and transformed into Arabidopsis. The mRNA and protein accumulation of *ZmMIPS* in Arabidopsis was determined by RT-PCR and Western Blot,

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respectively (Fig. 7A). Over-expressing *ZmMIPS* substantially increased *myo*-inositol content in leaves under both normal and drought condition (Fig. 7B). The 3 *ZmMIPS*-overexpressing Arabidopsis lines were more resistant to drought stress than any of the control lines (Fig. 7C). After re-watering, the survival percentage of the 3 *ZmMIPS* expressing lines were significantly higher than that of control lines (Fig. 7D). These results demonstrated that increasing *myo*-inositol amounts was able to enhance plant drought tolerance.

DISCUSSION

Raffinose, but not galactinol, directly contributes to plant drought stress tolerance

The function of raffinose and galactinol in plant drought stress tolerance and the molecular mechanism by which they act remains unclear despite extensive studies. Galactinol is a direct substrate for raffinose biosynthesis (Fig. 8). Both galactinol and raffinose are accumulated to higher levels in plants in response to abiotic-stresses (12). Knockout mutants of relevant genes involved in raffinose biosynthesis have failed to show any abnormalities in *Arabidopsis* during normal growth other than perturbations in raffinose amounts and decreased completion of germination in the dark (5,16,29). Over-expression of *GOLS* in Arabidopsis increased both galactinol and raffinose content, decreased *myo*-inositol (Fig. 3D), while concurrently enhancing the abiotic stress tolerance of plants (7,10-12). Until now, no evidence had shown that

over-expression of *RAFS* could regulate the raffinose content in plant vegetative tissues. In short, despite extensive studies, the function of raffinose in response to plant abiotic stress has not been distinguished from that of galactinol and the role raffinose plays in plant drought stress tolerance remains unknown. By characterization of the maize knock out mutant (*zmrafs*) and *ZmRAFS*-expressing Arabidopsis plants, we found that raffinose, but not galactinol, directly and positively regulates plant drought stress tolerance.

myo-inositol generated from ZmRAFS mediated galactinol hydrolysis in *ZmRAFS* overexpressing Arabidopsis enhances plant drought tolerance

ZmGOLS2 overexpressing Arabidopsis plants with increased galactinol and raffinose enhanced plant drought stress tolerance (Fig. 3 and Fig. 6) (12). However, *ZmRAFS* overexpressing Arabidopsis plant leaves contained much less raffinose and galactinol, yet the plants were more resistant to drought stress compared with the WT or GFP plants (Fig. 3A-D). These results indicate that *ZmGOLS2*- and *ZmRAFS*-overexpressing Arabidopsis plants utilize different mechanism to protect plants from drought stress. ZmRAFS protein in leaf extracts of *ZmRAFS* overexpressing Arabidopsis plants was able catalyze two different reactions, 1) to synthesize raffinose using exogenous sucrose and galactinol, or 2) to hydrolyze galactinol to produce *myo*-inositol and galactose

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when sucrose is absent (Fig. 3E-F). The *myo*-inositol was significantly increased in *ZmRAFS* overexpressing plants under drought stress (Fig. 3D), indicating that ZmRAFS hydrolyzed galactinol instead of synthesizing raffinose in *ZmRAFS* overexpressing Arabidopsis leaves. It has been proposed that overexpression of *myo-inositol phosphate synthase* (*MIPS*) enhanced dehydration, salt and cold stress tolerance in several plant species (13,30-33). Overexpression of two *maize MIPSs* in Arabidopsis increased *myo*-inositol accumulation and enhanced the plant drought tolerance (Fig. 7). These data support that *myo*-inositol contribute to the plant drought tolerance. *myo*-inositol, either synthesized from G-6-P by MIPS in *ZmMIPS* overexpressing plants, or generated by the hydrolysis of galactinol in *ZmRAFS* overexpressing plant leaves, contributes to the plant drought tolerance. Galactinol functions as a storage or a supply of *myo*-inositol or galactose. ZmRAFS may either hydrolyze galactinol to generate *myo*-inositol in *ZmRAFS*-expressing lines, or use galactinol to synthesize raffinose in *ZmGOLS2* lines, and in both cases, enhance the plant drought stress tolerance.

Taken together, we propose a model to elucidate the function and mechanism of raffinose metabolism in plant drought stress tolerance (Fig. 8). The drought stress signal activates transcription factors responsible for upregulating genes encoding key enzymes, such as *GOLS*, *RAFS*, or *MIPS* in the raffinose biosynthetic pathway, and their action stimulates

the accumulation of *myo*-inositol, galactinol and raffinose. Both *myo*-inositol and raffinose directly and positively regulate plant drought stress tolerance, at least in Arabidopsis and maize.

Availability of sucrose determines whether ZmRAFS is to synthesize raffinose or to hydrolyze galactinol.

The ZmRAFS has been proven to have both raffinose synthetic and galactinol hydrolytic activity as determined either *in vitro* or *in vivo* (Fig. 3-5) (19). Either synthesis of raffinose or hydrolysis of galactinol by ZmRAFS would generate *myo*-inositol. Based on our data and the findings that the pea RAFS showed attributes of catalyzing a ping-pong bi-reaction (4), we propose here that RAFS binds and hydrolyzes galactinol first, releasing *myo*-inositol and retaining the galactose for some period until RAFS either: 1) binds sucrose and makes raffinose or; 2) releases free galactose exhibiting a galactinol hydrolytic activity. The lower the sucrose concentration, the greater the probability of RAFS hydrolyses galactinol and the less opportunity RAFS has to make raffinose (Fig. 5B). With increased sucrose, ZmRAFS tends to synthesize raffinose rather than to hydrolyze galactinol (Fig. 5B). This hypothesis is also supported by the point mutation data of ZmRAFS protein. Mutation of certain AAs of ZmRAFS abolished both galactinol hydrolytic and raffinose synthetic activity of ZmRAFS (Fig. 4). These sites are possibly critical for galactinol

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binding because the RAFS would not synthesize raffinose if it loses the ability of binding and hydrolyzing galactinol. Mutation of AA 264 from aspartic acid (D) to alanine (A) abolished the raffinose synthetic activity entirely while enhancing the galactinol hydrolytic activity about 2 folds, indicating that this site is likely critical either for sucrose binding or for catalytic activity of RAFFINOSE SYNTHASE, but not for galactinol binding. Supplementation of sucrose converted the ZmRAFS from hydrolyzing galactinol to synthesizing raffinose in *ZmGOLS2/ZmRAFS* double expressors (Fig. 6G). These data also suggest that ZmRAFS hydrolyzes galactinol first and then starts synthesizing raffinose if free sucrose is available.

Over-expression of *GOLS* has been reported to enhance the galactinol and raffinose content and abiotic stress tolerance of the plants (10,12,34). In those studies, more galactinol was produced by a combination of endogenous and heterologous *GOLS* gene expression, while raffinose was produced by endogenous RAFS. These data suggest that the substrate galactinol is a limiting factor for RAFS to synthesize raffinose *in vivo* and the higher molar ratio of galactinol to RAFS would stimulate raffinose biosynthesis. The *myo*-inositol, which was generated from galactinol hydrolysis, regardless of raffinose synthesis, was quickly recycled back to galactinol by a greater titer of GOLS enzyme.

ZmRAFS exhibited lower affinity to sucrose than it did with galactinol

(Supplementary Table S2). AtRS5 has dramatically higher affinity to sucrose than it with galactinol (Supplementary Table S2) (29). Thus, the high level of galactinol could promote raffinose synthesis catalyzed by AtRS5 without being hampered by sucrose insufficiency in *GOLS*-overexpressing Arabidopsis line (Fig. 3D and Fig. 6D) (10,12,34). The sucrose content in Arabidopsis leaves is very high regardless of genotype (Fig. 3D), however, it is preferentially exported to the sink tissues loaded into companion cells or sieve elements by sucrose transporters. The Km of AtSUC9 for sucrose is 0.006 (35) while the Km of AtRS5 for sucrose is 0.35 (29) and the ZmRAFS to sucrose is 7.40 (Supplementary Table S2). These data show that the sucrose transporter in leaves has a much higher affinity for sucrose than RAFS. Once sucrose is synthesized in leaves, it is immediately bound by the sucrose transporters and transported to the sink tissues. The remaining sucrose in leaves is presumably insufficient for RAFS to synthesize raffinose in *ZmRAFS*-overexpressing Arabidopsis leaves. Once sucrose is unloaded at the sink tissue, it is free for raffinose synthesis. We have previously reported that *ZmRAFS*-expressing Arabidopsis seeds has much greater raffinose content than that of control lines (19).

To our knowledge, the effects of over-expressing *RAFS* have not been reported except in one case of a cucumber *RAFS* gene, along with a muskmelon *GOLS* gene and the *Alonsoa meridionalis STACHYOSE SYNTHASE* (*STS*)

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gene all co-transformed into Arabidopsis plants. Transgenic lines homozygous for the 3 genes accumulated substantial galactinol, raffinose and stachyose (36). Since over-expression of the *GOLS* gene itself may also cause these biological effects, it is unclear whether or not the foreign *RAFS* gene functions and it is difficult to draw a conclusion as to its contribution to raffinose production. The scarcity of publications of overexpression of *RAFS* genes might be due to its unanticipated function hydrolyzing galactinol instead of synthesizing raffinose in plant leaves by RAFS when it is overexpressed in plants.

Regulation of Raffinose Metabolism Pathway for Improving Plant Drought Stress Tolerance.

Our data indicate that raffinose is beneficial to plant drought stress tolerance. We and other groups have confirmed that overexpression of *GOLS* genes enhanced the abiotic stress tolerance of transgenic plants (11,12,34,37). It is worthwhile mentioning that constitutive expression of *ZmGOLS2* genes in Arabidopsis did not cause any adverse effects to the plant under normal conditions (12). Similarly, overexpression of *ZmRAFS* enhanced plant drought stress tolerance without causing adverse effects to the plants under normal conditions (Fig. 3A-C). Furthermore, seed vigor can also be improved through manipulation of raffinose family oligosaccharides metabolic pathway (19). Taken together, it has become promising to manipulate raffinose in crop plants to enhance

both seed longevity and plant drought stress tolerance.

EXPERIMENTAL PROCEDURES

Plant material.

Maize (*Zea mays* L.) inbred line B73 was maintained in the lab. The method of genotyping, *Mu*-insertional site identification, and characterization of seed sugar profile of the *zmrafs* mutants and null segregant counterparts (NS) were described previously (19). The Arabidopsis mutant *atrs5* (CS318250, T-DNA inserted in At5g40390) was purchased from Arabidopsis Biological Resource Center (ABRC) through the Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org/). The transgenic Arabidopsis lines expressing *ZmGOLS2*, *ZmRAFS* and *ZmMIPS* were obtained by using floral dip method as previously described (19).

Vector construction.

For construction of the ZmRAFS-YFP fusion protein expression vector, the *Rluc* CDS, the second 2×35S promoter and the *Fluc* CDS in a dual luciferase control vector (38) were replaced by a MCS sequence (containing *Xho*I, *Pst*I, *Nhe*I, *Hind*III, *Sac*I, *Mlu*I, *Bgl*II, *Nco*I, *Bam*HI, *Eco*RI and *Xba*I). The *YFP* CDS was obtained by PCR from pmEYFP-1 vector (# 38773, Addgene) using a pair of primers (PYFP-F and PYFP-R; Supplementary Table S3) and then inserted between the *Bam*HI and *Eco*RI sites. Upstream

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of the *YFP* CDS, a double-stranded linker DNA fragment (5'-[CCATGG]GGAGGATCTGGAGGAGGAGG ATCTGGAGGA[GGATCC]-3') was inserted between *Nco*I and *Bam*HI sites. The *ZmRAFS* CDS, without the stop codon, was amplified using a pair of primers (ZmRAFS-CRF-HindIII and ZmRAFS-CRR-MluI; Supplementary Table S3) together with the template, pET-ZmRAFS, and inserted between the *Hind*III and *Mlu*I sites. The construction of expression vectors for expressing *ZmRAFS* in Arabidopsis followed the published protocol (19). For construction of bacterial expression vector of mutated ZmRAFS protein, a QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, USA) was used according to the instruction manual utilizing the ZmRAFS: His6 expressing vector pET-ZmRAFS (19) as the PCR template. Listed in Supplementary Table S3, the primers were named by both the original AA, its position in the ZmRAFS protein, and the mutated AA (e.g. S115A-F and S115A-R are the forward and reverse primers, respectively for mutating serine 115 of the ZmRAFS to alanine).

For expression of ZmMIPS protein in bacteria, prokaryotic expression vectors were constructed (*pET-ZmMIPS1* and *pET-ZmMIPS2*). The cDNA of *ZmMIPS1* and *ZmMIPS2* were amplified from cDNA synthesized from mRNA that was isolated from maize leaf (B73 inbred line) using a pair of primers (for *ZmMIPS1*, use pCSGFPBT-MIPS1-F×pCSGFPBT-MIPS1-R; for *ZmMIPS2*, use pCSGFPBT-MIPS2-

F×pCSGFPBT-MIPS2-R). Then the coding region of *ZmMIPS1* and *ZmMIPS2* were amplified from the first PCR amplicon using primers pET-ZmMIPS-F and pET-ZmMIPS-R (conservative sequence of both ZmMIPS1 and ZmMIPS2). The coding region of *ZmMIPS1* or *ZmMIPS2* was ligated into *EcoR*I-*Hind*III site of *pET-21d* vector. For construction of Arabidopsis over-expression vector of *ZmMIPS*, *pET-ZmMIPS1* and *pET-ZmMIPS2* were used as templates for amplification of coding region of *ZmMIPS1* or *ZmMIPS2* using the same primer pair (MIPS1/2-ara-F and MIPS1/2-ara-R). The PCR products were ligated into *Mlu*I-*Xba*I sites of *pCsGFPBT*.

Abiotic stress treatment and sampling.

The three-leaf stage of hydroponically-grown B73 maize seedlings (under 12 h light [890±54 µmol m^{-2} sec^{-1}] and 12 h darkness; constant temperature of 28±l ℃) were used for abiotic stress treatment. For heat shock treatment, plants were transferred to 42 ℃ for 0, 2, 4, and 8 h. For dehydration treatment, plants were transferred to filter paper and dried for 0, 2, 4, 8, and 16 h. For salt stress treatment, plants were transferred to 200 mM NaCl solution and grown for 0, 1.5, 3, 6, 12, and 24 h. For cold stress treatment, plants were transferred to 4 ℃ for 0, 2, 4, and 8 h. The second leaf was harvested for RNA and protein analysis. For testing maize drought stress tolerance, one *zmrafs* mutant and one NS plant were grown in the same pot (15,000 cm^3) in a greenhouse for 50 days (V15 stage) under 18 h

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light $[900\pm54$ μmol m^{-2} sec^{-1}] at ambient temperature (about 20 ℃ at night and 25 to 30 ℃ during the day). Starting on day 51, water was withheld from the experimental group (10 randomly chosen pots) while the control group (the other 10 pots) was grown with continued watering to the same content (65% weight ratio of water/soil). After 10 days of dehydration, 3 to 8 replications (pots) were randomly chosen and the blade in the same position of NS and *zmrafs* plants were photographed. The middle part of blades (about 8 cm long) were collected and tested for the electrolyte leakage following a published protocol (27). Leaf samples for sugar detection (HPLC) were collected at same time of the day (12:00 noon) every two days during dehydration stress treatment. For testing the drought stress tolerance of Arabidopsis plants, seeds of different genotypes were germinated on growth medium (GM) agar (MS salt, 2.2 g Ł$^{-1}$; MES, 0.4 g Ł$^{-1}$, pH 5.8; sucrose, 10 g Ł$^{-1}$) plates at 22 ℃ for 2 weeks, then the plants were transferred to soil and grown at 22 ℃ for 2 weeks. Water was then withheld for 2 weeks before the plants were re-watered and allowed to recover. Just before re-watering, the electrolyte leakage were determined as previously described (27). After re-watering, the percentage of survival of each line was calculated from 8 plants per pot for each genotype. The experiment was repeated 3 times.

RNA extraction and qPCR.

RNA isolation and qPCR were performed following a published protocol (12). The expression level of tested genes was normalized to the expression level of constitutively expressed genes, specifically *AtACTIN2* in Arabidopsis and *ZmGAPDH* in maize (Supplementary Table S3). The experiment was repeated at least three times with independent biological samples. The real time RT-PCR was performed using primers as listed in Supplementary Table S3.

Purification of ZmMIPS protein and its enzyme activity assay.

Prokaryotic expression vector of ZmMIPS1 (*pET-ZmMIPS1*) and ZmMIPS2 (*pET-ZmMIPS2*) were transformed into *Escherichia coli* (Rosetta gami2, DE3, EMD Millipore) cells. The bacterial cultures were grown in Luria-Bertani medium to OD$_{600 nm}$ = 0.6 under 37°C, then supplemented with 0.4 mM isopropyl β-D-thiogalactoside (IPTG) and growth continued for 12 hours under 18°C. Bacteria was collected by centrifugation (4°C, 8000 ×g, 10 min) and then lysed in Tris-HCl buffer (20 mM, pH 7.5) using ultrasonication, and the homogenates were centrifuged (4°C, 12000 × g, 20 min). The clear supernatants were loaded onto pre-equilibrated (20 mM Tris-HCl, pH 7.5, 100 mM imidazole) Ni-NTA columns. The columns were then washed by 10 folds column volumes (CV) of 20 mM Tris-HCl (pH 7.5, containing 100 mM imidazole). The ZmMIPS-His$_6$ fusion proteins were eluted by 20 mM Tris-HCl (pH 7.5, containing 400 mM imidazole, 10 folds CV).

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Protein concentration was determined by Bradford assay (39).

Activity assay was performed by a published method (40). The 100 μL reaction systems containing 100 mM Tris-acetate (pH 7.5), 28 mM NH_4Cl, 1.6 mM NAD^+, 10 mM β-mercaptoethanol, 10 mM G-6-P and 2 μg ZmMIPS-His$_6$ fusion protein or BSA, were incubated under 37 °C for 1 hour. Reactions were terminated by addition of 40 μL 20% TCA (w/v). After centrifugation (12000 ×g, 10 min), the supernatants were supplied by equivalent volumes (130 μL) of $NaIO_4$ (200 mM) and incubated under 37 °C for 1 hour for liberating inorganic phosphate. Redundant $NaIO_4$ was eliminated by application of 140 μL 1 M Na_2SO_3. The released inorganic phosphates were measured by a colorimetric method (41). A 0.56 mL P_i reagent (prepared by adding 1 volume of 3M H_2SO_4, 1 volume of 2.5% $(NH_4)_6Mo_7O_{24}$ and 2 volume of H_2O followed by 1 volume of 10% ascorbic acid) was added into sample and re-incubated under 37 °C for 1 hour. OD820 nm of samples were detected and used for quantify the released phosphates using the inorganic phosphate standard curve.

Western blot analysis of ZmGOLS2, ZmRAFS and ZmMIPS protein accumulation.

Polyclonal antiserum against ZmGOLS2 and ZmMIPS was generated in immunized rabbits. ZmGOLS2-His$_6$ fusion protein expressed in *E. coli* BL21 (DE3) cells was purified using an electro dialysis method as previously described

(19). The equal volume mixture of Freund's complete adjuvant (Sigma, USA) purified ZmGOLS2-/ZmMIPS2-His6 fusion protein (150 μg) was then used for the first immunization. Two weeks after the first immunization, equal volume mixture of Freund's incomplete adjuvant (Sigma, USA) and purified ZmGOLS2-/ZmMIPS2-His6 fusion protein (150 μg) were used to inject the rabbit every two weeks for a total of 4 times antigen injections. Serum was extracted two weeks following the final boost and used for western blot hybridization. The polyclonal antibody for ZmRAFS was prepared as previously described (19). The polyclonal antibody for ZmGAPDH was purchased from CWBIO (China). For determining whether ZmRAFS protein accumulation was regulated by abiotic stress, the second leaf of the B73 seedlings treated by the different abiotic stresses were used for protein extraction. For determination of ZmGOLS2, ZmRAFS and ZmMIPS protein expression in transgenic Arabidopsis lines, the 2 week-old seedlings were used for protein extraction and subsequent Western blots as previously performed (12).

Localization of ZmRAFS-YFP fusion proteins in leaf protoplasts.

The ZmRAFS-YFP fusion expression vector was transformed into maize (B73) leaf protoplasts using PEG-Ca^{2+} method **(38)**. The transformed protoplasts were visualized with a confocal microscope (Nikon A1R, Japan). ZmRAFS-YFP protein production in the transformed protoplasts

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was also probed using ZmRAFS antibody.

Soluble carbohydrate extraction and HPLC-ELSD analysis of sugar components.

Soluble sugar extraction was performed following a published protocol with minor revision (23). A Waters X-bridge amide column (Waters, USA) was used with methanol: H_2O (90:10) as the mobile phase at speed of 0.5 mL $\cdot$ min^{-1} for separation of soluble sugar components. An evaporative light-scattering detector (ELSD, Waters 2424) monitored the sugar signal.

Enzyme activity assay of ZmRAFS.

The expression vector of *ZmRAFS* or its mutants (*mZmRAFS*) was transformed into *Escherichia coli* (Rosetta-gami™ 2 (DE3), EMD Millipore) cells. The protein expression was induced by IPTG and the activity reaction was performed as previously described **(19)**. The lysate of *ZmRAFS* or *mZmRAFS* expressing strain were used for western blot to confirm the soluble expression of the protein. The products catalyzed by each ZmRAFS or mZmRAFS were detected by HPLC-ELSD. The enzyme activity was calculated by the products divided by the intensity of the RAFS protein as determined by western blot analysis. The relative activity of mZmRAFS was normalized to wild type ZmRAFS. For kinetic analysis of ZmRAFS in raffinose synthesis, the substrate galactinol was saturated (16 mM), while the concentration of sucrose was provided from 2.8 to 28 mM; or the substrate sucrose was saturated (28 mM) while the concentration of galactinol ranged from 1 to 16 mM. For kinetic analysis of ZmRAFS in galactinol hydrolysis, the concentration of galactinol was ranged from 1 to 16 mM while no sucrose was supplied in the reaction system. For testing for raffinose-synthetic and galactinol-hydrolytic activities in leaves of over-expressing *ZmRAFS* Arabidopsis, 0.5 g of fresh leaves was ground into a powder in liquid nitrogen and transferred into 1 mL extraction buffer (50 mM HEPES, 5 mM $MgCl_2$, 1 mM EDTA, 20 mM DTT, 50 mM Na-Ascorbate, 0.1 % Triton-X100, 2% PVP-40 and 1 mM PMSF, pH 7.5 adjusted with KOH). After incubating for 30 min on ice, the supernatant was collected by centrifuging for 20 min at 12, 000 $\times$g at 4 °C. The protein concentration was determined by Bradford assay (39). The transferase and hydrolase reactions (50 μL) consisted of 25 μL crude extract together with 20 mM HEPES-KOH (pH 7.0), 16 mM galactinol with and without 28 mM sucrose, respectively. The reaction mixtures were incubated at 37 °C for 2 h prior to the addition of 500 μL 80% ethanol and boiling for 10 min to terminate the reaction. After recovery of the supernatant from a centrifugation of 10 min at 15, 000 $\times$g at room temperature, a dilution of the ethanol with 2.75 ml H_2O permitted the extract to freeze prior to lyophilizing to dryness under vacuum. The sugar powder was dissolved in 100 μL of water for HPLC-ELSD detection.

Infiltration of Exogenous Sugar.

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To investigate whether the sucrose concentration can determine the completion of the ZmRAFS transferase activity, exogenous sucrose were applied to the leaves from 4 week-old *WT, OEGFP, OEZmRAFS-25, OEZmGOLS2* and three *ZmGOLS2/ZmRAFS* Arabidopsis plants. Leaves of different lines were submerged in 30 mM sucrose solution or H_2O and were then infiltrated for 1 h. All samples in their respective liquids were incubated under light (100 μmol·m^{-2} s^{-1}) at 23 ℃ for 24 h before being washed with deionized water for 3 times and stored at -80 ℃ until HPLC-ELSD analysis of sugar content.

Statistical Analysis.

The significance among means was analyzed by the student's T-test. In either case, if the comparison contained multiple datasets, ANOVA test and Tukey's test (α=0.05) were used to determine the significance. The significance analysis was processed by using software IBM SPSS Statistics 19.

Data availability

All data are included in the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

T.L., Y.Z., Y.L., X.L., G.H., L.G., Q.H., L.D., B.D., and J.W. performed research, T.L, L.D., Y.R. and B.D. analyzed the data. T.Z., B.D., Y.R. and G.W. conceived the experiments and wrote the article.

ACCESSION NUMBERS

Sequence data can be found in Maize GDB or GenBank under the following accession numbers: *ZmRAFS:* GRMZM2G150906 or BT063253; *ZmGOLS2*: GRMZM5G872256 or AF497509; *ZmGAPDH*: GRMZM2G046804 or XM_008679567; *AtRS5*: NM_123403.

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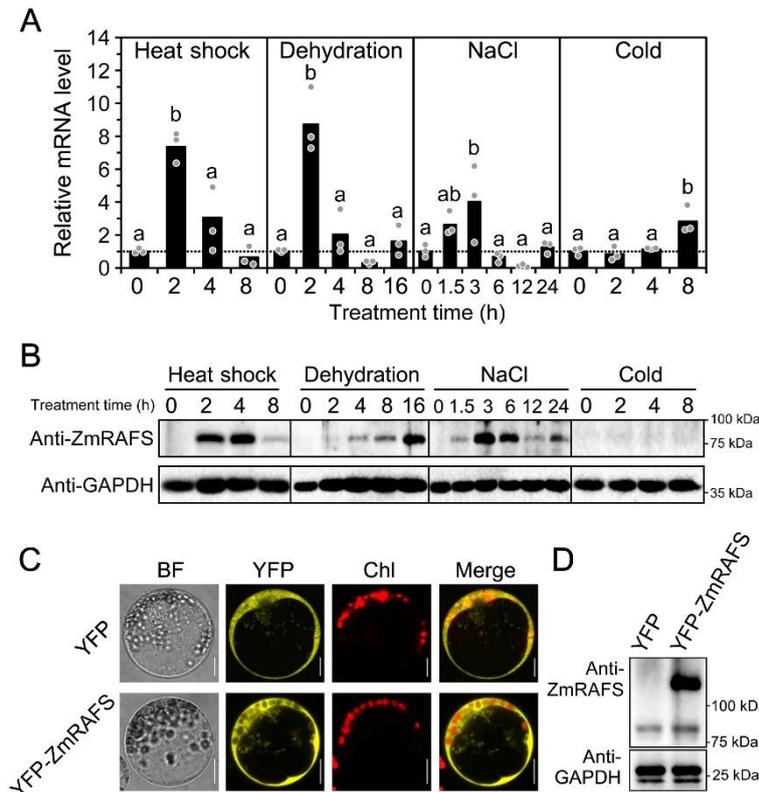
FIGURE 1: *ZmRAFS* is responsive to abiotic stress and its encoded protein is localized to the cytosol.

(A-B) *ZmRAFS* expression was induced by different abiotic-stresses in maize B73 seedling leaves (V3 stage). The maize seedlings were treated with heat shock (42 ℃), dehydration (dry on blot paper), NaCl (200 mM) and cold stress (4 ℃), respectively, for certain period of time as indicated in the graph and the leaf samples were collected for detection of the mRNA or protein expression of *ZmRAFS*. (**A**) The *ZmRAFS* mRNA accumulation in the second leaf was determined by real time RT-PCR. The expression of *ZmRAFS* mRNA was normalized to the expression of maize *GAPDH* and is presented relative to the untreated, control samples (0 h). The X-axis represents the time course of treatment. The Y-axis indicates relative *ZmRAFS* expression. The mixture of the 2nd leaf of three different seedlings were applied for RNA extraction and qRT-PCR as one biological replicate, there are three biological replicates. Each circle represents one biological replicate, and the horizontal bars denote the means. Different lowercase letters above the bars denote significant differences among different times of treatments (ANOVA test, $p<0.05$). (**B**) ZmRAFS protein accumulation was determined by western blot analysis. Top panel, western blot analysis of ZmRAFS protein; Bottom panel, western blot analysis of GAPDH protein demonstrating equal protein loading. **(C)** ZmRAFS was localized to the cytoplasm of maize protoplast cells. A YFP expression vector or a ZmRAFS-YFP fusion protein expression vector was transiently transformed into maize protoplasts. The YFP fluorescence and chloroplast auto-fluorescence signals were detected by confocal microscopy and then merged (Bar=10 μm). BF: Bright field; YFP: YFP signal; Chl: Chloroplast auto-fluorescence signal; Merge: Merged signal of YFP and Chl. (**D**) Western blot analysis of ZmRAFS-YFP fusion protein in transiently transformed maize protoplast cells as in C using ZmRAFS antibody. Both protoplast samples have endogenous RAFS (Native) but only the transiently transformed cells also have signal from the chimeric ZmRAFS-YFP fusion. From the same samples, Western blot analysis of ZmGAPDH protein in the lower panels demonstrating equal protein loading.
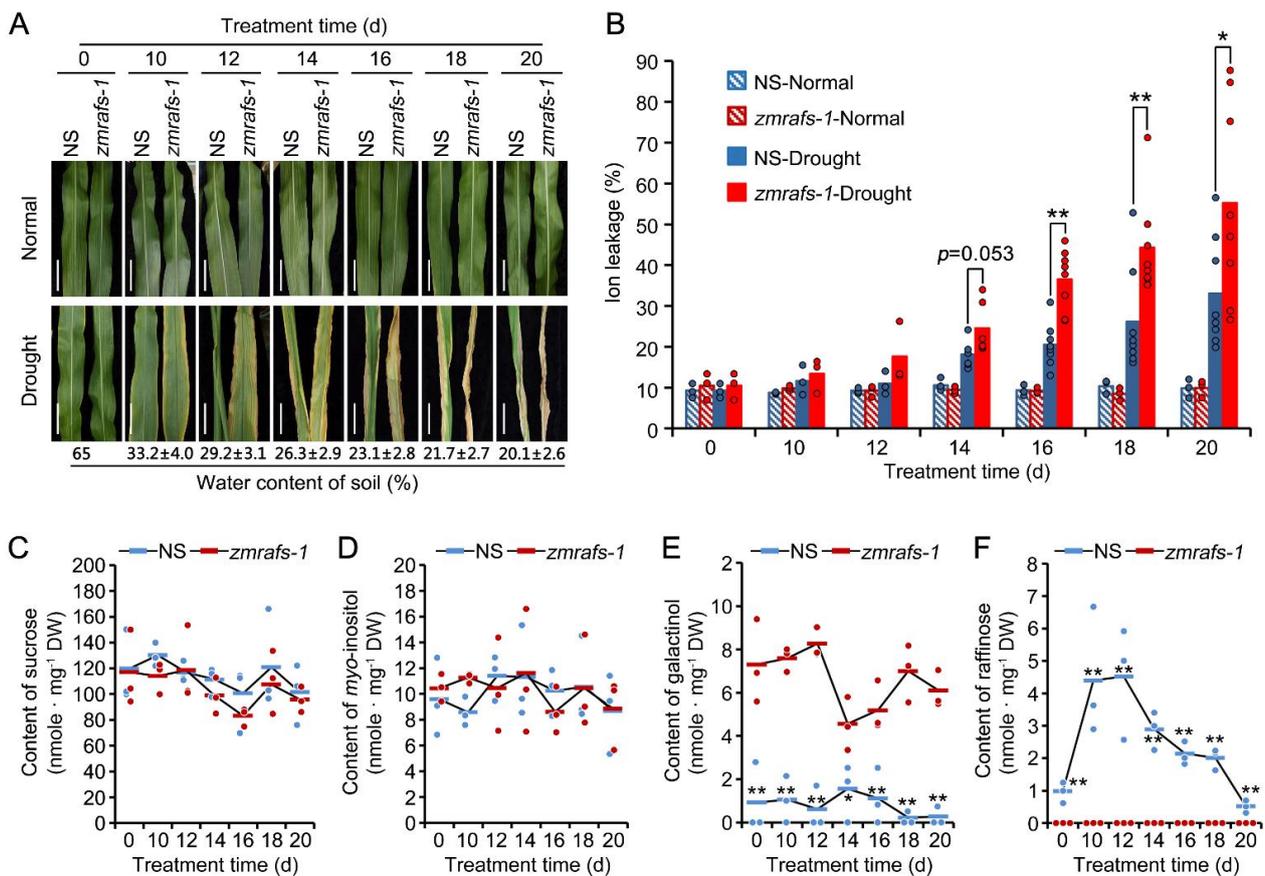
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FIGURE 2: *Mutator*-Interrupted *zmrafs-1* mutant plants were more sensitive to drought stress than null segregant plants.

(A) Representative leaves of null segregant (NS) or *zmrafs-1* plants, which were grown under normal or drought stress conditions, were photographed at the start of and during the treatment. Bar = 10 cm. One NS and one mutant plant were grown in the same pot under normal conditions in a greenhouse for 50 days (V15 stage). Plants were then grown under 65% water content of the soil's capacity or under drought stress conditions (water withheld) for 20 days. **(B)** A comparison of electrolyte leakage of leaves between NS (W22 background; blue) and *zmrafs-1* (red) plants which were grown under normal (hatched bars) or drought stress (filled bars) conditions as in A. The middle part of 7th leaves from different plants were collected for testing of the electrolyte leakage. Each circle represents one biological replicate (one single plant), there are three to eight biological replicates for each treatment. The horizontal bars denote the means. *p<0.05; **p<0.01 as compared by Student's t-test. (C-F) Soluble sugar profiles of leaves from NS and *zmrafs-1* plants at the start and during drought stress. Sucrose **(C),** *myo*-inositol **(D),** galactinol **(E),** and raffinose **(F)** amounts were measured for the null segregant (NS; blue circles) and the maize *raffinose synthase mutant-1* (*zmrafs-1*; red diamonds). The middle part (one gram) of 7th leaves from different plants were collected for sugar extraction and HPLC-ELSD detection. Each circle represents one biological replicate (one single plant), there are three biological replicates. The horizontal bars denote the means. *p<0.05, **p<0.01 denotes a significant difference between NS and *zmrafs-1* (Student's t-test). There was no significant difference of sucrose and *myo-inositol* between NS and *zmrafs-1* mutant.
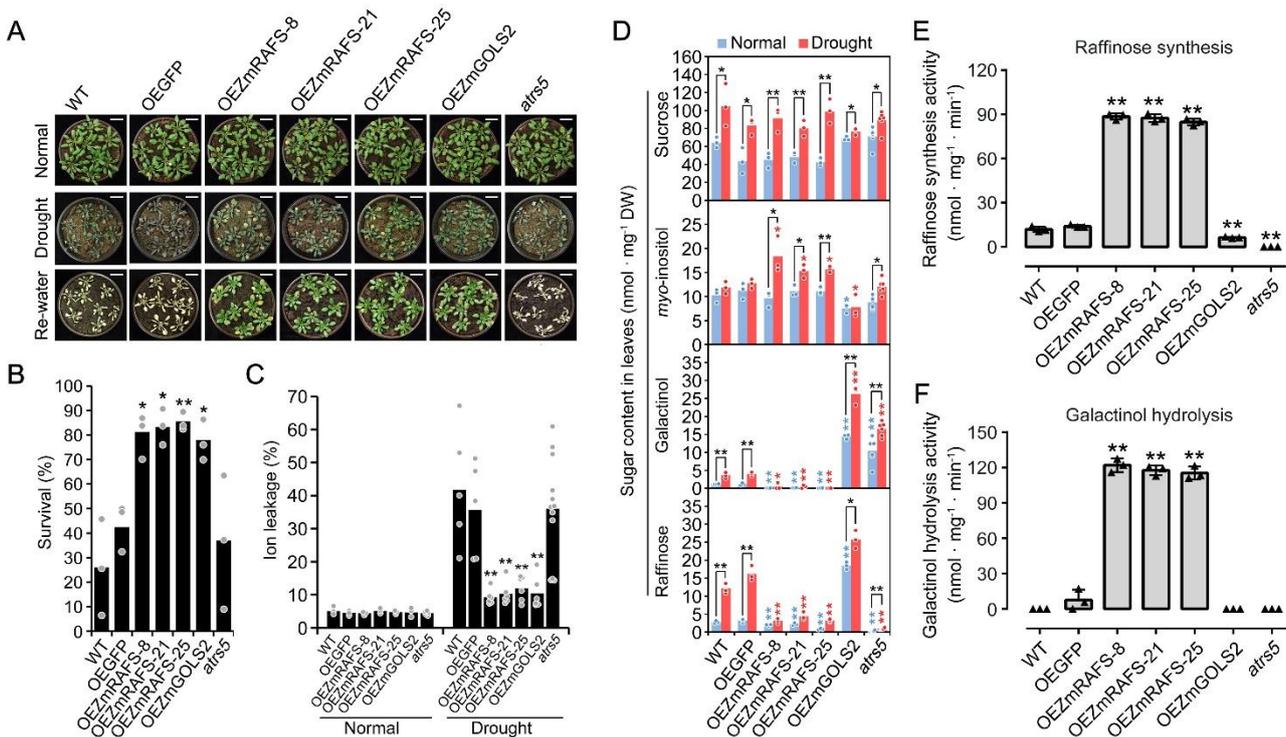
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FIGURE 3: In Arabidopsis, overexpression of *ZmGOLS2* and *ZmRAFS* increased and decreased, respectively, the galactinol and raffinose contents; and yet, both enhanced the drought stress tolerance of those Arabidopsis plants.

(**A**) Photographs of Arabidopsis plants without drought stress (Normal), at the end of the drought stress (Drought; 2 weeks without watering), and after re-watering (Re-water). Bar = 2 cm. Abbreviations are as follows: WT: Wild type Col-0 Arabidopsis plants; OEGFP: *GFP*-overexpressing Arabidopsis plants; OEZmRAFS-8, 21, or 25: *ZmRAFS*-overexpressing Arabidopsis plants; ZmGOLS2: *ZmGOLS2*-overexpressing Arabidopsis plants and; *atrs5*: *Arabidopsis raffinose synthase5* knockout mutant plants. (**B**) The percentage survival of both the *ZmRAFS*- and the *ZmGOLS2*-overexpressing Arabidopsis plants was greater than that of WT plants when water was withheld from the plants. Survival rate was calculated from three to six pots of plants for each Arabidopsis line in one independent experiment. There are three independent experiments. Each circle represents the data of one independent experiment. The horizontal bars denote the means. *p<0.05; **p<0.01, as compared to WT control (ANOVA test). (**C**) The leaf ion leakage of all three *ZmRAFS*- and the *ZmGOLS2*-overexpressing Arabidopsis plants was less than that of WT plants at the end of the drought stress treatment. Leaves form different plants that were grown under normal and drought conditions were collected for ion leakage test. Each circle represents one biological replicate (one single plant), there are three to twelve biological replicates for each tested line. The horizontal bars denote the means. **p<0.01 compared to WT control (ANOVA test). (**D**) Comparison of soluble sugar related to raffinose biosynthesis of the different Arabidopsis plants as in (A) which were grown under well-watered or drought conditions. Total soluble sugar was extracted from 0.5 g of leaves that were collected from different Arabidopsis plants for each line (one biological replicate) for HPLC-ELSD detection. Each circle represents one biological replicate and there are three to six biological replicates for each tested line. The horizontal bars denote the means. The colored asterisks are indicative of significant differences from WT under normal (blue) or drought (red) conditions (*p<0.05 and ** p<0.01, ANOVA test). A black asterisk (*p<0.05 and ** p<0.01) indicates significant differences between the normal and drought condition of each line (Student's t-test). (**E**) Characterization of the raffinose synthetic activity of the leaf extracts from different Arabidopsis plants as described in (A). Raffinose and *myo*-inositol were determined using cell-free leaf extracts of

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three *ZmRAFS*-overexpressing lines incubated with galactinol and sucrose, as detected by HPLC-ELSD. The enzymatic activity was determined by raffinose amounts (nmol) produced per mg protein per minute. The crude enzyme extracts that were extracted from the leaf mixture of three different plants were determined for its raffinose synthetic activity (one biological replicate). There are three biological replicates. Each triangle represents one biological replicate, and the horizontal bars denote the means. **$p<0.01$ compared to WT (ANOVA test). (**F**) Characterization of the galactinol hydrolytic activity of the leaf extracts from the Arabidopsis plants as described in (A). Galactose and *myo*-inositol were determined using cell-free leaf extracts of three *ZmRAFS*- overexpressing lines incubated with galactinol as detected by HPLC-ELSD. The enzymatic activity was determined by *myo*-inositol amounts (nmol) produced per mg protein per minute. The crude enzyme extracts that were extracted from the leaf mixture of three different plants were determined for its galactinol hydrolytic activity (one biological replicate). There are three biological replicates. Each triangle represents one biological replicate, and the horizontal bars denote the means. **$p<0.01$ compared to WT (ANOVA test).
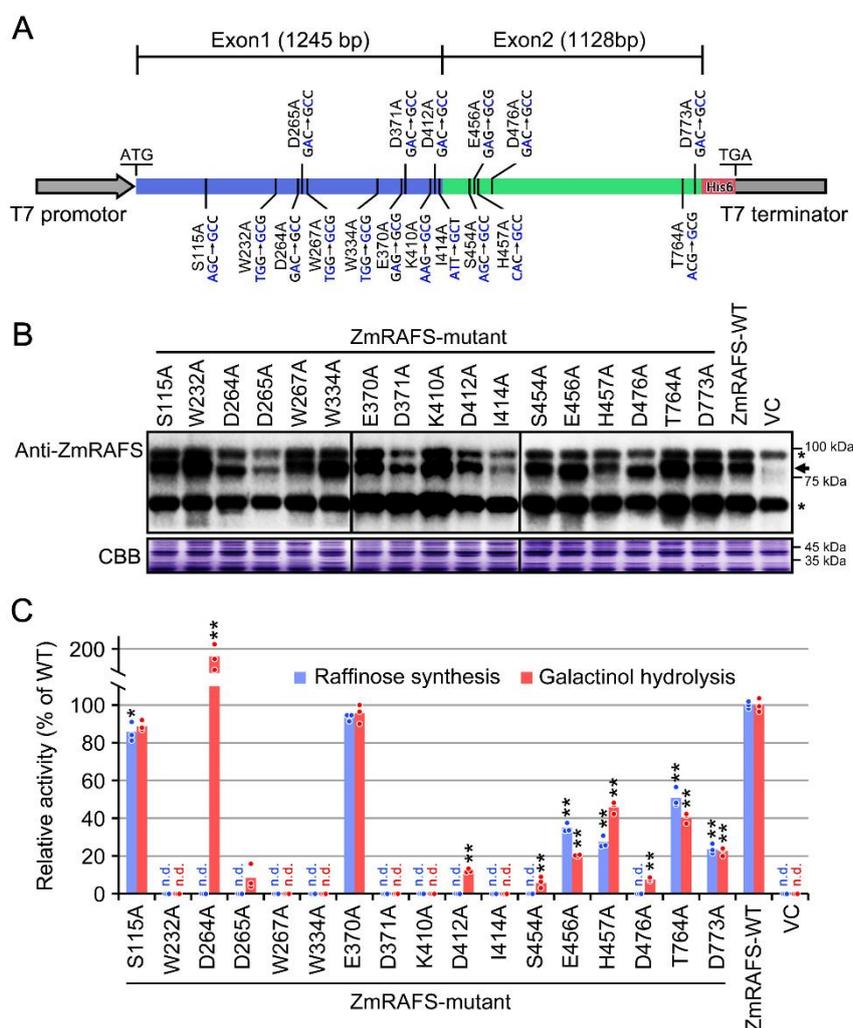
FIGURE 4: Characterization of enzyme activity of ZmRAFS and its amino acid-point mutants. (**A**) Schematic representation of the bacterial expression vector of *ZmRAFS* and its mutants. The residue number of each of the alanine point mutations is indicated. Letters in red and blue indicate the mutation of the AA and the corresponding nucleotide(s), respectively. (**B**) Using the ZmRAFS antibody, Western blot characterization of the presence of each

mutated ZmRAFS protein (arrow denotes the expected signal; * denotes a cross-reacting protein) in the soluble *E. coli* extract. A sister gel was stained with Coomassie Brilliant Blue-250 (CBB). Abbreviations are as follows: VC, pET-21d empty vector control; ZmRAFS-WT, unmutated ZmRAFS-His6 fusion protein; and all designated residue, alanine-mutants. (**C**) In vitro assay of the raffinose synthetic- and galactinol hydrolytic-activity of ZmRAFS and its alanine-mutated forms calculated as a ratio of the amount product to enzyme, then converted to a percentage of the wild type ZmRAFS activity. Crude enzyme extracts were extracted from bacteria cultures of ZmRAFS-WT, VC or ZmRAFS mutant clones for the enzymatic activity assay. Each circle represents one biological replicate (cultures from one flask), there are three biological replicates. The horizontal bars denote the means. Using the ANOVA test, significant differences as compared to the wild type ZmRAFS are denoted with * and ** representing $p < 0.05$ and < 0.01, respectively). n.d. indicates not detected under the conditions used. VC designates the pET-21d empty vector control.
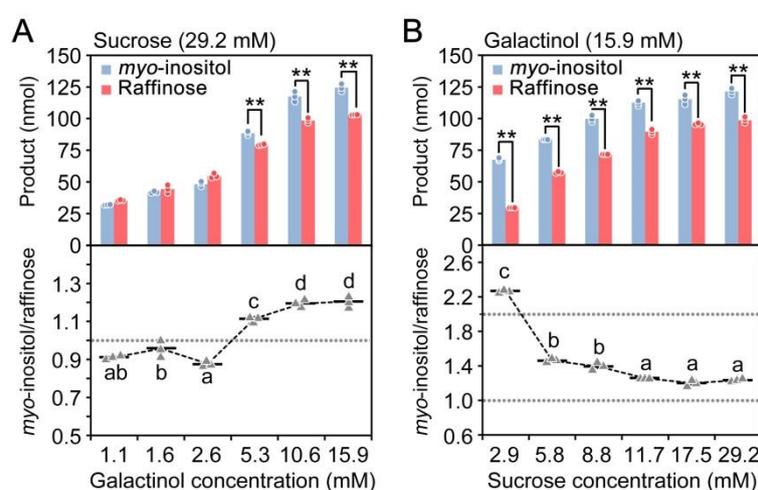

FIGURE 5: The relative amounts of sucrose and galactinol determines whether RAFS completed the transferase reaction.

(**A**) Amount of sugar products of the transferase reaction as detected by HPLC-ELSD (top panel) and the molar ratio of those products (*myo*-inositol/raffinose; bottom panel). Sucrose was kept at saturating levels (29.2 mM) as galactinol concentration was varied as noted on the x-axis. Crude enzyme extracts were extracted from BL21 (expressing *ZmRAFS*) bacteria cultures for the enzymatic activity assay. Each circle (in the top panel) or triangle (in the bottom panel) represents one biological replicate (cultures from one flask), there are three biological replicates. Sugar amount data represent the mean of three replicates and significant differences between the two products were indicated with ** for a $p < 0.01$ using a Student's t-test. Different lowercase letters denote significant differences among the ratios within each data set (Tukey's test). (**B**) All are the same as in (**A**) except galactinol concentration was kept constant and saturating (15.9 mM) as sucrose concentration was varied.
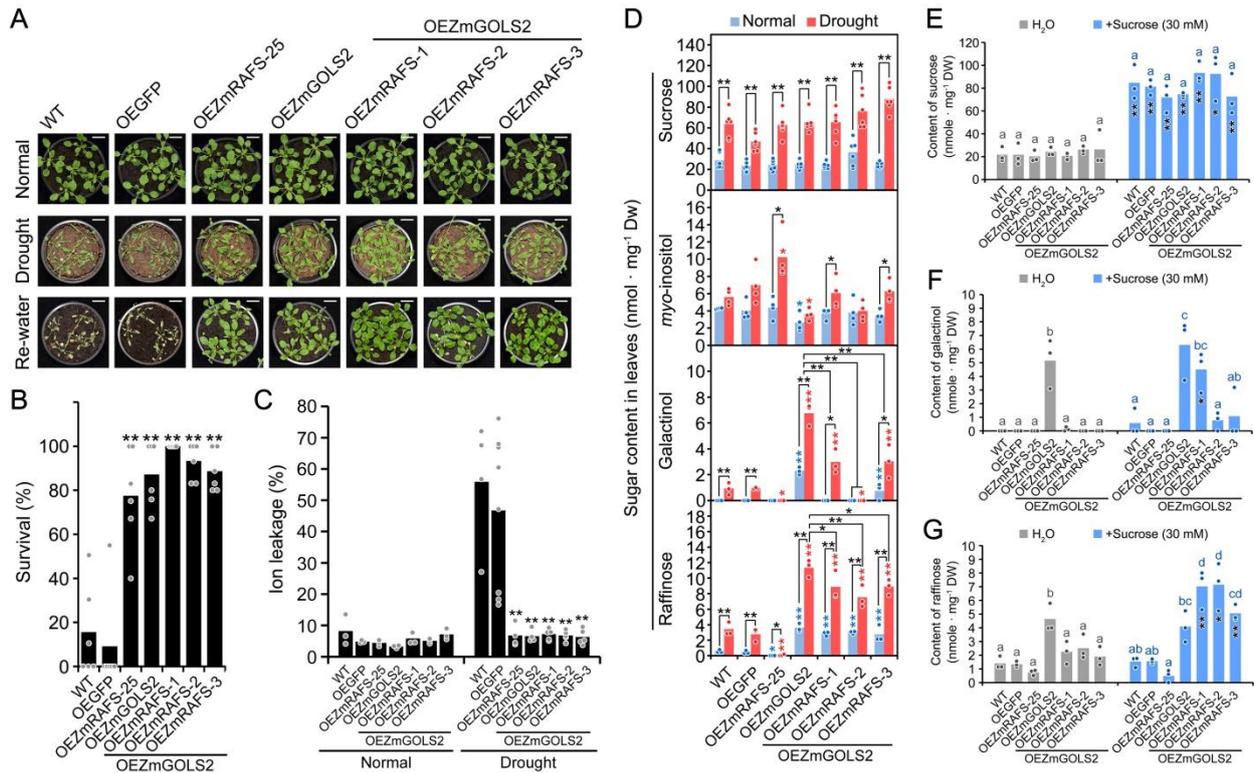
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FIGURE 6: Supplementation of sucrose to the detached leaves of *ZmGOLS2/ZmRAFS*-overexpressing Arabidopsis plants permitted ZmRAFS to complete the transferase activity and form raffinose.

(A) Photographs of Arabidopsis plants before drought stress, after drought stress and after re-watering. Plant genotypes, as in Fig. 3, included WT, OEGFP, OEZmRAFS-25 and OEZmGOLS2 and the additional OEZmGOLS2/OEZmRAFS-double overexpressing Arabidopsis plants. **(B)** The percentage survival of different Arabidopsis lines after re-watering following drought stress. Survival rate was determined by counting the plants from five pots of each Arabidopsis line for one independent experiment. There are six independent experiments. Each circle represents data from one independent experiment. The horizontal bars denote the means. Significance as compared to the WT control was determined using ANOVA test and is denoted using ** for a p<0.01). **(C)** Comparison of the leaf ion leakage of different Arabidopsis lines which were under normal growth condition or after drought stress (before re-watering) treatment. Leaves form different individual plant under normal and drought condition were collected for ion leakage test. Each circle represents one biological replicate (one plant) and there are three to eight biological replicates for each Arabidopsis line. The horizontal bars denote the means. Significance is indicated by ** for a p<0.01 for the comparisons made against WT plants (ANOVA test). **(D)** Comparison of sucrose, *myo*-inositol, galactinol and raffinose content of leaves among different Arabidopsis lines which were grown under normal or drought conditions. Total soluble sugar was extracted from 0.5 g leaves from different plant and the the sugar content was detected by HPLC-ELSD. Each circle represents one biological replicate (independent sugar sample from different individual plant), there are three to six biological replicates for each Arabidopsis line. The horizontal bars denote the means. ANOVA test was performed to detect significant difference between different lines, *p<0.05, **p<0.01, showed by colored asterisks. Student's t-test was using for analyze significant differences between the normal and drought condition of each line, *p<0.05, **p<0.01, showed by black asterisk. **(E-G)** Comparison of the sucrose (E), galactinol (F) and raffinose (G) content in detached leaves, infiltrated with either water or 30 mM sucrose for 1 h, among the various Arabidopsis genotypes. The leaves of different Arabidopsis lines

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(4 weeks-old) were then subsequently infiltrated in the same solution for 2 h and then incubated for another 22 h under light, the sugar content of leaves was determined by HPLC-ELSD. Each circle represents one biological replicate (leaves from 5 plants of each Arabidopsis line for each treatment), there are there biological replicates. The horizontal bars denote the means; $*p<0.05$; $**p<0.01$ (Student's t-test). An asterisk indicates a significant difference between sugar- and water-treated groups of each plant genotype. Different lowercase letters denotes significance among different plant genotypes within the treatment group (Tukey's test).
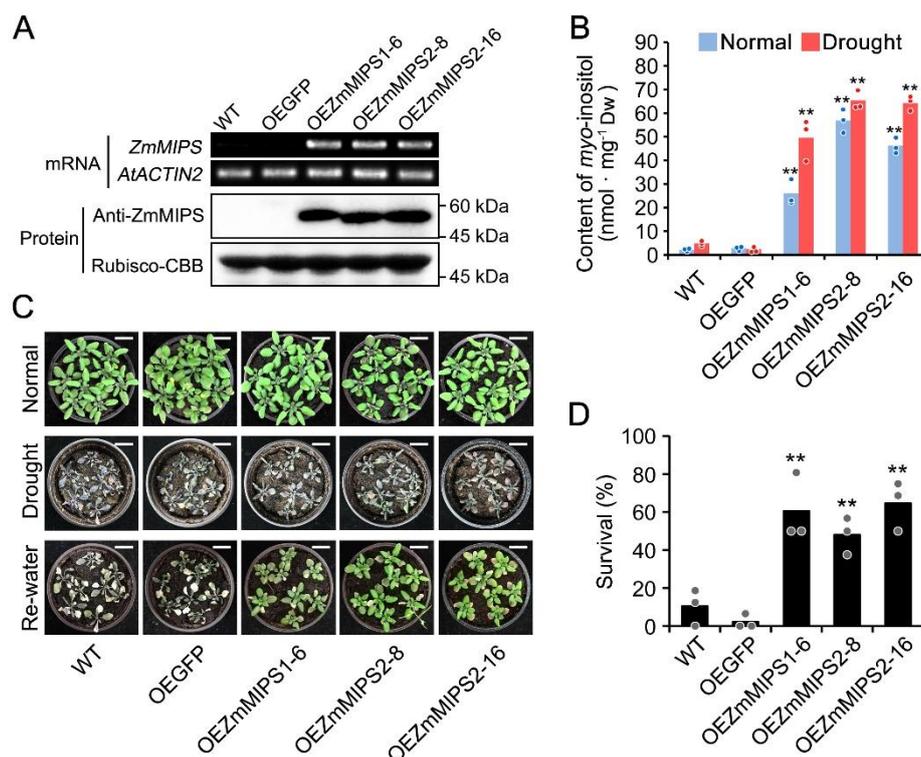

FIGURE 7: Over-expression of *ZmMIPS1* or *ZmMIPS2* increased *myo*-inositol accumulation in Arabidopsis leaves and enhanced plant drought tolerance. **(A)** Characterization of *ZmMIPS1* and *ZmMIPS2* overexpressing Arabidopsis plants. Top panel, RT-PCR analysis of mRNA accumulation of *ZmMIPS*, *AtACTIN2* expression was used as a control; bottom panel: Western Blot analysis of ZmMIPS protein accumulation. RUBISCO CBB staining was used as a loading control. WT: Wild type Col-0 Arabidopsis plants; OEGFP: *GFP*-overexpressing Arabidopsis plants; OEZmMIPS1-6, *ZmMIPS1* over-expressing line; OEZmMIPS2-8/-16, *ZmMIPS2* over-expressing line. **(B)** Comparison of *myo*-inositol content in leaves among different Arabidopsis lines which were grown under normal and drought conditions. Total soluble sugar was extracted from 0.5 g leaves from different plants for *myo*-inositol measurement by HPLC-ELSD. Each circle represents one biological replicate (sugars extracted from different plant of the same Arabidopsis line), there are there biological replicates. The horizontal bars denote the means. $**p<0.01$ (compared to WT, ANOVA test). **(C)** Photographs of Arabidopsis plants before drought stress, after drought stress and after re-watering. Bar=2 cm. **(D)** The percentage survival of different Arabidopsis lines after re-watering following the drought stress. Survival rate was determined by counting the plants from five pots of each Arabidopsis line for one independent experiment. There are three independent experiments. Each circle represents data from one independent experiment. The horizontal bars denote the means. $*p<0.05$; $**p<0.01$ (ANOVA test).
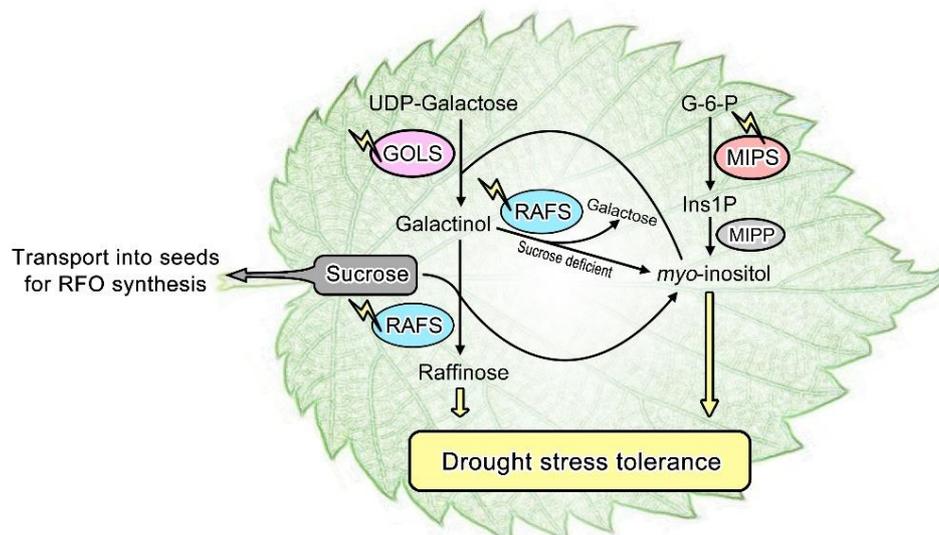
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FIGURE 8: Simplified raffinose synthetic metabolism and its effects on drought stress tolerance in maize and Arabidopsis. RAFFINOSE SYNTHASE enhances plant drought tolerance by either synthesizing raffinose, or by hydrolyzing galactinol which will generate more *myo*-inositol, depending on the relative amount of its substrates, galactinol and sucrose. GOLS, GALACTINOL SYNTHASE; RAFS, RAFFINOSE SYNTHASE; MIPS, MYO-INOSITOL PHOSPHATE SYNTHASE. RFO, Raffinose family oligosaccharides.

Raffinose synthase enhances drought tolerance through raffinose synthesis or galactinol hydrolysis in maize and Arabidopsis plants

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