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Characterisation of genes involved in galactolipids and sulfolipids metabolism in maize and *Arabidopsis* and their differential responses to phosphate deficiency

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Abstract. Galactolipids (MGDG and DGDG) and sulfolipids (SQDG) are key components of plastidic membranes, and play important roles in plant development and photosynthesis. In this study, the whole families of *MGD*, *DGD* and *SQD* were identified in maize genome, and were designated as *ZmMGD1-3*, *ZmDGD1-5* and *ZmSQD1-5* respectively. Based on the phylogenetic analyses, maize and *Arabidopsis* MGDs, DGDs and SQDs were clearly divided into two major categories (Type A and Type B) along with their orthologous genes from other plant species. Under low-phosphorus condition, the expression of Type B *MGD*, *DGD* and *SQD* genes of maize and *Arabidopsis* were significantly elevated in both leaf and root tissues. The lipid analysis was also conducted, and an overall increase in non-phosphorus lipids (MGDG, DGDG and SQDG), and a decrease in phosphorus lipids (PC, PE and PA) were observed in maize leaves and roots under phosphate deficiency. Several maize *MGD* and *SQD* genes were found involved in various abiotic stress responses. These findings will help for better understanding the specific functions of MGDs, DGDs and SQDs in 18:3 plants and for the generation of improved crops adapted to phosphate starvation and other abiotic stresses.

Additional keywords: abiotic stress, DGD, digalactosyldiacylglycerol synthase, low-phosphate, maize, MGD, monogalactosyldiacylglycerol synthase, SQD, sulfoquinovosyldiacylglycerol synthase, *Zea mays*.

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Introduction

The photosynthetic membranes surrounding the chloroplast and thylakoid of higher plants are lipid bilayers with embedded proteins, which provide a platform for photosynthesis (Kobayashi *et al*. 2007; Rocha *et al*. 2018). The four main types of glycerolipids that make up the photosynthetic membrane are monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), and phosphatidylglycerol (PG), and the outer envelope of chloroplast membrane includes a higher scale of typical eukaryotic lipids for instance the phospholipid phosphatidylcholine (PC) (Denev and Minkov 2001; Narayanan *et al*. 2018). Studies have shown that glycerolipid synthesis in chloroplast inner envelope is of critical importance for proper thylakoid biogenesis and photosynthesis (Kelly *et al*. 2016; Li *et al*. 2018).

The galactolipids MGDG and DGDG are synthesised in the chloroplast membrane with synthesis catalysed by two galactosyltransferases – MGDG synthase (MGD) and DGDG synthase (DGD) respectively (Denev and Minkov 2001; Rocha *et al*. 2018). MGD catalyses the galactose conversion from UDP-galactose to the diacylglycerol (DAG) backbone to form MGDG, and then the second galactose is transferred from UDP-galactose to MGDG by DGD for the final formation of DGDG (Li *et al*. 2016*a*). In *Arabidopsis*, MGDG synthase is divided into two subtypes: Type A (AtMGD1) and Type B (AtMGD2 and AtMGD3) (Awai *et al*. 2001). *AtMGD1* maintains high expression during the growth and development of *Arabidopsis*, but no significant expression of *AtMGD2* has been detected (Kobayashi *et al*. 2006). Studies of *AtMGD1* mutant(*mgd1-2*) showed that the photosynthetic membrane was disrupted, resulting in complete loss of photosynthetic capacity and halting photosynthetic autotrophic growth

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(Dörmann *et al*. 1995; Fujii *et al*. 2018). Furthermore, deletion of *AtDGD1* showed abnormal chloroplast phenotypes, which suggested that MGD and DGD has important effects on the function of photosynthetic membranes (Dörmann *et al*. 1995; Fujii *et al*. 2018).

A phosphate-saving mechanism involving the enhanced accumulation of DGDGs has been discovered in plants under phosphate deficient conditions. This mechanism could produce extra-plastidial membranes where they substitute phospholipids (Kobayashi *et al*. 2006; Rocha *et al*. 2018). The proportion of non-phospholipids to phospholipids in *Arabidopsis* increases sharply under phosphate stress (Härtel *et al*. 2000). Under normal conditions, the enzymes MGD1 and DGD1 produce large amount of galactolipids for the expansion of the thylakoid membrane; however, under phosphate stress, the activities of AtMGD2 and AtMGD3 are strongly induced (Härtel *et al*. 2000; Kobayashi *et al*. 2006). When AtMGD2 and AtMGD3 were activated, two DGDG synthase genes, *DGD1* and *DGD2*, were also triggered, resulting in an increase of DGDG content (Nussaume *et al*. 2011). The same mechanism has also been reported in marine heterotrophic bacteria (Sebastián *et al*. 2015).

The sulfur-containing anionic glycerolipid SQDG is situated only in thylakoid membranes, and is generated by sulfoquinovosyldiacylglycerol synthase (SQD) catalysing the conversion of the sulfoquinovose moiety to diacylglycerol (DAG) (Yu *et al*. 2002; Zhan *et al*. 2017). In *Arabidopsis*, SQD1 is situated to the chloroplast matrix and is soluble, whereas SQD2 is localised to the inner envelope of the chloroplast (Essigmann *et al*. 1998; Yu *et al*. 2002). *Arabidopsis* plants that have mutations in *SQD1* or *SQD2* genes exhibit delayed growth under phosphate stress condition, although significant growth defects and a lack of the novel anionic glycolipid glucuronide diacylglycerol (GlcADG) was detected only in the *sqd2* mutant (Okazaki *et al*. 2013; Zhan *et al*. 2017). In bacteria, sulfolipid-deficient null mutants stop growing earlier than wild types under phosphate stress (Benning *et al*. 1993; Güler *et al*. 1996).

In plant cells, the synthesis of glycerolipids could be processed via plastid/chloroplast compartmentalised prokaryotic pathway, and endoplasmic reticulum localised eukaryotic pathway respectively (Ohlrogge *et al*. 1991). Some plants, such as *Arabidopsis*, whose major chloroplast lipids (MGDG and DGDG) are generated jointly by both pathways, are typically characterised by the high levels of 16:3 fatty acids, are termed 16:3 plants (Heinz and Rougham 1983; Dörmann *et al*. 1995), whereas the others, such as maize, whose MGDG and DGDG synthesis relies almost entirely on the ER pathway, have products with high levels of 18:3 fatty acids, so they are called 18:3 plants (Wada and Murata 2010; Gu *et al*. 2017). Previous studies on the metabolism and function of galactolipids have focussed mainly on the 16:3 plant *Arabidopsis*, but only few studies have been conducted on 18:3 plant species (Chen and Thelen 2013; Gu *et al*. 2017).

Phosphate deficiency of soil and subsequent abiotic stresses are major factors that limit crop production in many regions of the world. In the present study, the MGD, DGD and SQD families were characterised from maize genome, and bioinformatics analyses were conducted to reveal their evolutionary and structural features. The expression profiles of these genes under low-phosphorus and various abiotic stress conditions were determined and a comparative analysis between maize and *Arabidopsis* were conducted. This study provides valuable information on deciphering the roles of MGDs, DGDs and SQDs in typical 18:3 plants and important field crop maize.

Materials and methods

Identification and classification of MGD, DGD and SQD genes in maize and Arabidopsis

The protein sequences of *Arabidopsis* MGD, DGD and SQD family members were obtained from TAIR and NCBI database, and were used as queries to search the MaizeGDB and NCBI database by sequence alignment using the BLASTp program. The screening criteria were $E \leq 1e^{-10}$ and protein length $\geq$200 amino acids (aa). The candidate genes encoding maize MGDs, DGDs and SQDs were retrieved from maize genome, and were confirmed at the online Pfam and SMART websites using the specific gene name and typical functional domains. A total of 13 non-redundant genes were reserved for further analysis.

Phylogenetic analysis, chromosomal location and syntenic analysis of the MGD, DGD and SQD families

The full-length amino acid sequences of MGD, DGD and SQD families from *Arabidopsis. Thaliana* (L.) Heynh), maize (*Zea mays* L.), sorghum (*Sorghum bicolor* (L.) Moench), soybean (*Glycine max* (L.) Merr., rice (*Oryza sativa*) L.), *Brachypodium sylvaticum* (Huds.) Beauv.)) and *Medicago truncatula* Gaertn. were obtained from online database using NCBI BLASTP tools. The protein sequences from different plant species were aligned by MEGA5.0 software (https://www.megasoftware.net/, accessed 28 January 2020). The phylogenetic trees for each family were constructed via the un-rooted neighbour joining method with the bootstrap values set at 1000 replicates using MEGA5.0. The chromosomal location of those genes was depicted using the MapInspect software. The syntenic blocks among *Arabidopsis*, maize, sorghum, soybean and rice homologous genes were generated according to the PGD Database, and only those had a linear relationship were shown (Xu *et al*. 2018).

Analyses of gene structure, functional domains and cis-elements in the promoters of the MGD, DGD and SQD families

The exon-intron structures of *MGD*, *DGD* and *SQD* genes in maize and *Arabidopsis* were acquired from the Gene Structure Display Server. The information on conserved functional domains was derived from Phytozome and SMART. The 1.5-kb upstream regions of *MGD*, *DGD* and *SQD* genes were derived from the transcription start site based on the maize and *Arabidopsis* genome. They were then searched against the PlantCARE database to determine the putative *cis*-elements.

Expression profiles of the maize MGD, DGD and SQD genes in diverse tissues and under different stress treatments

The expression data of *MGD*, *DGD* and *SQD* in maize sampled at different developing stages or various tissues (60 different tissues, including 11 major maize organ systems) were acquired

from the online Maize eFP database. The expression data of *MGD*, *DGD* and *SQD* genes in 40 different *Arabidopsis* tissues were collected from TAIR database. The heat-map representing the gene expression intensities were generated and cluster analysis was completed by Tree View ver. 1.6 software (see Table S1, available as Supplementary Material to this paper). For expression analysis of maize *MGD*, *DGD* and *SQD* genes under abiotic stresses, the publicly available maize RNA-seq transcriptome datasets deposited in the NCBI SRA database were used. The transcriptome data includes cold (SRX2672484) (Gu *et al.* 2017), heat (SRR1238715, SRR1819196 and SRR1819198), salt (SRR1238719) and UV (SRR1238720) (Table S2) (Makarevitch *et al*. 2015). For cold stress, seeds of maize (He 344) were grown at 22°C and 16 h/8 h (light/dark) daily photoperiodic cycle. The 2-week-old seedlings were removed to another growth chamber set at 5°C for cold treatment (other conditions remained the same), and the seedlings grown at 22°C were used as controls. Samples of maize leaf were collected at 3-day time point under cold treatment (Gu *et al.* 2017). Heat, salt and UV stresses were applied to maize variety B73: maize seeds were grown at 24°C in 1 : 1 mix of autoclaved field soil and MetroMix, and the light conditions under all conditions are natural light conditions For heat stress, seedlings were placed at 50°C for 4 h. For high salt stress, plants were watered with 300 mmol NaCl 20 h before collecting. UV stress was applied in the growth room using UV-B lamps for 2 h (Makarevitch *et al*. 2015).

Quantitative real-time RT-PCR analyses of maize and Arabidopsis *MGD, DGD and SQD genes in response to phosphate deficiency*

The maize seedlings (variety He 344) were cultured in the nutrient solution supplied with sufficient phosphate (+P, 1000 µmol KH_2PO_4) 3 days after germination. The basic nutrient solution contains: 2 mmol $Ca(NO_3)_2$•$4H_2O$, 1.25 mmol NH_4NO_3, 0.1 mmol KCl, 0.65 mmol K_2SO_4, 0.65 mmol $MgSO_4$, 10 mmol H_3BO_3, 0.5 mmol $(NH_4)_6Mo_7O_{24}$, 1 mmol $MnSO_4$, 0.1 mmol $CuSO_4$•$5H_2O$, 1 mmol $ZnSO_4$•$7H_2O$ and 0.1 mmol Fe-EDTA. After 2 weeks, some maize seedlings (with 2–3 leaves) were transferred to nutrient solution containing very low concentration of phosphate (–P, 5 µmol KH_2PO_4, and 1 mmol KCl was added to complement the concentration of K^+), and maize seedlings remained in sufficient phosphate nutrient solution(+P, 1000 µmol KH_2PO_4) were used as control. The greenhouse condition was set as: temperature 28°C (day) and 25°C (night), regime at a photon flux density (PFD) of 1000 µmol m^{-2} $s^{-1}$with a 16/8 h light/dark cycle and ~65% RH. Leaf and root samples were collected at 0, 24 and 72 h under both +P and –P treatment. *Arabidopsis* (wild type *Colombia*) seeds were germinated on MS medium and transplanted into nutrient solution after 5 days containing 1000 µmol KH_2PO_4 (+P). After 15 days of growing, some of the plants were transferred into low phosphate culture (–P, 5 µmol KH_2PO_4), and *Arabidopsis* kept growing in +P solution were used as control. All samples had at least three replicates and the collected samples were quickly wrapped in tin foil, and frozen in liquid nitrogen immediately and stored at –80°C.

The maize *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *Actin* genes were used as endogenous controls. Each 10 µL PCR reaction included 3.4 µL ddH_2O, 1.0 µL cDNA template and 0.3 µL each primer, and 5.0 µL buffer Mix. The PCR reaction started with a 95°C/3 min initial step; followed by 35 cycles of 94°C/60 s, 94°C/15 s and 55°C/30 s; and terminated at 72°C for 60 s. Quantitative real-time RT-PCR was implemented in an optical 96-well plate using the SYBR Select Master Mix RT-PCR system. Primers used for quantitative real-time PCR are shown in Table S3.Three independent biological replicates were performed and the results of quantitative real-time RT-PCR were analysed using $2^{-\Delta\Delta CT}$ method.

Maize lipid extraction and analysis

The total lipids were extracted from leaves and roots that collected at 72 h under both +P and –P treatment. The total lipid extraction method could refer to the previous study (Narayanan *et al.* 2016). Remove ~200 mg of sample and quickly place it in a Teflon-lined 50 mL glass tubes (with screw-cap) containing 3.0 mL 75°C is opropanol (with 0.01% BHT) for 15 min. After cooling to 25°C, 1.5 mL chloroform and 0.6 mL water were added and vortexed rapidly, followed by shaking and mixing for 1 h, and then the lipid extract was transferred to a new glass tube. The extraction step was repeated with chloroform/methanol (2 : 1), and the combined lipid extracts were cleaned with 1.0 mL 1 mol KCl. The lipid extracts were then dried with nitrogen and stored at –80°C. Mass spectrometry and lipid analysis were carried out by the Kansas Lipidomics Research Centre (KLRC, Kansas State University, Manhattan, KS, USA).

Statistical analysis

The statistical analysis was conducted by SPSS statistics 22.0, and the Student's *t*-test was used to determine the significance levels. The significant level was $P < 0.05$. Data are presented as means ± s.d. The bars without error lines were control, and different lower case letters means there are significant differences among the bars.

Results

Genome-wide identification of MGD, DGD and SQD gene family members from maize

The protein sequences of the *Arabidopsis MGD*, *DGD* and *SQD* families were used as queries for the BLASTp searching against the maize genome, phytozome, and NCBI databases. A total of three *MGD* genes, five *DGD* genes, and five *SQD* genes were identified in maize genome, and were designated as *ZmMGD1-3*, *ZmDGD1-5* and *ZmSQD1-5*, respectively, after their corresponding *Arabidopsis* orthologs (Table S4). As shown in Table S4, the *Arabidopsis MGD* family members have a coding region between 1398 and 1602 bp, and the encoded proteins have 465 to 533 amino acids with a molecular weight ranging from 52.73 to 58.54 kDa, and the predicted isoelectric point (pI) values are 6.30 to 9.30. In maize, the coding region of *ZmMGDs* is between 1434 to 1590 bp, encoding 477 to 529 amino acids, and the molecular weight is between 53.01 to 56.90 kDa with a predicted isoelectric point ranging from 6.84 to 9.55. Two *DGD* genes in *Arabidopsis* are

quite different in size, *AtDGD1* has a coding span of 2427 bp encoding a protein of 808 amino acids; whereas *AtDGD2* has a coding span of 1422 bp encoding a protein of 473 amino acids. Similarly, the five *DGD* members in maize showed large size difference. *ZmDGD1-3* have a coding region over 2200 bp, and the encoded proteins are close to 800 amino acids; whereas *ZmDGD4-5* have a coding region around 1400 bp, and the encoded proteins are less than 500 amino acids. The coding regions of the two *Arabidopsis SQD* genes are 1434 bp (*AtSQD1*) and 1533 bp (*AtSQD2*), and encode proteins of 477 and 510 amino acids respectively. The maize *SQD* family also has five members, having a coding region among 1245 and 1494 bp, encoding 414 to 497 amino acids.

The chromosomal localisation analysis demonstrated that the 13 maize genes of three families located on seven maize chromosomes except chromosomes 3, 6 and 10 (see Fig. S1, available as Supplementary Material to this paper). Chromosome 1 harboured four genes, including *ZmDGD4*, *5*, *ZmMGD2* and *ZmSQD5*. To understand the replication relationship of these genes in maize and other plants genome, the tandem duplicated analysis among maize, rice, soybean, sorghum and *Arabidopsis* was conducted, and a syntenic block was generated. As shown in Fig. 1*d*, a total of six repetitive gene pairs (10 repeat genes) were found, including *OsDGD1*/*OsDGD4*/*OsDGD5*, *OsMGD1*/*ZmMGD1*, *OsMGD3*/*ZmMGD3*, *OsSQD1*/*ZmSQD3*, *OsSQD3*/*ZmSQD1* and *OsSQD2*/*ZmSQD4*/*ZmSQD5*. They are primarily segmental repeat events, and no tandem replication events occur. Repetitive genes exemplified their common genomic origin and potential functional similarity.

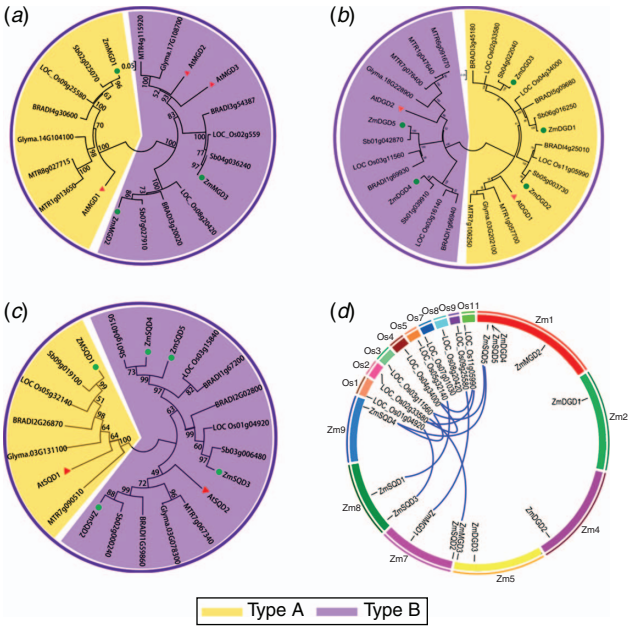

Fig. 1. Phylogenetic tree of MGD, DGD and SQD proteins from maize (*Zm*, green dots), *Arabidopsis* (*At*, red triangle), rice (*Os*), sorghum (*Sb*), soybean (*Glyma*), *Brachypodium* (*Bra*) and *Medicago* (*MTR*). Phylogenetic trees constructed the full-length amino acid sequences of these proteins using MEGA5.0 (*a*–*c*). Syntenic analysis of *MGD*, *DGD* and *SQD* genes. The chromosomes are described as a circle (*d*). The coloured cambers represent the syntenic relationship of the *MGD*, *DGD* and *SQD* genes. (Additional information on these genes can be found in Tables S1, S2, S5, available as Supplementary Material to this paper)

Phylogenetic analyses of the MGD, DGD and SQD gene families

The full-length amino acid sequences of MGD, DGD and SQD families from maize (*Z. mays*), *A. thaliana*, rice (*O. sativa*), sorghum (*S. bicolor*), soybean (*G. max*), *B. sylvaticum* and *M. truncatula* were obtained from online database using NCBI BLASTP tools (Fig. 1). In previous studies, the *Arabidopsis* MGD family has been divided into two subtypes, Type A (AtMGD1) and Type B (AtMGD2 and AtMGD3), based on their differential response to Pi-deprived conditions (Mie *et al.* 2013). In the present study, the phylogenetic analysis of MGD orthologues genes divided them into two distinct subgroups: one subgroup containing the Type A AtMGD1, ZmMGD1, and their orthologs; and another subgroup containing the Type B MGDs form *Arabidopsis*, maize and other plant species (Fig. 1*a*). In each subfamily, MGDs from monocotyledons and dicotyledons were further clustered into different branches. AtMGDs displayed high homology with MGDs from other monocotyledons, including soybean and *Medicago*; whereas ZmMGDs were clustered with MGDs from other dicotyledons, including rice, sorghum and *Brachypodium*. ZmMGDs and their sorghum orthologs had much closer relationship. Similar phylogenetic relationships were also found in the DGD and SQD families, and two clearly separated subgroups were revealed and classified as Type A and Type B (Fig. 1*b*, *c*). The Type A DGDs contained AtDGD1 and ZmDGD1-3 and their orthologs from other plant species, whereas Type B DGDs included AtDGD2, ZmDGD4-5 and their orthologs. Similar to MGDs, in each subgroup, AtDGD displayed high homology with DGDs from other monocotyledons, while ZmDGDs were clustered with DGDs from other dicotyledons (Fig. 1*b*). SQDs were also divided into Type A and Type B subgroups. AtSQD1 and ZmSQD1 were classed into Type A subgroup, whereas AtSQD2 and ZmSQD2-5 were classed into Type B subgroup. Among them, ZmSQD4 and ZmSQD5 were more closely related to each other. In each cluster, maize SQDs and their sorghum orthologs formed phylogenetic branches at higher values, indicating that they have a high degree of sequence homology.

Gene architecture and conserved functional domains in MGD, DGD and SQD families

An exon–intron distribution diagram of 20 *MGD*, *DGD* and *SQD* genes from *Arabidopsis* and maize was generated according to their genomic and coding sequences, and an un-rooted phylogenetic tree of the 20 protein sequences was constructed to show their evolutionary relationships (Fig. 2). The results showed that the number of introns in the same subgroup was relatively consistent, but the difference between Type A and Type B subgroups was obvious. As can be seen in Fig. 2, configuration analysis showed that the number of exons/introns in *Arabidopsis* or maize *MGD* genes differed little, ranging from 5 to 7, and similar gene structure patterns were observed in each of the two subgroups. The similarity of the exon/intron structure of the MGD genes suggested that they had

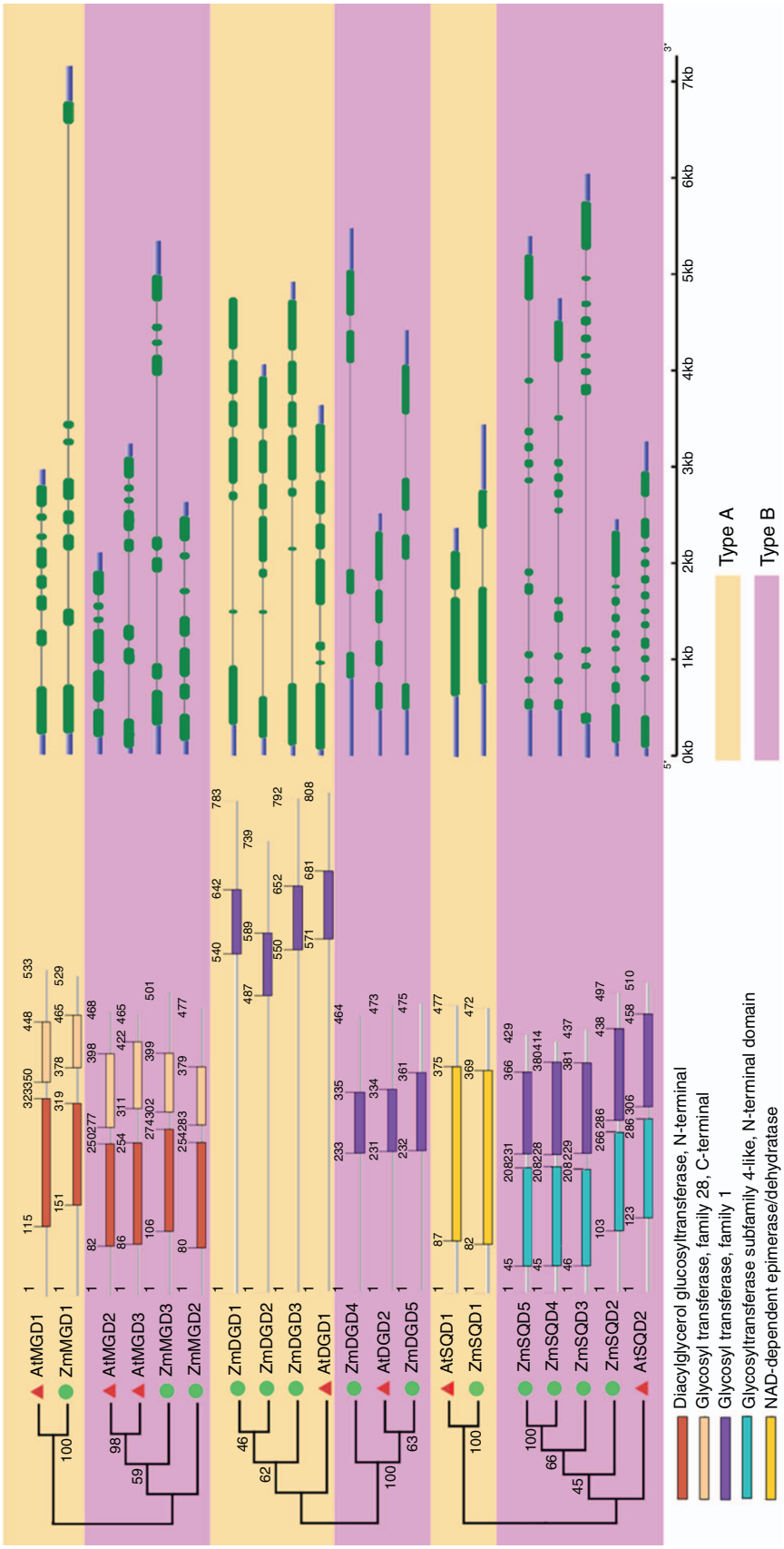

Fig. 2. Analysis of conserved functional domains and the exon-intron organisation of the of MGD, DGD and SQD families from maize and *Arabidopsis*. The phylogenetic tree of MGD, DGD and SQD proteins is shown on the left. The rectangles in the middle panel represent the predicted signal peptides, with positions of amino acids shown by numbers. In the right panel, the cylinders represent exons, and the lines in between represent introns.

undergone gene duplication during the evolutionary processes. The *Arabidopsis* and maize *DGD* genes also showed extremely high structural similarity in each subgroup. Type A *DGDs* contain six introns, but Type B *DGDs* have only three introns. In the *SQD* gene family, there is a big difference in the number of introns between Type A and Type B *SQDs*. The Type A *SQD* genes contained only one intron, whereas the Type B *SQD* genes possessed 6–11 introns. Significant differences between the two types of *DGD* and *SQD* genes indicated that the genes might have undergone species specific evolutionary processes. Among the Type B *SQD* genes, *ZmSQD4* and *ZmSQD5* have very similar intron distribution, which suggests that these two genes had replicated during evolution.

As shown in Fig. 2, the functional domains of MGD, DGD and SQD proteins have been identified and illustrated. In the MGD protein family, highly conserved diacylglycerol glycosyltransferase (*N*-terminal) and glycosyltransferase (*C*-terminal) domains have been revealed. In the DGD protein family, only one glycosyltransferase family1 domain was identified, which was located closer to the *C*-terminus of the Type A DGDs compared with the smaller Type B DGD proteins. We note that the two types SQD proteins have completely different conserved domains. The Type A *AtSQD1* and *ZmSQD1* have NAD-dependent dehydratase polypeptides, whereas the Type B SQD proteins have one glycosyltransferase subfamily4-like domain and one glycosyltransferasefamily1 domain. In the SQD protein family, members of each type have a high degree of similarity in the conserved domain construction; however, the Type A and Type B SQDs differ greatly, indicating potential functional differences and differentiation of proteins.

Expression profiles of MGD, DGD and SQD genes in maize and Arabidopsis *tissues*

To determine the expression profiles of *MGD*, *DGD* and *SQD* genes in different developmental stages of maize and *Arabidopsis*, we collected data from maize eFP and TAIR database and performed gene expression analysis. As shown in Fig. 3*a*, Type A *Arabidopsis MGD* gene (*AtMGD1*) expressed

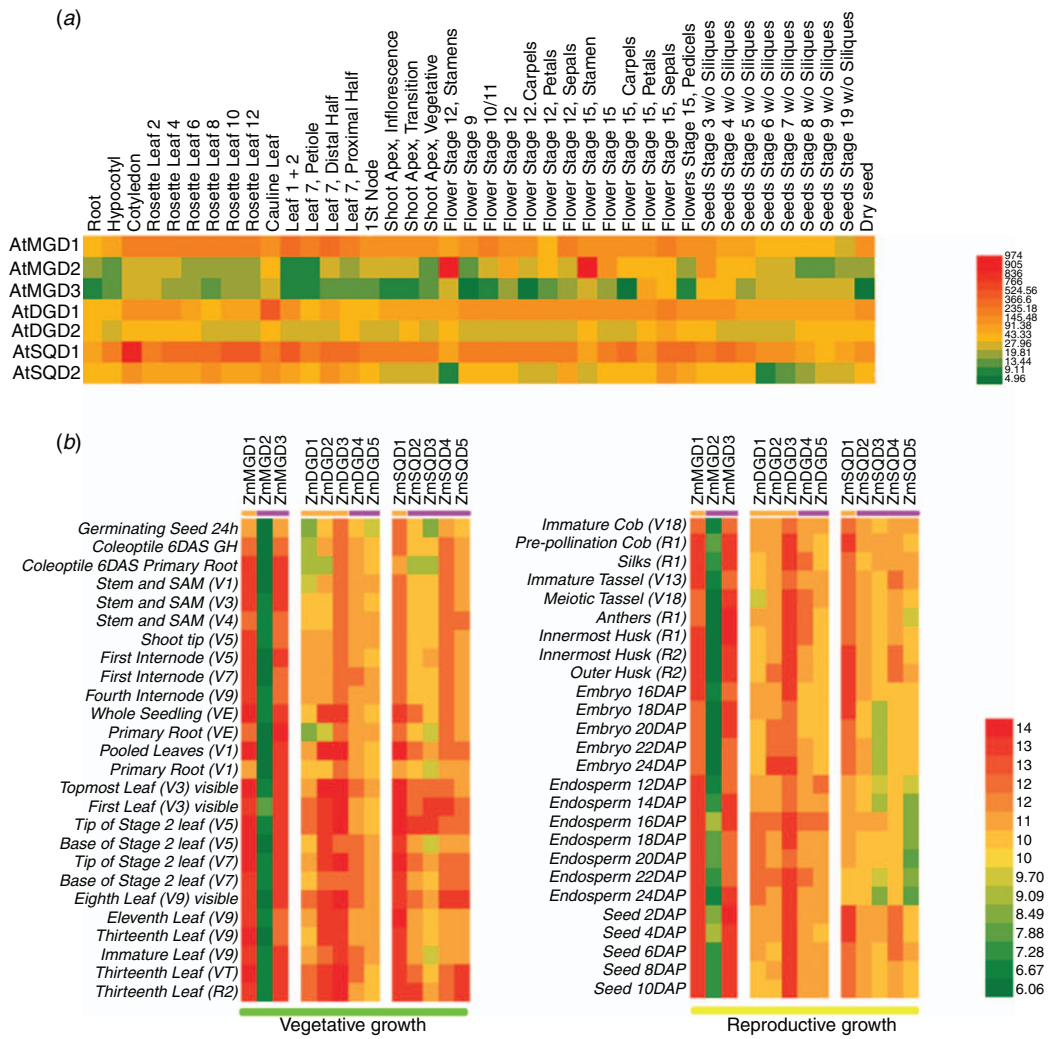

Fig. 3. Heat map of expression profiles of *Arabidopsis* (*a*) and maize (*b*) *MGD*, *DGD* and *SQD* genes in different tissues or at different growth stages. The red boxes indicate high transcript levels and green boxes indicate low transcript levels. (The heat-map data was shown in the Table S2, available as Supplementary Material to this paper)

at a higher level throughout the growth and development stages, but the expression level of Type B *AtMGD2* and *AtMGD3* was low. Similarly, the expression of Type A DGD (*AtDGD1*) was higher than that of Type B DGD (*AtDGD2*) during the whole growth and development stages. The expression of Type A SQD (*SQD1*) was also significantly higher than that of Type B SQD (*SQD2*). As shown in Fig. 3*b*, the expression of *ZmMGD1* and *ZmMGD3* was at a relative high level throughout both reproductive and vegetative growth stages, whereas the level of *ZmMGD2* was extremely low. Among the five *ZmDGD* genes, the expression of *ZmDGD3* was significantly higher than that of other *ZmDGD* genes. Furthermore, the expression of type A *ZmDGDs* (*ZmDGD1-3*) was higher in various leaf developmental stages. In the *ZmSQD* family, the expression of Type A *ZmSQD1* was obviously higher than Type B *ZmSQDs*, whereas the expression of Type B *ZmSQDs* was observed at early leaf developmental stages, which could reflect their diverse functions in chloroplast and photosynthesis.

Gene expression analyses of MGD, DGD and SQD genes in maize and Arabidopsis *against phosphate starvation*

In order to understand the response of *MGD*, *DGD*, and *SQD* genes to phosphorus deficiency, we performed quantitative real-time RT-PCR analysis on the gene expression of *Arabidopsis* and maize *MGD*, *DGD*, and *SQD* genes. As shown in Fig. 4, under low-phosphorus stress, the expression of *ZmMGDs* was induced after 72 h under low phosphate treatment, and the induction of Type B *ZmMGDs* was much significant in both leaf and root tissues. The obvious induction of Type B *AtMGDs* was also observed (Fig. 4*a*, *b*, right panel), and the induction was more pronounced in root tissues compared with the leaves. As for the *DGD* genes, the expression of Type A and Type B *ZmDGDs* was significantly upregulated after 24 h under low-phosphorus treatment, and the induction was more evident in maize leaves (Fig. 4*a*, *b*, left panel). In *Arabidopsis*, the Type B *AtDGD2* in leaves was apparently induced after 72 h of phosphate starvation, whereas the induction in roots was not significant. In both maize leaf and root tissues under phosphate starvation, a remarkable induced expression of Type B *ZmSQDs* (*ZmSQD2-5*) was observed in comparison to Type A *ZmSQD* (*ZmSQD1*) (Fig. 4*a*, *b*, left panel). In *Arabidopsis*, the expression of Type B *AtSQD2* was also found obviously upregulated under low phosphate, especially in roots(Fig. 4*a*, *b*, right panel).

The co-expression analysis of transcription factors and MGD and SQD genes in maize

The significant response of several maize *MGD*, *DGD* and *SQD* genes in response to phosphate starvation prompted us to explore their regulation at the transcriptional level. Co-expression analysis of transcription factors (TFs) and the most low-phosphate responsive genes (*ZmMGD3* and *ZmSQD3*) was performed using Cytoscape ver. 3.4.10. The transcription factors were screened from phosphorus stress transcriptome data using the plant TF database PlanTFDB. The strong low-phosphate responsive genes *ZmMGD3* and *ZmSQD3* were named as 'guide genes' and used as probes to perform co-expression relationship analysis. As shown in Fig. 5, a total of 11 transcription factors involved in the regulation of the guide genes, including MYB (GRMZM2G078820, GRMZM2G403620), ERF (GRMZM2G129777, GRMZM2G141219), Trihelix (GRMZM2G016649, GRMZM2G314660), BES1 (GRMZM2G102514), FAR1 (GRMZM2G034868)), G2 (GRMZM2G016370), LBD (GRMZM2G386674), and NF-YA (GRMZM2G165488). The co-expression network was generated for each individual guide gene, and was also established among both guide genes and the corresponding TFs (Fig. 5). Three transcription factors were found to be significantly associated with *ZmMGD3*, including MYB (GRMZM2G078820, GRMZM2G403620), and ERF (GRMZM2G129777 and GRMZM2G141219); whereas, Trihelix (GRMZM2G016649, GRMZM2G314660), and MYB(GRMZM2G078820, GRMZM2G403620) were significantly associated with *ZmSQD3*. Therefore, these transcription factors might be involved in the regulation of low-phosphorus stress response of *ZmMGD3* and *ZmSQD3*.

Metabolism of maize membrane glycerolipids under low phosphorus stress

In order to study the membrane lipid metabolism in maize under low-phosphorus stress, the changes of major glycerolipids in maize leaves and roots that under –P treatments for 72 h were measured by lipidomics analysis (Welti *et al*. 2002). As shown in Fig. 6*a*, in leaf tissues of the maize seedlings under low phosphorus stress, the molar percentage of the galactolipid (including MGDG and DGDG) and sulfolipid (SQDG) was significantly increased; while the exclusive plastidial phospholipid PG was slightly increased as well. Nevertheless, the content of most of the phospholipids, including PC, PE, PA and PI, was decreased. In root tissues (Fig. 6*b*), the phospholipids are dominant in proportions. Under low phosphorus condition, the level of PC was elevated; whereas PE and PA were decreased. The changes of the four major chloroplast bilayer lipids were not significant.

Expression profiles of maize MGD, DGD and SQD genes under environmental stresses

In order to investigate the potential roles of maize *MGD*, *DGD* and *SQD* genes in environmental stress responses, the transcriptional data of these genes under various stress treatment was obtained by searching the available RNA-seq database downloaded from the NCBI sequence-reading archive (SRA). The RNA-seq analysis was conducted on samples from the aerial parts of maize seedlings, and the abiotic stresses treatment includes: cold, heat, salt, and UV. As shown in Fig. 7, under cold stress, *ZmMGD1-3*, *ZmDGD3*, and *ZmSQD4–5* were upregulated, albeit only *ZmMGD2* was significantly differentially expressed ($|\text{Log2FC}| \geq 2$). Under heat stress, *ZmMGD1*, *ZmDGD1* and *ZmDGD3-5* were upregulated, among which *ZmMGD1* and *ZmDGD1* were differentially expressed. Salt treatment obviously induced the transcripts accumulation of *ZmDGD1* and *ZmDGD3* and *ZmSQD3*. However, under UV treatment, most genes were downregulated (Fig. 7). To further understand the regulation patterns, the *cis*-elements in the promoter regions of these genes were revealed. The results showed that there was large number of *cis*-elements related to various abiotic stresses and hormone

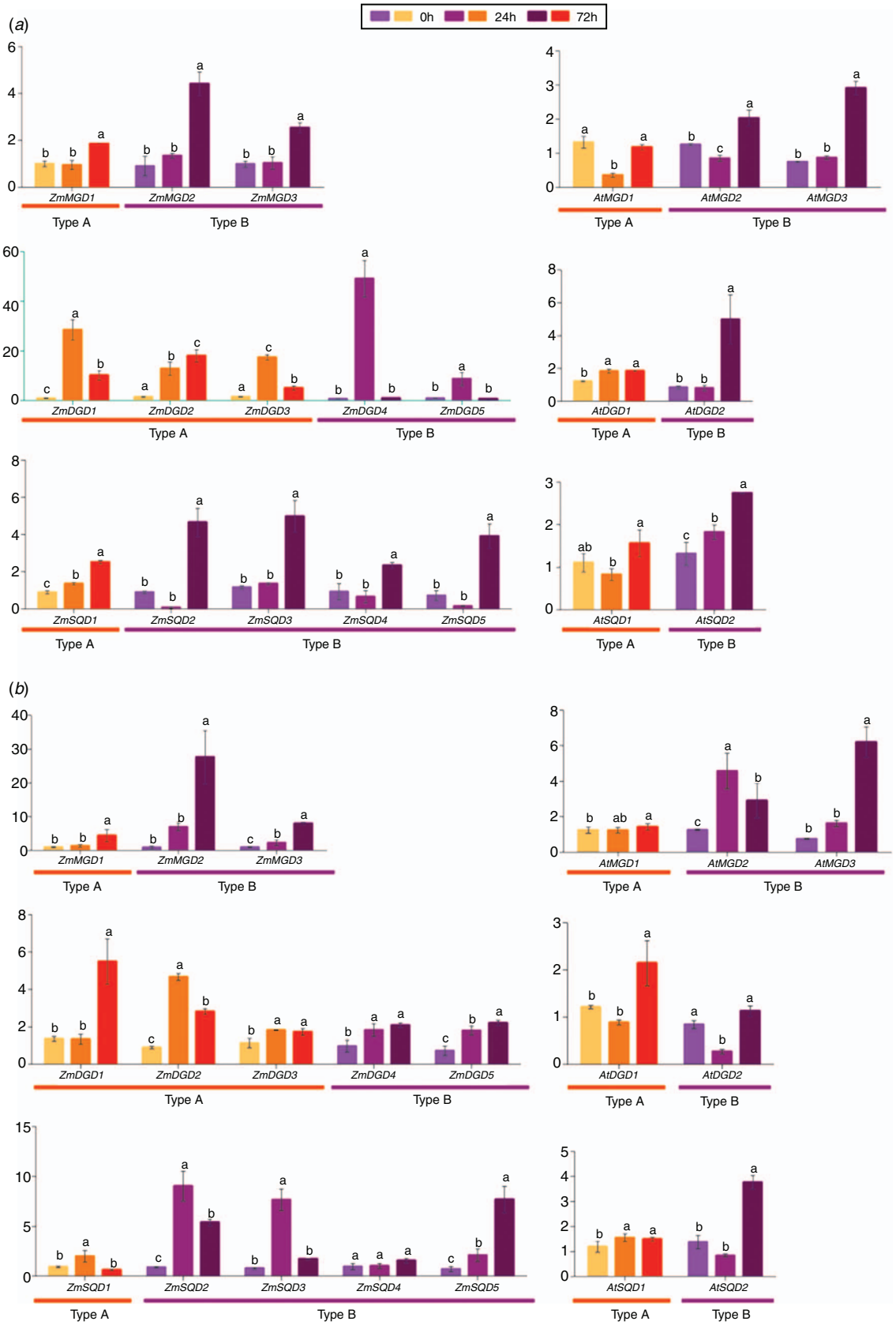

Fig. 4. The expression analysis of *MGD*, *DGD* and *SQD* genes in leaves (*a*) and roots (*b*) of maize and *Arabidopsis* under low-phosphorus stress by quantitative real-time RT–PCR. RNA was extracted from developing seeds at 0, 24 and 72 h after phosphorus stress. Quantitative real-time RT-PCR was performed using specific primers designed based on individual *MGD*, *DGD* and *SQD* gene sequences. Means with different lower case letters are significantly different ($P < 0.05$), with leaves and roots cultured in a nutrient solution with sufficient phosphate were used as control.

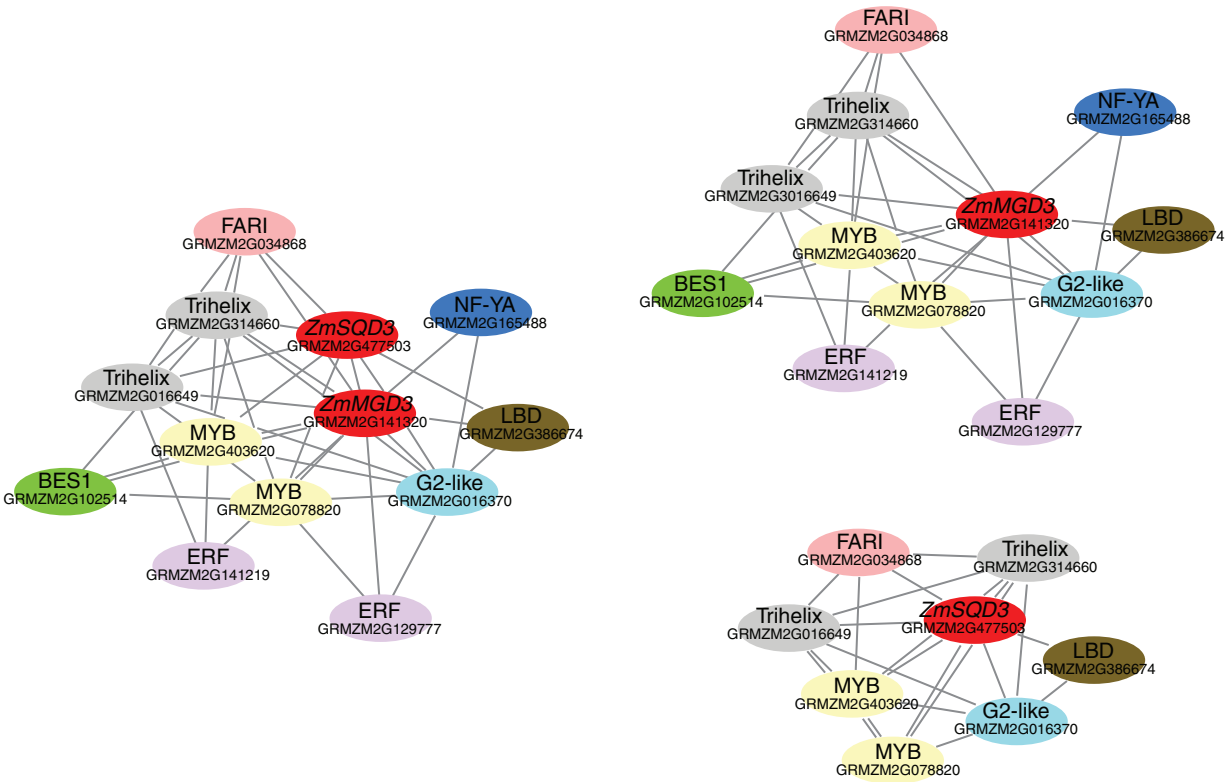

Fig. 5. A co-expression network of transcription factors and *ZmMGD3*, *ZmSQD2* under low-phosphorus stress. A co-expression relationship was established when the Pearson correlation was above 0.97, and a co-expression network was created using a Perl script with a default value of 0.6. Different coloured circles represent different transcription factors.

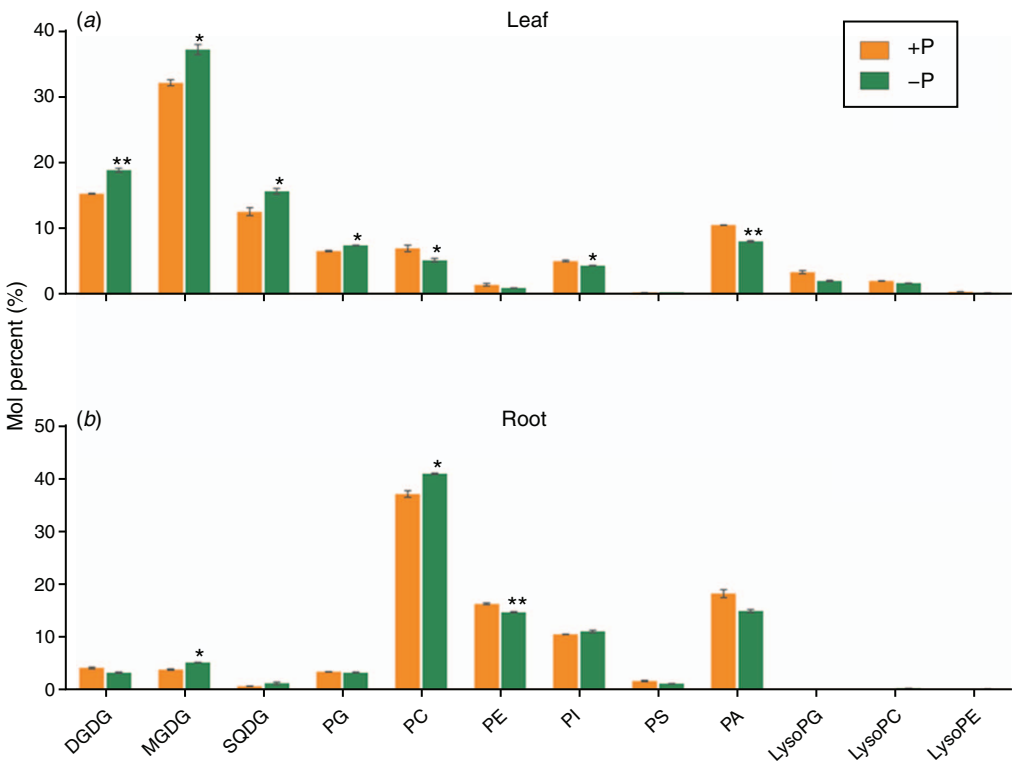

Fig. 6. Changes in glycerolipids in maize leaves (*a*) and roots (*b*) under low phosphorus stress. Values (mol %) are means ± s.d. (n = 5). Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; LPG, Lyso-PC; LPC, Lyso-PC; LPA, Lyso-PC. Values that are significantly different from the control are indicated: *, $P < 0.05$.

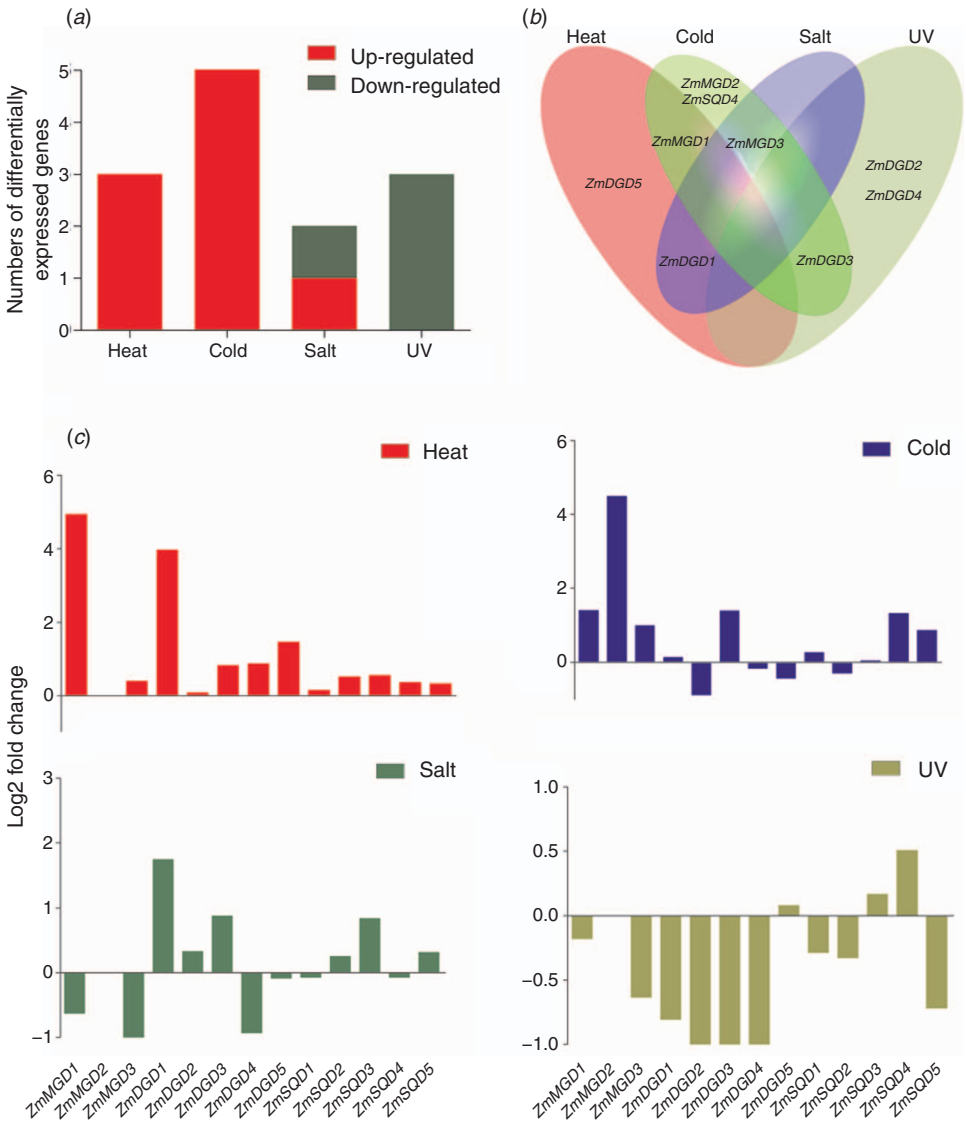

Fig. 7. Expression analysis of maize *MGD*, *DGD* and *SQD* genes under different environmental stresses. (*a*) Number of differentially expressed those genes under various environmental stresses. Compared with the control, the expression level of genes at |Log2 fold-change|≥1 was assigned as differentially expressed genes; (*b*) Venn diagram with overlapping genes under different environmental stresses; (*c*) expression profiles of those genes under cold, heat, salt and UV stresses.

responses identified and could be divided into 12 categories (Fig. S2). The number of *cis*-elements recognised by transcription factors MYB and WRKY was found relatively high in the promoter region of Type B *ZmMGD2* and *ZmMGD3* and the Type B *SQD* genes.

Discussion

In plants, galactolipids(MGDG and DGDG)and sulfolipids (SQDG) account for more than 80% of plastidic membrane lipids, and play a variety of roles on maintaining the structure of cells and organelles, converting light energy to form chemical energy and protecting cells against low-phosphate and adverse environmental conditions (Aronsson *et al*. 2008; Li *et al*. 2016*b*). The enzymes catalysing the formation of MGDG, DGDG and SQDG were termed MGD, DGD and SQD, respectively, and have been identified from various plant species (Awai *et al*. 2001; Kelly *et al*. 2003). In previous studies, differential regulation of two types of MGDs under phosphate-limited conditions has been observed in *Arabidopsis* and several other plants, and the low-phosphate induced MGD isoforms could lead to increased synthesis of non-phosphorus membrane lipid to replace the phosphorus-containing lipid under phosphate deficiency (Nakamura *et al*. 2009; Koichi *et al*. 2010).

To better understand the distinct characteristics of different isoforms of MGD, DGD and SQD in plants, we identified their family members in maize and *Arabidopsis*, and performed comparative bioinformatics and expression analysis. The

phylogenetic analysis divided MGD genes into two subgroups: one subgroup containing the AtMGD1 and its orthologs; and another subgroup containing the *Arabidopsis* AtMGD2 and AtMGD3 and their orthologs from maize and other plant species. In previous studies, the *Arabidopsis* MGDG synthase is divided into two subtypes: Type A (AtMGD1) and Type B (AtMGD2 and AtMGD3), which function differently in plant development and in their low-phosphate responses (Awai *et al*. 2001; Kobayashi *et al*. 2006). In the present study, MGDs from various plant species are also classified with *Arabidopsis* Type A and Type B MGD orthologs, which indicates that MGDs from other plant species also compliant this classification. Similarly, DGDs and SQDs from different plant species were clustered into two subgroups as well, and were marked as Type A and Type B with reference to the MGDs. Since there are no such classification of DGDs and SQDs reported, this finding suggested that distinct isoforms of these two families of genes might function differently and subjected to differential regulation under certain conditions similar to Type A and Type B MGDs. The functional domain analysis showed that the maize and *Arabidopsis* MGDs and DGDs shared high similarity in domain structure, whereas members from different groups of SQDs varied significantly, indicating the potential functional divergence. Both *AtSQD1* and *ZmSQD1* on the Type A branch have a NAD-dependent dehydratase domain, while other SQD proteins have both glycosyltransferase subfamily 4-like and glycosyl transferase family 1 domain. The *Arabidopsis* SQD2 has been implicated to be relevant to the accumulation a novel plant lipid–glucuronosyldiacylglycerol (GlcADG) under phosphorus depletion condition (Okazaki *et al*. 2013). This agrees with another study showing that *SQD2* participated in flavonoid glycosylation, regulating sugar metabolism and seed setting in rice (Zhan *et al*. 2017).

In the long-term evolution process, plants gradually formed a protective mechanism of the photosynthetic membrane under phosphorus deficiency, that is, the synthesis of non-phosphorus membrane lipid such as DGDG and SQDG to replace phosphorus-containing membrane lipids, to save phosphate and keep normal photosynthesis under adverse conditions (Andersson and Tjellstrom 2013; Shi *et al*. 2013). In *Arabidopsis*, the Type B AtMGD2 and AtMGD3 were strongly activated under phosphate starvation (Mie *et al*. 2013), and led to the induced MGDG and DGDG synthesis (Kobayashi *et al*. 2006; Nussaume *et al*. 2011). In plants, the synthesis of galactolipids MGDG and DGDG involves two different pathways: the plastid/chloroplast localised prokaryotic pathway and the endoplasmic reticulum compartmentalised eukaryotic pathway (Ohlrogge *et al*. 1991). In *Arabidopsis*, a typical 16:3 plant, the galactolipids synthesis relies on both eukaryotic pathway and prokaryotic pathway, whereas the 18:3 plant maize has been shown to mainly use the eukaryotic pathway for galactolipids synthesis (Li *et al*. 2016*a*; Gu *et al*. 2017). Since most of the previous studies on the galactolipids metabolism under phosphorous deficiency conditions have focussed on the 16:3 plant *Arabidopsis*, the information on the 18:3 plants has been lacking.

In the present study, the transcriptional response of all maize and *Arabidopsis MGD*, *DGD* and *SQD* genes against phosphate starvation was investigated using quantitative real-time RT-PCR, lipids accumulation was determined, and a comparative analysis was conducted and interpreted (Fig. 8). As shown in Fig. 8, under low phosphorus conditions, the expression of maize and *Arabidopsis* Type B *MGD* genes was significantly elevated in both leaf and root tissues, which is in agreement with the previous findings (Shi *et al*. 2013; Sarkis *et al*. 2014). The expression of both Type A and Type B *DGD* genes was induced by phosphorus starvation in maize and *Arabidopsis*. It has been reported that in *Arabidopsis*, although both *DGD1* and *DGD2* were induced by phosphate deprivation, DGD2 seems to be the major enzyme and preferably used MGDG generated by Type B MGD2/MGD3 to produce DGDG in the outer envelope of chloroplast (Härtel *et al*. 2000; Benning 2009). In comparison of Type A *SQDs*, the expression of maize and *Arabidopsis* Type B *SQD* genes were significantly induced in both leaf and root tissues. In *Arabidopsis*, *AtSQD2* is considered to be directly related to plant response to phosphate stress (Yu *et al*. 2002). These findings suggested that the Type B *MGD*, *DGD* and *SQD* genes in plants are involved in the synthesis of non-phosphorus lipids under low-phosphate conditions.

In the present study, the lipid analysis was also conducted in maize leaves and roots under low-phosphate conditions. An overall increase in non-phosphorus lipids (MGDG, DGDG and SQDG), and a decrease in phosphorus lipids (PC, PE, PI and PA) were observed, especially in leaf tissues. The results implied that under low-phosphorus stress, the degradation of phospholipids from eukaryotic pathway was enhanced to provide PA and DAG for the synthesis of MGDG, and then DGDG in the 18:3 plant maize. The DAG derived from ER eukaryotic pathway could also be used as substrate for the synthesis of SQDG, leading to increased generation of SQDG (Fig. 8). It has been reported that DGDG is the major bilayer lipid in chloroplast membrane and functions pivotally in maintaining the membrane integrity under stress conditions (Li *et al*. 2016*b*). Studies also showed that phospholipid synthesis in leaves is affected by the lack of phosphorus supply, whereas photosynthetic membrane lipids were significantly elevated to maintain membrane lipid stability (Benning 2009; Moellering and Benning 2011).

Phosphate starvation usually combines with other abiotic stresses. Plant *MGD*, *DGD* and *SQD* genes are found play certain roles in various environmental stress responses. The overexpression of rice MGDG synthase gene (*OsMGD*) in tobacco produces a large amount of galactolipid in leaves, resulting in enhanced salt tolerance of the tobacco plant (Wang *et al*. 2014). DGD gene could regulate the heat tolerance of *Arabidopsis* by regulating the ratio of the thylakoid MGDG and DGDG, and improved the heat resistance of the plant at the physiological level (Chen *et al*. 2006). In addition, the drought response of plants in maize is affected by the upregulation of galactolipid biosynthesis genes (Chen *et al*. 2018). In our study, differential expression profiles of maize *MGD*, *DGD* and *SQD* genes was observed under the heat, cold, salt and UV stresses. The expression of *ZmMGD* and *ZmDGD* genes was significantly affected by temperature stress, especially the Type A *ZmMGDs*. The Type B *ZmSQDs* were more responsive to temperature and salt stresses. It is worth noting that most maize *MGD*, *DGD* and *SQD* genes were repressed by UV treatment.

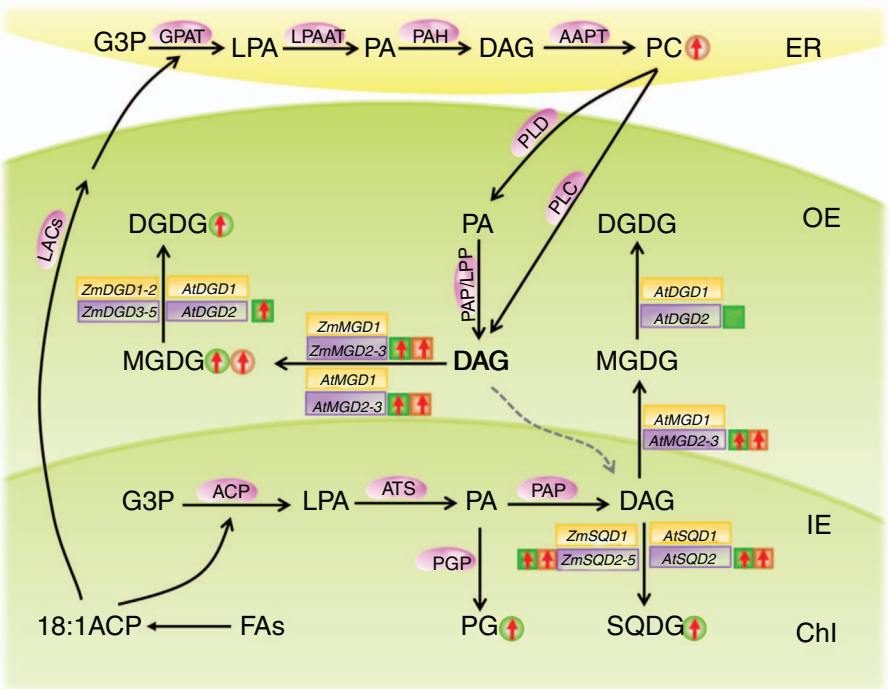

Fig. 8. Schematic diagram of lipid metabolism network of maize and *Arabidopsis*. The orange and purple rectangles represent the Type A and Type B *MGD*, *DGD* and *SQD* genes respectively. The coloured squares with the red upward arrows indicate upregulated genes under low-phosphorus conditions. The leaf tissues are shown in green and the roots are shown in brown squares. Green and brown circles with a red upward arrows represent the accumulation of lipid in maize leaves and roots under low-phosphorus stress respectively. Abbreviations: chl, chloroplast; IE, inner envelope; OE, outer envelope; ER, endoplasmic reticulum.

Phosphate deficiency of soil and abiotic stresses are major factors limit crop production in many regions of the world, As a result, plants have developed various biochemical adaptive mechanisms to deal with these stresses, including membrane lipid remodelling (Okazaki *et al*. 2013; Li *et al*. 2016*a*). In the present study, the bioinformatics analysis of maize *MGD*, *DGD* and *SQD* genes and their responses to phosphate depletion and abiotic stresses produced findings that will help for further characterisation of genes against phosphorus depletion and for the generation of improved crops adapted to phosphate starvation and other abiotic stresses.

Conflicts of interest

The authors declare no conflicts of interest.

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