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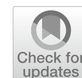
ORIGINAL PAPER



Expression of *AtGA2ox1* enhances drought tolerance in maize

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Abstract

Drought is a major limiting factor to maize (*Zea mays* L.) yield. Plant hormones, including gibberellins (GAs), play important roles in plant response to drought stress. In previous studies, significant reductions in GAs levels have been reported under drought stress. In maize, GA content is correlated to drought tolerance, but the molecular mechanism remains unclear. In the present study, *AtG2ox1*, a member of the GA2ox family with a clear function, was used to create GA deficiency maize. The transgenic maize had a higher chlorophyll content and faster growth rate, when compared to the wild type (WT) plants, under drought stress in a greenhouse. The physiological and biochemical test results revealed that transgenic maize had decreased levels of GA_1 and malondialdehyde (MDA), and increased content of proline and soluble sugars, and antioxidant enzyme activities, when compared to the WT. Furthermore, the transcriptomic analysis revealed that some differentially expressed genes involved in transcription factors correlated to drought stress and abiotic stress responses, and that signaling was enriched. All these results reveal the possible molecular mechanism of GA regulation in drought tolerance, in which the overexpression of *AtGA2ox1* altered the expression of multiple genes correlated to the internal antioxidant system and maintenance of cell osmotic potential. The present study demonstrates that the overexpression of *AtGA2ox1* could control GA content and improve drought tolerance in transgenic maize. Furthermore, this strategy represents a novel approach to address drought tolerance in maize breeding.

Keywords *AtGA2ox1* · Gibberellin · Drought tolerance · Maize

Introduction

Maize is one of the most widely consumed and valuable staple food worldwide. Drought is presently one of the major factors that severely limit maize growth and productivity (Edmeades et al. 2000), causing 15–20% loss of production annually. As global climate change progresses, drought due to severely arid climates has become a severe threat (FAOSTAT 2010), particularly in regions that rely on in-season rainfall. Therefore, improving drought tolerance in maize has been the most daunting challenge.

Plant hormones, including gibberellins (GAs), have long been known to be highly involved in the regulation of plant growth and development. Previous studies have extensively revealed GA metabolic patterns and mechanisms of action (Yamaguchi 2008), and further cloned the majority of GA-associated genes from *Arabidopsis thaliana* and rice (Thomas et al. 1999; Hedden and Phillips 2000; Sakamoto et al. 2004).

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Abiotic stresses, including drought, often elicit changes in GA metabolic pathways, leading to the promotion of specific protective mechanisms in plants. For instance, drought results in the rapid decrease in levels of GAs in maize leaves (Wang et al. 2008; Nelissen et al. 2018). Previous studies have revealed the enhancement of salt tolerance in plants by reducing the level of bioactive GAs. Magome et al. (2004) reported the higher survival rate (92.7%) of GA-deficient *Arabidopsis* mutant, *ga1-3*, in high-salinity stress conditions, when compared to wild-type (WT) plants (52.7%). Paclobutrazol (PBZ) is a GA inhibitor that is responsible for inducing stress tolerance in plants by increasing antioxidant enzyme activity (Somasundaram et al. 2009). Conversely, the external application of GAs would increase plant sensitivity to abiotic stress. Furthermore, the survival rate of *Arabidopsisga1-3* mutants sprayed with exogenous GAs decreased by 54.5% under high-salinity stress (Magome et al. 2004). These studies suggest the important role for GAs in plant response to stress conditions (Colebrook et al. 2014).

The overexpression of the *GA 2-oxidase* (*AtGA2ox*) gene has been proven to offer a direct approach to decrease endogenous levels of GAs in the model plant *A. thaliana* (Schomburg et al. 2003; Biemelt et al. 2004; Lee and Zeevaart 2005; Dijkstra et al. 2008; Huang et al. 2010a, b). Furthermore, previous studies have revealed the critical role of the *GA2ox* gene family in the GA metabolic pathway by metabolizing bioactive GAs into non-bioactive GAs in higher plants (Yamaguchi 2008; Han and Zhu 2011). Achard et al. (2008a, b) revealed that the overexpression of *AtGA2ox* increased the expression of genes related to reactive oxygen species (ROS)-detoxification enzymes, resulting in improved stress tolerance in *A. thaliana*. Shan et al. (2013) reported a significant increase in *GA2ox* expression level under drought stress in maize inbred line Zheng58, suggesting the active regulation of adaptation to drought by bioactive GAs (Biemelt et al. 2004; Lee and Zeevaart 2005; Dijkstra et al. 2008; Huang et al. 2010a, b). Furthermore, the expression of *Oryza sativa* GA 2-oxidase 5 (*OsGA2ox5*) in transgenic *Arabidopsis* and rice lines resulted in higher tolerance to high-salinity stress (Shan et al. 2014), indicating that the *GA2ox gene* is involved in abiotic stress resistance. Although many studies have shown that GA is closely correlated to drought, the molecular mechanisms remain unclear, in terms of whether the method of regulating endogenous GA content can affect the drought tolerance of maize.

In maize, 10 *ZmGA2ox* family members were found by bioinformatic analysis, and the expression patterns during seed germination were reported in a previous study (Song et al. 2011). To the best of our knowledge, none of ZmGA2ox's functions were analyzed in detail, and it remains unknown which function controls the bioactive GA levels. In the present study, GA deficiency model was created in maize by expressing a heterologous GA oxidase, *AtGA2ox1*, which is known to decrease endogenous GAs and regulate the architecture in *A. thaliana*. The molecular mechanisms of the endogenous GA content in conferring maize drought tolerance were further explored through a series of physiological and biochemical experiments, and differential transcriptome analysis. The present study provides a method for producing a GA-deficiency model in maize through the exogenous expression GA 2-oxidase, supporting the notion that the overexpressed *AtGA2ox* gene can substantially improve plant architecture and drought tolerance in maize. In addition, the possible molecular mechanisms of *AtGA2ox1* for improving maize drought resistance were also proposed in the present study.

Materials and methods

Construction of plant expression vectors and *Agrobacterium*-mediated transformation

The *AtGA2ox1* gene (GenBank accession no. AT1g78440) was cloned into the monocot expression vector pCAM3300, which was named as pCAM3300-GA2ox. The ubiquitin promoter was used to drive the expression of the *AtGA2ox1* gene (Fig. 1a). Transgenic maize plants were produced by using *Agrobacterium*-mediated transformation, as described by Ishida et al. (2007).

Measurement of GA_1 content

GA_1 was extracted and purified according to the protocol of Yang et al. (2014). Approximately 1 g of leaf tissues were sampled from 5-week-old seedlings. Then, these were homogenized with ice-cold 80% methanol at 4 °C for 12 h to extract the GAs. Next, the extracts were centrifuged at 8000 × g for 10 min at 4 °C. Then, the supernatants were allowed to pass through the Sep-Pak C_{18} columns (Waters Corporation) preconditioned with 100% and 80% methanol. Afterwards, the eluant was collected, dried and re-dissolved in a mixture of methanol–1% acetic acid (2:3, v/v) for the high-performance liquid chromatography analysis. The GA contents were assayed using the method of Urbanová et al. (2013). Three biological replicates were analyzed for each sample.

Phenotyping and relative chlorophyll content analysis

Plant height, which was defined as the length from the first internode to the top of the first leaf, was measured with five replicates from 3-week-old seedlings in the greenhouse. Relative chlorophyll content was estimated from the third

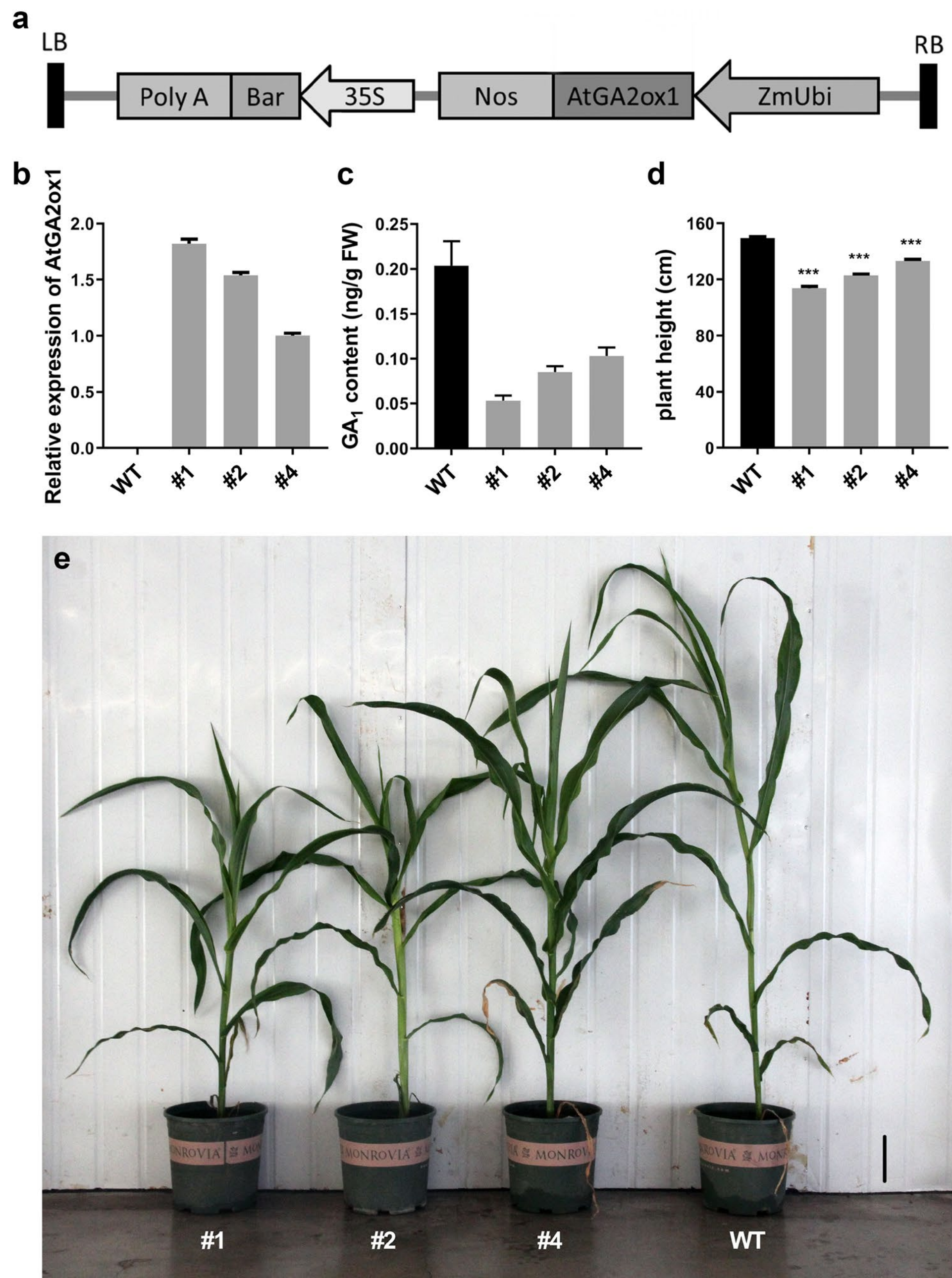

Fig. 1 Overexpression of *AtGA2ox1* in maize. **a** The map of pCAM3300-GA2ox vector, **b** expression levels of *AtGA2ox1* in different transgenic lines detected by qRT-PCR. All reactions were performed in three biological duplications, and each biological duplication had three technical replicates, **c** the GA_1 content in 5-week-old WT and T_2 generation transgenic plants, **d** the plant heights of WT and different transgenic lines at flowering stage, and **e** the plant phenotype at flowering stage. Bar = 10 cm. The data were presented as mean ± SE. The asterisks indicate the significant differences between transgenic lines (#1, #2 and #4) and WT plants (t-test, ***P < 0.001)

expanded leaf with three replicates using SPAD-502 (Konica Minolta, Japan).

Drought treatment for transgenic maize plants

T_2 generation transgenic lines (#1, #2 and #4) and WT plants were grown in pots (10 × 10 cm) containing peat-vermiculite (5:1, v/v) medium in a greenhouse at 27 ± 2 °C under natural light. PCR analysis was used to screen for positive transformants, from which watering was suspended for 10 plants from each line at 21 days old for 9 days for drought treatment. Then, the leaves were collected from each line at the end of treatment, and stored at − 80 °C for further analysis. The amount of substrate and water for each of the experimental materials were applied at equal levels.

Physiological and biochemical analysis

Proline content was analyzed by ninhydrin colorimetric assay (Bates et al. 1973). The soluble sugar content was measured by anthrone colorimetric assay, as previously described (Yemm and Willis 1954). Malondialdehyde (MDA) content was estimated following a previously described protocol (Gilbert et al. 1984). SOD, CAT and POD

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activity was measured, as previously described by Hodges et al. (1999), Beers and Sizer (1952), and Upadhyaya et al. (1985), respectively. For all the above measurements, three replicates per line were used. Statistical differences were determined using Student's two-tailed *t* test.

Statistical analysis

In experiments for the GA_1 measurement, phenotyping and relative chlorophyll content analysis, drought treatment, and physiological/biochemical analysis, the data were statistically analyzed, and the means of each transgenic line (#1, #2 and #4) and WT were compared using *t*-test, and were calculated using the statistical software GraphPad version 7.0 (San Diego, CA, USA). The data were presented as the mean and standard error (SE) of the mean. All tests had three replicates.

RNA-seq analysis

RNA sequencing was performed on T_2 plants from transgenic line #1, and WT plants were used as a control. Leaf tissues were harvested in plants at stages V5 (Vegetative 5, five fully extended leaves) for T_2 plants in a controlled chamber. The RNAs were pooled from 10 transgenic plants and 10 control plants, and these were randomly divided into three groups as three biological replicates in both the transgenic plants and control. The sequencing libraries were generated using a NEBNext UltraTM RNA Library Prep Kit for Illumina (NEB, USA), according to manufacturer's recommendations, and index codes were added to attribute the sequences to each of the samples. Clean reads (http://www.ncbi.nlm.nih.gov/bioproject/359734) were generated by removing the adaptor, low-quality sequence reads were obtained from the raw datasets, and these were mapped to the maize reference genome sequence (http//ftp.ensemblgenomes.org/pub/plants/release-32/fasta/zea_mays/) using Tophat2 software tools. Reads with only a perfect match or one mismatch with the reference genome were further analyzed and annotated based on the following databases: KEGG Ortholog (KO) database (https://www.kegg.jp/kegg/ko.html), and Gene Ontology (GO) database (http://www.geneontology.org). The gene expression levels were quantified to fragments per kilobase of the transcript per million fragments mapped. The read counts for each sequenced library were adjusted in the edgeR program package by one scaling normalized factor, and applied to the differential expression analysis using the DEGseq (2010) R package. The *P*-value was adjusted using the *q*-value (Storey and Tibshirani 2003). A Q-value of < 0.005 and a $\log_2$ (fold change) of ≥ 1 were set as the thresholds for significant differential expression.

Real-time PCR (qRT-PCR) analysis

Total RNA from T_2 transgenic event #1 was isolated from 0.1 g of flash-frozen, tissue samples using an EasyPure Plant RNA Kit (TransGen Biotech, China). Then, a TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, China) was used to synthesize the cDNA. The quantitative real-time gene expression was determined through gene-specific primers using TB Green™ Premix Ex Taq™ (Tli RNaseH Plus), ROX plus (Takara, China) on an ABI7900HT system (Applied Biosystems, USA). All reactions were performed in three biological duplications. The Zm*GAPDH* gene (GenBank accession no. X07157) was used as an internal control. Statistical analysis was performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The gene-specific primers are listed in Table S1.

Results

AtGA2ox1 gene overexpression results in the GA deficient phenotype in maize

GA deficiency may induce plant stress response. In order to determine whether reducing the bioactive GA content can improve the drought tolerance of maize, GA-deficient maize was created by overexpressing *AtGA2ox1* (Fig. 1a). Three individual transgenic events with different increased *AtGA2ox1* expression levels were identified (Fig. 1b). Among these, transgenic line #1 had the highest expression level of *AtGA2ox1*, followed by transgenic line #2 and #4. In order to further determine whether the overexpression of the *AtGA2ox1* gene affected the GA content in maize, GA_1 content was tested among the three independent transgenic events. Compared with the WT plants, the GA_1 content was reduced by 49.5–74% in transgenic maize (Fig. 1c). Compared to the WT, the activation of *AtGA2ox1* in transgenic maize created a dominant semi-dwarf phenotype, and there was a dramatic change in plant height from the jointing stage to the mature stage (Fig. 1d, e). Our results support the notion that the overexpression of *AtGA2ox1* could create a GA-deficient phenotype in maize.

AtGA2ox1 gene overexpression enhances tolerance to drought

In order to determine the effects of *AtGA2ox1* overexpression on drought tolerance, three T_2 transgenic maize lines (#1, #2 and #4) were evaluated in drought stressed conditions. After 3 weeks in the greenhouse under normal conditions, the plant height was found to be slightly different, and there were no other visible differences in phenotype between

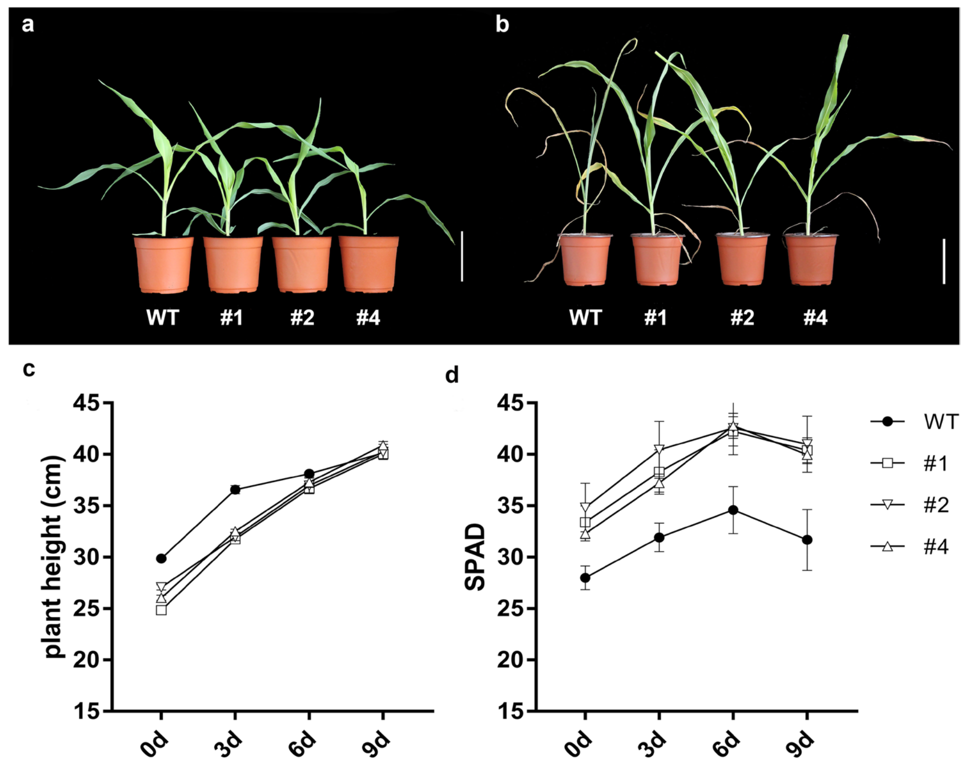

Fig. 2 The performance of transgenic maize with over-expressed *AtGA2ox1* under drought treatment. **a** The growth performance of WT and transgenic lines (#1, #2 and #4) before drought stress, **b** the growth performance of WT and transgenic lines (#1, #2 and #4) after 9 days drought stress, **c** the growth rate of WT and transgenic lines (#1, #2 and #4), and **d** chlorophyll SPAD values of the third expanded leaves of WT and transgenic lines (#1, #2 and #4). WT and T_2 generation transgenic plants were grown in a greenhouse for 21 days and then stopped to water for 9 days. The values were measured during the 9 days of drought treatment. Bar = 10 cm. The data were presented as mean ± SE (n = 3)

the transgenic plants and control plants (Fig. 2a). When water was withheld for 9 days, the control plants exhibited a visibly wilting phenotype, while plants of the three transgenic events suffered less damage (Fig. 2b). In drought stress conditions, the chlorophyll content and plant growth rate were dynamically detected. Before drought stress, the transgenic maize had a higher chlorophyll content and shorter plant height, when compared to the WT. When water was withheld, the WT plants grew slower than the transgenic maize under drought stress (Fig. 2c), and the rate of decline in chlorophyll content was significantly slower than the WT plants (Fig. 2d). These results reveal that the transgenic maize had less impact on growth and development under drought stress, when compared to the WT.

AtGA2ox1 gene overexpression increases proline and soluble sugar content, reduces MDA content, and enhances antioxidant enzyme activities under drought stress

Amino acid proline, soluble sugar, MDA and antioxidant enzymes have been identified as crucial indicators in many plant species in response to environmental stresses, and these have been proposed to play an important role in the adjustment of osmotic and oxidative stresses caused by drought tolerance. In the present study, these indicators were tested. As shown in Fig. 3a, b, all stressed plants contained higher concentrations of proline and soluble sugars, and the levels in the transgenic lines were 1.54–2.58- and 1.4–1.79-fold of those in control plants, respectively. These results indicate that the overexpression of *AtGA2ox1* could increase the accumulation capacity of proline and soluble sugars in transgenic maize. The MDA content of transgenic maize after 9 days of drought stress treatment was reduced by 20.7–38.3%, when compared to the WT (Fig. 3c), leading to high levels of tolerance to drought. Antioxidant reductases, including SOD, CAT and POD, have been reported to be associated with the elimination of ROS. In the present study, compared to the WT, the activity levels of SOD, CAT and POD in transgenic maize increased to 1.98–2.49-fold (Fig. 3d), 1.29–2.22-fold (Fig. 3e), and 1.68–2.27-fold (Fig. 3f), respectively, when under drought stress conditions. The present result supports the notion that AtGA2ox1-OE increases antioxidant enzyme activity, reduces the accumulation of ROS, and decreases oxidative damage to the cell membrane in maize.

Differential transcriptome analysis between transgenic maize and control plants

GA is an essential hormone that regulates growth and development throughout the entire life cycle of plants (Yamaguchi 2008). Altered GA content may impact many aspects of plant growth and developmental processes. In order to better understand how GA_1 deficiency improves maize drought tolerance, a whole genomic transcriptomics profiling was

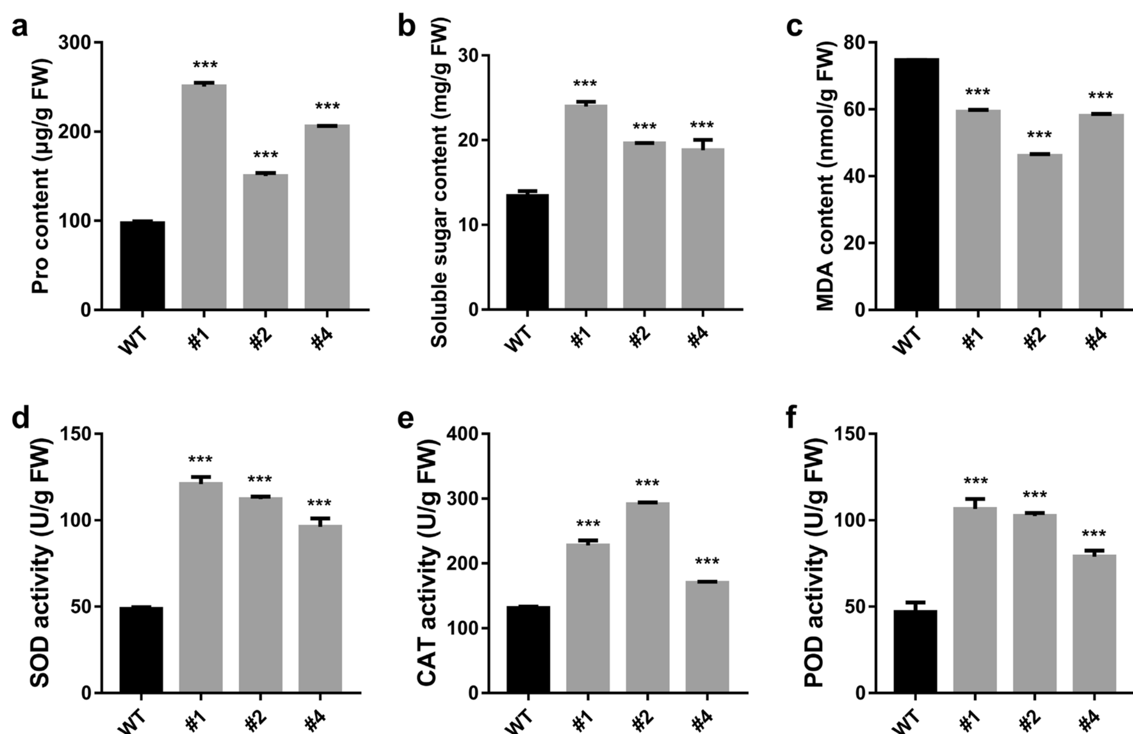

Fig. 3 Comparisons of solute content and antioxidant enzyme activity in transgenic maize and the WT under drought treatment. WT and T_2 generation transgenic plants were grown in a greenhouse for 21 days and then stopped to water for 9 days. The proline contents (**a**), soluble sugar contents (**b**), MDA (**c**), SOD (**d**), CAT (**e**), and POD (**f**) were measured in WT and transgenic plants after 9 days drought treatment. The data were presented as the mean ± SE (n = 3). The asterisks indicate the significant differences between transgenic lines (#1, #2 and #4) and the WT (t-test, ***P < 0.001)

performed for the leaf of transgenic line #1 and the WT plants as a control. Then, the RNA-seq data were further analyzed, and the differentially expressed genes (DEGs) were identified. The consistency was observed between three replications, and the large transcriptional differences were found between transgenic and WT plants. Based on stringent statistics and filtering, the expression of 3064 genes changed due to the ectopic expression of *AtGA2ox1*, with a total 1943 and 1121 genes being upregulated and downregulated at the V5 leaf stages in transgenic maize. In order to confirm the transcriptomic data, 16 genes, including eight upregulated genes and eight downregulated genes, were selected (Table S4), and the expression was validated by qRT-PCR. The expression patterns of these 16 genes were consistent with the trend of the transcriptome data, proving that the transcriptome data was accurate (Fig. S1).

GO functional enrichment analysis was performed with the DEGs from the comparisons between transgenic line #1 and the WT (Table S2). A total of 2464 DEGs were annotated, and 35 pathways associated with drought stress in the GO biological processes (Fig. 4), including response to stress (GO:0006950), response to heat (GO:0009408), response to hydrogen peroxide (GO:0042542), fatty acid beta-oxidation (GO:0006635), toxin catabolic process (GO:0009407), cellular response to water deprivation (GO:0042631), heat acclimation (GO:0010286), response to ROS (GO:0000302), response to water (GO:0009415) and GA catabolic process (GO:0045487), were significantly upregulated. However, the gibberellic acid mediated signaling pathway (GO:0009740) and GA biosynthetic process (GO:0009686) were significantly downregulated. In addition, a KEGG analysis was performed in the present study. It was revealed that the enrichment of 333 DEGs in 24 pathways were combined with drought stress, and more than two-thirds of these DEGs were enriched in the pathway of protein processing, endoplasmic reticulum, plant hormone signal transduction, biosynthesis of amino acids, starch and sucrose metabolism, glutathione metabolism, glycerophospholipid metabolism, and carbon metabolism (Table 1). Furthermore, five of these pathways, including protein processing in the endoplasmic reticulum, galactose metabolism, fatty acid metabolism, fatty acid biosynthesis, and biosynthesis of unsaturated fatty acids, were upregulated.

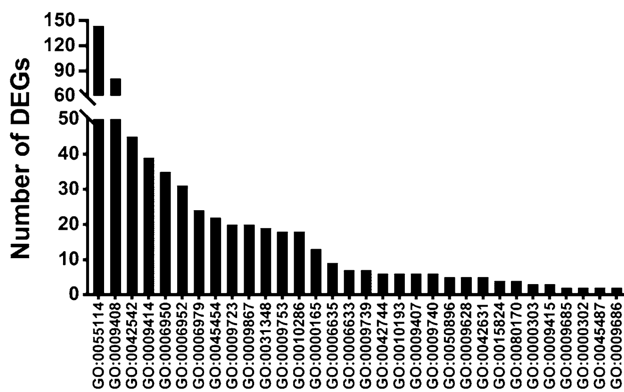

Fig. 4 The enrichment of DEGs in the biological process GO terms. The enrichment of DEGs in the biological process GO terms: (GO:0055114): oxidation–reduction process, (GO:0009408): response to heat, (GO:0042542): response to hydrogen peroxide, (GO:0009414): response to water deprivation, (GO:0006950): response to stress, (GO:0006952): defense response, (GO:0006979): response to oxidative stress, (GO:0045454): cell redox homeostasis, (GO:0009723): response to ethylene, (GO:0009867): jasmonic acid mediated signaling pathway, (GO:0031348): negative regulation of defense response, (GO:0009753): response to jasmonic acid, (GO:0010286): heat acclimation, (GO:0000165): MAPK cascade, (GO:0006635): fatty acid beta-oxidation, (GO:0006633): fatty acid biosynthetic process, (GO:0009739): response to GA, (GO:0042744): hydrogen peroxide catabolic process, (GO:0010193): response to ozone, (GO:0009407): toxin catabolic process, (GO:0009740): gibberellic acid mediated signaling pathway, (GO:0050896): response to stimulus, (GO:0009628): response to abiotic stimulus, (GO:0042631): cellular response to water deprivation, (GO:0015824): proline transport, (GO:0080170): hydrogen peroxide transmembrane transport, (GO:0000303): response to superoxide, (GO:0009415): response to water, (GO:0009685): GA metabolic process, (GO:0000302): response to reactive oxygen species, (GO:0045487): GA catabolic process, and (GO:0009686): GA biosynthetic process

Molecular mechanisms of AtGA2ox1 created GA-deficit phenotype and improved maize drought resistance

AtGA2ox1-OE creates a semi-dwarf and drought tolerance enhance phenotype, and the possible molecular mechanisms are proposed in Fig. 6 based on the physiological and biochemical indicators and transcriptome analysis. The qRT-PCR analysis verified that two GA2ox family members, Zm00001d039394 and Zm00001d043411, were upregulated in the AtGA2ox1-OE maize plant, while the GA20ox family member, Zm00001d042611, was significantly downregulated (Fig. 5a; Table S3). These revealed that AtGA2ox1-OE produces a GA-deficit phenotype not only through the deactivation of bioactive GA pathway, but also through the inhibition of the endogenous GA biosynthesis pathway.

In addition, other DEGs involved in ROS signaling and related to proline metabolism were also enriched, such as CAT, glutaredoxins (Grxs), thioredoxins (Trx), ferredoxins (Fds) and proline-rich protein (Table S3). These genes are involved in ROS removal, and improve the stability of the plasma membrane biochemical process. Zm00001d014848, which is a catalase isozyme gene, Zm00001d017240, which is a Grx-like gene, Zm00001d028992, which is a Trx family member, Zm00001d034760, which is a member of the Fds family, and Zm00001d033079, which is a proline-rich protein gene, are the five DEGs selected for qPCR verification. These were all upregulated in the AtGA2ox1-OE maize, and the results were consistent with those in the transcriptome data (Fig. 5b; Table S3). Furthermore, these results were in accordance with the physiological and biochemical findings of transgenic maize under drought stress, suggesting that these DEGs may play an important role in ROS detoxification enzyme activity, proline content increase, plasma membrane stability, and osmotic balance of transgenic maize under drought stress.

Next, it was also found that the DEGs of various transcription factor (TF) families were enriched, including HSF, NAC, WRKY, AP2/ERF, bHLH, MYB, and bZIP (Tables 2, S3). In the present study, seven DEGs were selected from each TF family, and the expression patterns were also verified by qRT-PCR. Zm00001d034433 (HSF), Zm00001d043877 (NAC), Zm00001d009103 (AP2/ERF), Zm00001d013130 (bHLH), Zm00001d012379 (MYB) and Zm00001d039658 (bZIP) were upregulated in AtGA2ox1-OE maize, while Zm00001d023332 (WRKY) was downregulated in AtGA2ox1-OE maize. These results were consistent with those of the transcriptome data (Fig. 5c; Table 2). Therefore, this indicates that these TFs play an important role in the tolerance of transgenic maize to drought stress.

Discussion

GAs have been proven to be important components of stress tolerance in plants (Vettakkorumakankav et al. 1999). To date, some genes in the GA2ox family have been reported to be involved in stress responses in different plant species (Achard et al. 2008a, b; Magome et al. 2004, 2008). In order to explore the potential molecular mechanism, and verify whether the strategy of improving drought resistance of maize by accelerating the inactivation of bioactive GA into inactive GA is feasible, *AtGA2ox1* was overexpressed in maize, and the transgenic maize presented a GA deficiency phenotype (Fig. 1e). *AtGA2ox1* has a clear function (Thomas et al. 1999; Yamaguchi 2008), and has been validated in *Bahiagrass* to obtain a GA deficient transgenic plant (Agharkar et al. 2007). In addition, the results of nucleotide sequence alignments and phylogenetic analysis (data from the database of MaizeGDB) indicated no gene directly homologous to *AtGA2ox1* in maize. Hence, *AtGA2ox1* was preliminarily selected to generate the GA-deficient

Table 1 KEGG enrichment related to drought stress

Pathway	ko_ID	Number of DEGs	Expression level
Protein processing in endoplasmic reticulum	ko04141	56	Up
Plant hormone signal transduction	ko04075	53	Up, down
Biosynthesis of amino acids	ko01230	27	Up, down
Starch and sucrose metabolism	ko00500	20	Up, down
Glutathione metabolism	ko00480	20	Up, down
Glycerophospholipid metabolism	ko00564	19	Up, down
Carbon metabolism	ko01200	17	Up, down
Glycolysis/gluconeogenesis	ko00010	15	Up, down
Arginine and proline metabolism	ko00330	14	Up, down
Phosphatidylinositol signaling system	ko04070	11	Up, down
Glycerolipid metabolism	ko00561	11	Up, down
Inositol phosphate metabolism	ko00562	10	Up, down
Peroxisome	ko04146	8	Up, down
Pyruvate metabolism	ko00620	8	Up, down
Fatty acid degradation	ko00071	8	Up, down
Galactose metabolism	ko00052	6	Up
Oxidative phosphorylation	ko00190	5	Up, down
Pentose and glucuronate interconversions	ko00040	5	Up, down
Fatty acid metabolism	ko01212	4	Up
Citrate cycle (TCA cycle)	ko00020	4	Up, down
Diterpenoid biosynthesis	ko00904	4	Up, down
Fatty acid biosynthesis	ko00061	3	Up
ABC transporters	ko02010	3	Up, down
Biosynthesis of unsaturated fatty acids	ko01040	2	Up

Pathway name of the KEGG pathway, *ko_ID* KEGG orthologous gene ID

transgenic maize. The present results have proven the contribution of reduced levels of bioactive GAs in improving drought tolerance, strengthening the understanding of the molecular mechanism underlying drought tolerance in maize.

The physiological and biochemical indicators and transcriptome analysis revealed that the remarkable role of *AtGA2ox1* overexpression in transgenic maize for the enhancement of drought tolerance were via two pathways (Fig. 6). The first pathway was the ROS-detoxification pathway, which enhances antioxidant enzyme activities in transgenic maize under drought stress conditions. In the present study, SOD, CAT and POD enzyme activities were enhanced in transgenic maize plants (Fig. 3d–f), resulting in the reduction of the accumulation of ROS to inhibit membrane lipid peroxidation under drought stress conditions. The transcriptome analysis revealed that the DEGs were significantly enriched in the pathways of the oxidation–reduction process (Fig. 4; Tables 1, S3). Furthermore, these DEGs potentially enhanced the ability of ROS-detoxification in transgenic maize. Under drought stress, reductions in carbon assimilation resulted in an imbalance between electron excitation and utilization through photosynthesis, which lead to the production and accumulation of ROS. Then, ROS damages cell membranes, proteins and nucleic acids, causing oxidative stress (Sharma et al. 2012). The present study confirms that the reduced GA content in transgenic maize could activate internal antioxidant systems to eliminate ROS during drought stress. This support the results of previous studies (Achard et al. 2008a, b; Zhong et al. 2014; Lo et al. 2017), which reported the improvement of stress tolerance by reducing GA content to enhance the activity of ROS-detoxification enzymes for decreasing the levels of ROS. Due to the ROS-detoxification, the MDA content was reduced in transgenic maize (Fig. 3c). MDA is the catabolic end product of membrane lipid peroxidation, which serves as an indicator of the degree of abiotic stress-induced damage in plants. The functions of MDA were altered by reacting with proteins and nucleic acids, weakening the intermolecular bridges and bonds between cellulose molecules, and inhibiting protein synthesis (Moore and Roberts 1998).

The second pathway is involved in the drought-induced increase in proline and soluble sugar content in transgenic maize (Fig. 3a, b). The transcriptome analysis revealed that some DEGs involved in the proline metabolic pathway were also enriched (Table S3). Proline is an essential osmolyte

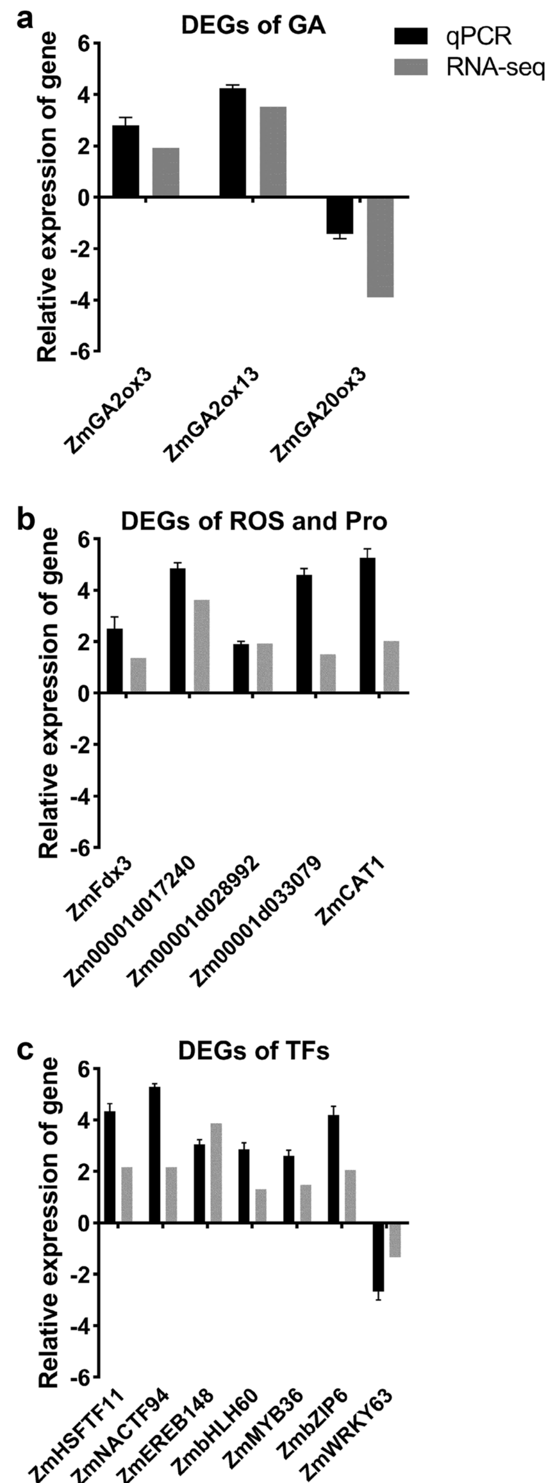

Fig. 5 The expression pattern of DEGs involved in GA, ROS, Pro and TFs identified by RNA-seq in transgenic maize line #1. **a** The relative expression of DEGs in the GA metabolism pathway. Zm00001d043411 (*GA2ox3*), Zm00001d039394 (*GA2ox13*), Zm00001d042611 (*GA20ox3*). **b** The relative expression of DEGs in the ROS scavenging and Pro metabolism pathway. Zm00001d034760 (*Fdx3*), Zm00001d017240 (unannotated), Zm00001d028992 (unannotated), Zm00001d033079 (unannotated), Zm00001d014848 (*CAT1*). **c** The relative expression of DEGs in TFs. Zm00001d034433 (*HSFTF11*), Zm00001d043877 (*NACTF94*), Zm00001d009103 (*EREB148*), Zm00001d013130 (*bHLH60*), Zm00001d012379 (*MYB36*), Zm00001d039658 (*bZIP6*), Zm00001d023332 (*WRKY63*). All reactions were performed in three biological duplications, and each biological duplication had three technical replicates. Statistical analysis was performed using the $2^{-\Delta\Delta CT}$ method. The error bars are presented as mean ± standard error (SE)

that allows plants to maintain the cell osmotic potential, cellular protection, and osmotic balance (Sperdouli and Moustakas 2012). The present study confirmed that the activation of the internal antioxidant system increased the accumulation of osmolytes, which mediated osmotic adjustment by reducing GA content, leading to the enhancement of tolerance to drought.

Furthermore, some TFs involved in abiotic stress responses and signaling were highly enriched, such as NAC, HSF, WRKY, AP2/ERF, bHLH, MYB and bZIP (Tables 2, S3). The TF family has been reported to join in the osmotic equilibrium, ROS scavenge, and protein–protein interaction between TFs. *AtJUB1* (*JUNGBRUNNEN 1*) is a hydrogen peroxide (H_2O_2)-induced NAC TF. *AtJUB1-OE* plants have been identified to have higher proline content, with improved drought tolerance in *Arabidopsis* and tomato (Wu et al. 2012; Thirumalaikumar et al. 2018). Interestingly, Zm00001d043877, the homology of *AtJUB1*, was also found to be upregulated in transgenic maize (Table 2). AtERF71/HRE2-overexpressing transgenic plants exhibited tolerance to salt stress, and had lower levels of ROS under high salt treatment (Park et al. 2011). A similar phenomenon was also found, in which Zm00001d009103, a transcriptional factor AP2/ERF, increased its expression in transgenic maize. In the present study, Zm00001d012379, as a member of the MYB TF family, was upregulated in transgenic maize (Table 2). A previous study revealed that in *AtMYB44* overexpressed plants, the WRKY70 and PR genes were upregulated, and this lead to the enhanced resistance to biotic stress in transgenic tomato (Shim et al. 2013). Zm00001d039658, as a HY5 and a member of the bZIP TF family, was upregulated in transgenic maize (Table 2). Related studies have revealed that HY5 binds to the promoters of many WRKY genes that encode for TFs involved in defense responses (Lee et al. 2007). HSF is an important TF family, which is involved in various abiotic stresses in plants (Guo et al. 2016). In HSP-related genes and cloning from *Arabidopsis*, chickpea and sunflower, these were overexpressed in various plants, and significantly improved heat, salt and drought tolerance (Wu et al. 2012; Yokotani et al. 2008; Liu et al. 2011; Ma et al. 2016; Ogawa et al. 2007). In the present study, it was observed that HSF and HSP had the most significant number among all groups (Table S2), and that these might have had a considerable contribution to the drought tolerance of transgenic maize. Zm00001d034433 is

Table 2 DEGs of the transcription factors involved in abiotic stress responses

Group	Gene ID	log_2FC	Swiss-Prot_annotation	NR_annotation	Reference
HSF	Zm00001d034433	2.17	Heat stress transcription factor A-2e OS = *Oryza sativa* subsp. *japonica* (rice) PE = 2 SV = 1	Heat shock factor protein HSF30 [*Zea mays*]	Yokotani et al. (2008)
NAC	Zm00001d043877	2.17	Transcription factor JUNGBRUNNEN 1 GN = F23E6.1 OS = *Arabidopsis thaliana* (Mouse-ear cress) PE = 1 SV = 1	TPA: putative NAC domain transcription factor superfamily protein [*Zea mays*]	Wu et al. (2012)
WRKY	Zm00001d023332	− 1.34	Probable WRKY transcription factor 54 GN = WRKY54 OS = *Arabidopsis thaliana* (Mouse-ear cress) PE = 2 SV = 2	Putative WRKY DNA-binding domain superfamily protein [*Zea mays*]	Chen et al. (2017)
AP2/ERF	Zm00001d009103	3.88	Ethylene-responsive transcription factor ERF071 GN = ERF071 OS = *Arabidopsis thaliana* (Mouse-ear cress) PE = 2 SV = 1	PREDICTED: ethylene-responsive factor-like protein 1 isoform X1 [*Zea mays*]	Park et al. (2011)
bHLH	Zm00001d013130	1.30	Transcription factor PIF5 GN = F17J16.110 OS = *Arabidopsis thaliana* (Mouse-ear cress) PE = 1 SV = 1	Putative HLH DNA-binding domain superfamily protein [*Zea mays*]	Oh et al. (2007)
MYB	Zm00001d012379	1.48	Transcription factor MYB44 GN = K8K14.2 OS = *Arabidopsis thaliana* (Mouse-ear cress) PE = 2 SV = 1	MYB transcription factor [*Zea mays*]	Shim et al. (2013)
bZIP	Zm00001d039658	2.05	Transcription factor HY5 GN = HY5 OS = *Solanum lycopersicum* (tomato) PE = 2 SV = 1	PREDICTED: putative bZIP transcription factor superfamily protein isoform X1 [*Zea mays*]	Lee et al. (2007)

The first column represents the group name of DEGs involved in abiotic stress responses, the second column represents the gene ID, and the subsequent columns represent the fold change (log_2FC) of the DEGs. The last column represents the annotation information of DEGs in the Swiss-Prot and NR databases

a member of HSFs, and is upregulated in transgenic maize (Table 2). Some of the reported TFs observed to have a decreased expression under drought stress were also found in the present. Furthermore, Zm00001d023332 was annotated as a WRKY, and was downregulated in transgenic maize (Table 2). The homology of Zm00001d023332 in *Arabidopsis* was identified to be correlated to drought tolerance. *WRKY54* negatively regulates drought tolerance by inhibiting dehydration-inducible gene expression, and the *wrky46/wrky54/wrky76* triple mutant had a stronger drought-resistant phenotype, when compared to the WT (Chen et al. 2017). In previous studies, reduced endogenous GA levels were found to directly or indirectly induce the expression of a series of abiotic stress-related TFs (Gallego-Bartolome et al. 2011; Qi et al. 2014). PIF5, a bHLH transcriptional factor, inhibits GA metabolism in seed germination by indirectly repressing GA synthetic genes *GA3ox1* and *GA3ox2*, and activating the GA catabolic gene *GA2ox2*, increasing ABA levels (Oh et al. 2007). Zm00001d013130 and PHYTOCHROME-INTERACTING FACTOR3-LIKE5 (PIF5) were also found to be upregulated in OE maize plants (Table 2). This supports that the change in GA content and regulation of the GA pathway metabolism could improve drought tolerance in maize. The present study reports the improvement of drought tolerance by overexpressing the *AtGA2ox1* gene to reduce the content of bioactive GAs in maize. Combined with previous studies, it was further confirmed that the improvement of drought resistance of genetically modified maize was through the enhancement of antioxidant enzyme activities and accumulation of osmolytes.

Although the regulated GA pathway can improve drought tolerance, one problem that should be noted is that it usually produces a dwarf phenotype, which affects the yield. The change in GAs content in plants could affect plant height, and the overexpression of the *GA2ox* gene would reduce plant height. However, the degree of plant height reduction is often uncertain, resulting in extreme or semi-dwarfing traits (Lo et al. 2008). In a previous study, it was found that drought tolerance can be enhanced through the application of a growth regulator and chlormequat chloride, limiting GA synthesis and production in maize, without producing an extreme-dwarfing phenotype (Spitzer et al. 2015). It is well-known that an appropriate GA content is very important to keep the balance among plant height, stress resistance and yield. The

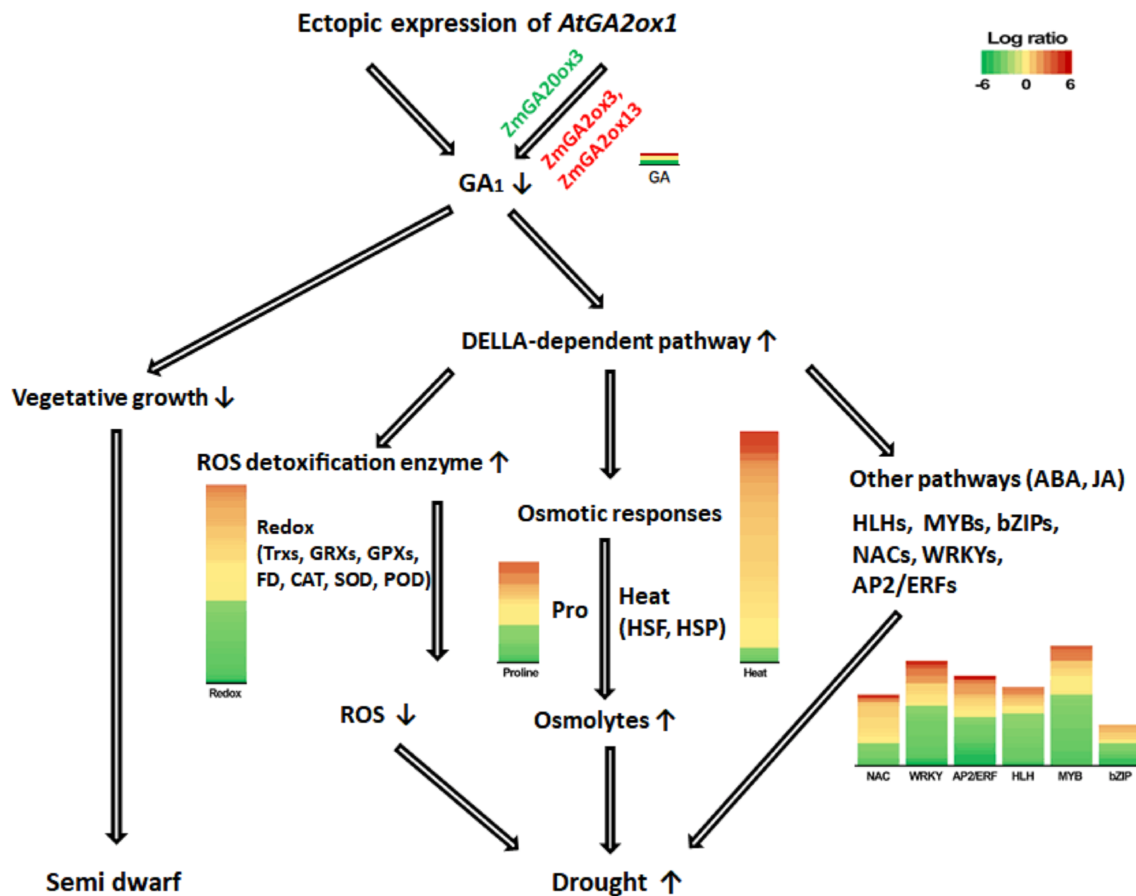

Fig. 6 Pathways that lead to increased drought stress tolerance. The solid arrowheads indicate the upregulation and downregulation of events. The open arrowheads indicate the suggested sequence of events. The upregulated and downregulated clustered genes are marked in red and green, respectively. The detailed list of genes in each group and the extent of changes are illustrated in Table S3. (Color figure online)

present study provides a new strategy to improve drought tolerance in maize by *AtGA2ox1-OE* biotechnology. Fortunately, an extreme dwarfing phenotype of maize was not obtained, but a semi-dwarfing phenotype. This provides a new approach for the biotechnological improvement of drought tolerance in maize. However, it should be noted that the influence of the overexpression of *AtGA2ox1* on the other growth and development pathways of transgenic maize remains unclear, especially the impact on yield and other traits with the reduction of endogenous bioactive GAs and plant height, which needs to be further researched. A greater tissue specificity of the *GA2ox* gene transgene expression would be needed to maximize the benefits, while avoiding negative impacts on maize development. Moreover, the genes in the GA metabolism pathways can also be mutated by gene editing and other techniques, in order to obtain the degradation pathway with a weakened function. It is hoped that this work may provide new insight for future studies on the roles of the GA2ox family members in maize.

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Author contribution ZC and YL contributed equally to the study. CG and XL designed the experiments. ZC, YY, YL, QL, NL, WH and DH performed the experiments. ZC and XL analyzed the data. ZC and XL wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest in this work.

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